1-2020

Basic Cell and Molecular Biology 4e: What We Know and How Found Out

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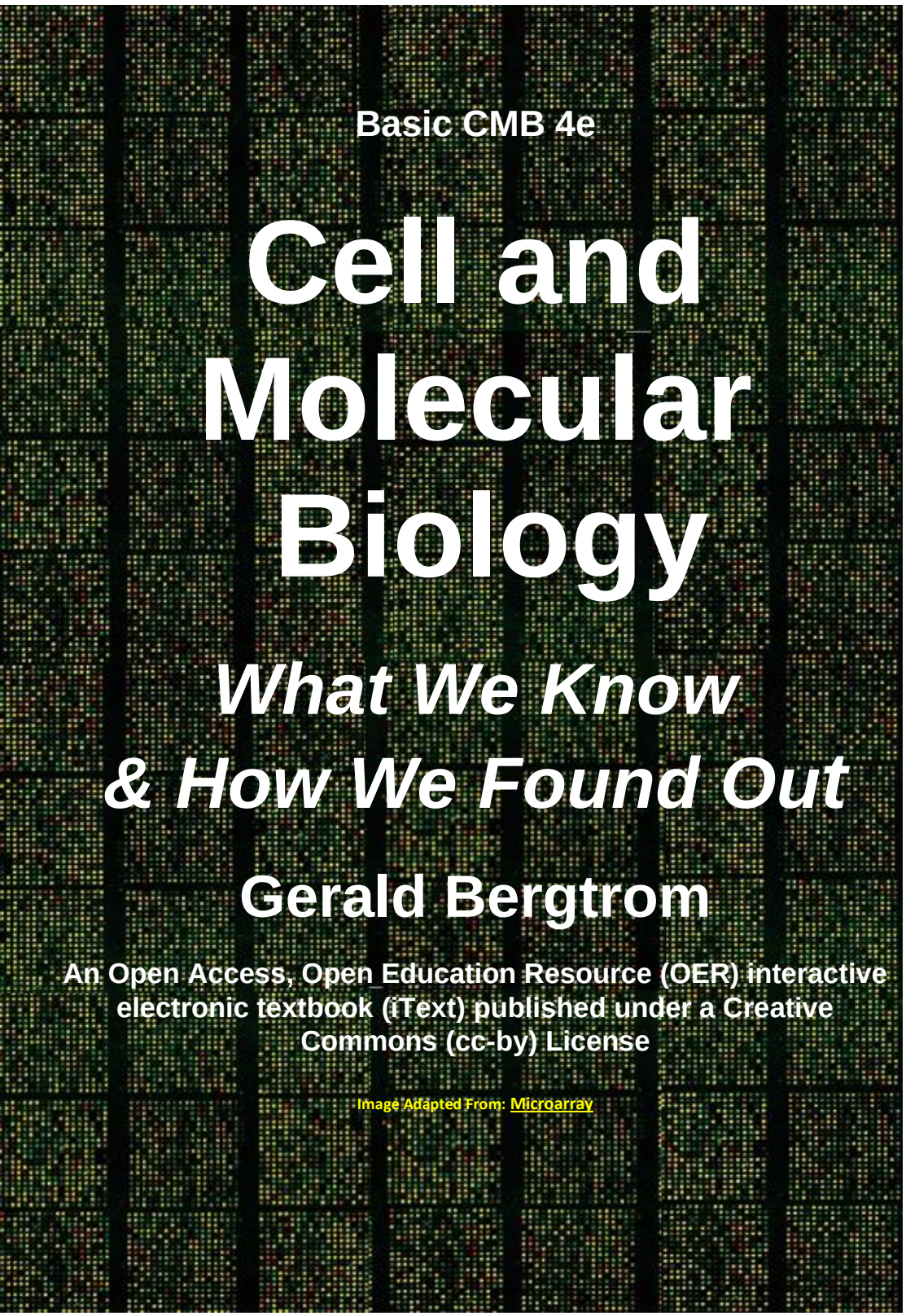

Basic CMB 4e

Cell and Molecular Biology

What We Know & How We Found Out

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Image Adapted From: Microarray

Cell and Molecular Biology

What We Know & How We Found Out

Annotated CMB 4e

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By

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New in CMB4e:

- ✓ *Reformatting, including*
  - *An expanded 'active' Table of Contents (the original Table of Contents remains as the Table of Chapters)*
  - *numbered figures with figure legends*
  - *New List of Figures and sources (Appendix I; other appendices renumbered)*
  - *An epilog!*
- ✓ *Many content updates (new illustrations, figures, links)*
- ✓ *More than 50 pages of new and reformatted content, with new sections on: Viruses, Proprioreception, Schrödinger's cat (!), history of CRISPR/Cas, 'next gen' DNA sequencing, directed evolution and more*
- ✓ *New **Challenge** boxes (Annotated & Instructors' editions)*

Dedicated to:

Sydell, Aaron, Aviva, Edan, Oren and our extended family

whose patience and encouragement made this work possible,

my students from whose curiosity I received as much as I gave,

and the memory of my mentor Herbert Oberlander,

who gave me the time, opportunity

and tools to do science.

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4th Edition, Published 2020

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M.T. Bott, Senior Lecturer, Biological Sciences, University of Wisconsin-Milwaukee

Preface to CMB4e

A grasp of the logic and practice of science is essential to understand the rest of the world around us. To that end, the **CMB4e** *iText* remains focused on experimental support for what we know about cell and molecular biology, and on showing students the relationship of cell structure and function.

Rather than trying to be a comprehensive reference book, the ***Basic* CMB4e** itext selectively details investigative questions, methods and experiments that lead to our understanding of cell biology. This focus is nowhere more obvious than in the chapter ***learning objectives*** and in *web-links. In addition to external online resources, links to the author's short YouTube voice-over PowerPoint (VOP) videos with optional closed captions are embedded near relevant text. Each video is identified by a *play-video* symbol and can be opened by clicking a descriptive title or using *QR bar codes*, such as the example below:

 **102 Golgi Vesicles & the Endomembrane System** 

The *Learning objectives* align with content and ask students to use new knowledge to make connections and deepen their understanding of concept and experiment. All external links are intended to expand or explain textual content and concepts and to engage student curiosity. All images in the iText and *just-in-time* VOPs are by the author or are from public domain or Creative Commons (CC) licensed sources.

Beyond the ***Basic CMB4e*,** a freely available ***Annotated*** version of the iText contains interactive links and formative assessments in the form of ***Challenge*** boxes. The ***Instructors* CMB4e** version models additional interactive features, including short ***25 Words or Less*** writing assignments that can be incorporated into almost any course management system, many of which the author has assigned as homework in his *flipped*, *blended* course. These assessments aim to reinforce writing as well as critical thinking skills. As a Sample Chapter, Chapter 1 of the ***Instructors*** version of CMB4e is freely available for download; the complete ***Instructors*** version is available on request.

My goal in writing and updating this *iText* is to make the content engaging, free and comparable in accuracy and currency to commercial textbooks. I encourage instructors to use the interactive features of the iText (critical thought questions, YouTube videos, etc.) to challenge their students.

***Note:** Web links to the author's own resources may occasionally be updated, but should remain active. Links to resources selected (but not created) by the author were live at the time of publication of the iText, but may disappear without notice!

With all of these enhancements, I encourage students to think about

- how good and great experiments were inspired and designed,
- how alternative experimental results were predicted,
- how data was interpreted and finally,
- how investigators (and we!) arrive at the most interesting "next questions".
-

The online *iText* is the most efficient way to access links and complete online assignments. Nevertheless, you can download, read, study, and access many links with a smart phone or tablet. And you can add your own annotations digitally or write in the margins of a printout the old-fashioned way! Your instructor may provide additional instructions for using your ***iText***.

Special to Instructors from the Author

All versions of the ***Basic*** and ***Annotated*** versions of **CMB4e** are freely available as pdf files to you and your students. To get the ***Instructors*** version you will need to fill out a short form identifying yourself as an instructor. When you submit the form, you will get pdf as well as MS-Word files for the *Basic*, *Annotated* and *Instructor's* ***CMB4e.*** Once you download the ***CMB4e*** *iText(s)* of your choice, you should find it an easy matter to use the MS-Word file to add, subtract, modify or embellish any parts of it to suit your purposes (in accordance with the Creative Commons CC-BY license under which it is published). Common modifications are adding content of your own, or even that students uncover as part of their studies (or as an assignment!). A useful enhancement of the iText is to add links to your own assessments (quizzes, writing assignments) that take students directly to a Quiz, Discussion Forum, DropBox, etc. in your Learning Management System, e.g., D2L, BlackBoard, Canvas, etc.) course site. This is seamless if students open the iText from within your course site, but also works as long as both the iText and course site are open at the same time. You can provide a customized version of the iText to your students as a smaller pdf file (recommended) or as an MS-Word document.

As implied above, you ask your students participate in the improvement of the iText (for fun or for credit!) and to share the results with others! One final caveat: whereas I provide content updates, that have significant potential subject to confirmation, very current research is not necessarily definitive. I hope that you (and perhaps your students!) will enjoy creating and customizing interactive elements and digging in to some of the most recent research included in the *iText*. Above all, I hope that your students will achieve a better understanding of how scientists use skills of inductive and inferential logic to ask questions and formulate hypotheses…, and how they apply concept and method to testing those hypotheses.

Acknowledgements

Many thanks go to my erstwhile LTC (now CETL) colleagues Matthew Russell, Megan Haak, Melissa Davey Castillo, Jessica Hutchings and Dylan Barth for inspiration in suggesting ways to model how open course content can be made interactive and engaging. I also thank my colleagues Kristin Woodward and Ann Hanlon in the Golda Meir Library for their help in publishing the various online editions and versions of ***CMB*** on the University of Wisconsin-Milwaukee Digital Commons open access platform (http://dc.uwm.edu). I am most grateful to Ms. M. Terry Bott for reviewing and vetting the images used in this iText as either in the public domain or designated with a Creative Commons (CC) license as an open resource (see *Creative Commons License* page, above). Most recently, I owe a debt of gratitude to our departmental lab manager (and just down the hall neighbor) Jordan Gonnering for lots of hardware and software assistance during the preparation of CMB4e. Last but not least I must acknowledge the opportunity I was given at the University of Wisconsin-Milwaukee to teach, study and do research in science and interactive pedagogy for more than 35 years. My research and collegial experience at UW-M have left their mark on the content, concept and purpose of this digital *Open Education Resource* (OER).

About the Author

Dr. Bergtrom is Professor (Emeritus) of Biological Sciences and a former Learning Technology Consultant in the UW-Milwaukee *Center for Excellence in Teaching and Learning*. Scientific interests include cell and molecular biology and evolution. Pedagogic interests are blended and online instruction and the use of technology to serve more active and engaged teaching and learning. He has taught face-to-face, fully online, *blended* and *flipped* classes at both undergraduate and graduate levels. He also developed and co-instructed *Teaching with Technology*, an interdisciplinary course aimed at graduate students that they might someday find themselves teaching. In his 40+ years of teaching and research experience, he has tested and incorporated pedagogically proven teaching technologies into his courses. His research papers have been supplemented with publications on active blended, online and flipped classroom methods[1-3].

The first edition of his *Open Access/Creative Commons* electronic iText, ***Cell and Molecular Biology***–*What We Know & How We Found Out* first appeared in 2015[4]. Subsequent editions and versions followed in 2016[5], 2018[6] and 2019[7]. The latest editions are available at http://dc.uwm.edu/biosci_facbooks_bergtrom/. Older editions (and versions) will remain available by request to the author.

1. Bergtrom, G. (2006) *Clicker Sets as Learning Objects*. Int. J. Knowl. & Learn. Obj. 2:105-110. (http://www.ijello.org/Volume2/v2p105-110Bergtrom.pdf)
2. Bergtrom, G. (2009) *On Offering a Blended Cell Biology Course*. J. Res. Center Ed. Tech. 5(1) (http://www.rcetj.org/?type=art&id=91609&).
3. Bergtrom, G. (2011) *Content vs. Learning: An Old Dichotomy in Science Courses*. J. Asynchr. Learning Networks 15:33-44 (http://jaln_v15n1_bergtrom.pdf)
4. Bergtrom, G. (2015) *Cell and Molecular Biology: What We Know & How We Found Out* [***CMB***] (If necessary, please contact the author to access this edition)
5. Bergtrom, G. (2016) *Cell and Molecular Biology: What We Know & How We Found Out* [**CMB2e**] (If necessary, please contact the author to access this edition)
6. Bergtrom, G. (2018) *Cell and Molecular Biology: What We Know & How We Found Out* [***CMB3e***] (If necessary, please contact the author to access this edition)
7. Bergtrom, G. (2020) *Cell and Molecular Biology: What We Know & How We Found Out* [***CMB4e***] (http://dc.uwm.edu/biosci_facbooks_bergtrom/)

Table of Chapters

(Click title to see first page of chapter)

Table of Contents

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Cell and Molecular Biology; What We Know & How We Found Out

Commented [GKB1]: Use this space for your notes, questions and comments.

Chapter 1: Cell Tour, Life's Properties and Evolution, Studying Cells

Life's domains, Scientific method, Cell structures, Study methods (microscopy, cell fractionation, functional analyses); Common ancestry, Genetic variation, Evolution, Species diversity

CELLS: *LEFT: Robert Hooke's drawing of cork slices seen through a microscope froom his 1665) Micrographia;* MIDDLE: Row of windows to monks' chambers (cells) as Hooke may have seen them. a monk is drawn in the window at the left; RIGHT: a monk's cell.

1.1 Introduction

You will read in this book about experiments that revealed secrets of cell and molecular biology, many of which earned their researchers Nobel and other prizes. But let's begin here with a *Tale of Roberts*, two among many giants of science in the renaissance and age of enlightenment whose seminal studies came too early to win a Nobel Prize.

One of these, **Robert Boyle**, was born in 1627 to wealthy, aristocrat parents. In his teens, after the customary *Grand Tour* of renaissance Europe (France, Greece, Italy…) and the death of his father, he returned to England in 1644, heir to great wealth. In the mid-1650s he moved from his estates where he had set about studying physics and chemistry, to Oxford. There he built a laboratory with his own money to do experiments on the behavior of gasses under pressure. With a little help, he discovered *Boyle's Law*, confirming that the gasses obey mathematical rules. He is also credited with showing that light and sound could travel through a vacuum, that something in air enables combustion, that sound travels through air in waves, that heat and particulate motion were related, and that the practice of alchemy was bogus! In fact, Boyle pretty much

converted alchemy to chemistry by doing *chemical analysis*, a term he coined. As a chemist, he also rejected the old Greek concept of earth, air, fire and water elements. Instead, he defined elements as we still do today: the element is the smallest component of a substance that cannot be further chemically subdivided.

He did all this a century before Antoine Lavoisier listed and define the first elements! Based on his physical studies and chemical analyses, Boyle even believed that the indivisible units of elements were atoms, and that the behavior of elements could be explained by the motion of atoms. Boyle later codified in print the scientific method that made him a successful experimental scientist.

The second of our renaissance Roberts was **Robert Hooke**, born in 1635. In contrast to Boyle parents, Hooke's were of modest means. They managed nonetheless to nurture their son's interest in things mechanical. While he never took the Grand Tour, he learned well and began studies of chemistry and astronomy at Christ Church College, Oxford in 1653. To earn a living, he took a position as Robert Boyle's assistant. It was with Hooke's assistance that Boyle did the experiments leading to the formulation of *Boyle's Law*. While at Oxford, he made friends and useful connections.

One friend was the architect Christopher Wren. In 1662, Boyle, a founding member of the Royal Society of London, supported Hooke to become the society's *curator of experiments*. However, to support himself, Hooke hired on as professor of geometry at Gresham College (London). After "the great fire" of London in 1666, Hooke, as city surveyor and builder, participated with Christopher Wren in the design and reconstruction of the city. Interested in things mechanical, he also studied the elastic property of springs, leading him to *Hooke's Law*, namely that the force required to compress a spring was proportional to the length that the spring was compressed. In later years these studies led Hooke to imagine how a coil spring might be used instead of a pendulum to regulate a clock. While he never invented such a clock, he was appointed to a Royal Commission to find the first reliable method to determine longitude at sea. He must have been gratified to know that the solution to accurate determination of longitude at sea turned out to involve a coil-spring clock! Along the way in his 'practical' studies, he also looked at little things; he published his observations in *Micrographia* in 1665. Therein he described microscopic structures of animal parts and even snowflakes. He also described fossils as having once been alive and compared microscopic structures he saw in thin slices of cork to monks' cells (rooms, chambers) in a monastery. Hooke is best remembered for his law of elasticity and of course, for coining the word *cell*, which we now know as the smallest unit of living things.

Now fast-forward almost 200 years to observations of plant and animal cells early in the 19th century. Many of these studies revealed common structural features including a nucleus, a boundary wall and a common organization of cells into groups to form

multicellular structures of plants and animals and even lower life forms. These studies led to the first two precepts of ***Cell Theory***: (1) *Cells are the basic unit of living things*; (2) *Cells can have an independent existence*. Later in the century when Louis Pasteur disproved notions of *spontaneous generation*, and German histologists observed *mitosis* and *meiosis* (the underlying events of cell division in eukaryotes) a third precept rounded out Cell Theory: (3) *Cells come from pre-existing cells*. That is, they reproduce. We begin this chapter with a reminder of the ***scientific method***, that way of thinking about our world that emerged formally in the 17th century. Then we take a tour of the cell, reminding ourselves of basic structures and organelles. After the 'tour', we consider the ***origin of life*** from a common ancestral cell and the subsequent ***evolution*** of cellular complexity and the incredible diversity of life forms. Finally, we consider some of the *methods* we use to study cells. Since cells are small, several techniques of microscopy, cell dissection and functional/biochemical analysis are described to illustrate how we come to understand cell function.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast *hypotheses* and *theories* and place them and other elements of the scientific enterprise into their place in the cycle of the *scientific method.*
2. compare and contrast structures common to and that distinguish *prokaryotes*, *eukaryotes* and *archaea*, and groups within these *domains.*
3. articulate the function of different cellular substructures.
4. explain how *prokaryotes* and *eukaryotes* accomplish the same functions, i.e. have the same *properties of life*, even though prokaryotes lack most of the structures.
5. outline a procedure to study a specific cell *organelle* or other substructure.
6. describe how the different structures (particularly in eukaryotic cells) relate/interact with each other to accomplish specific functions.
7. describe some structural and functional features that distinguish prokaryotes (eubacteria), eukaryotes and archaea.
8. place cellular organelles and other substructures in their evolutionary context, i.e., describe their origins and the selective pressures that led to their *evolution.*
9. distinguish between the roles of random *mutations* and *natural selection* in evolution.
10. relate archaea to other life forms and speculate on their origins in evolution.
11. suggest why evolution leads to more complex ways of sustaining life.
12. explain how *fungi* are more like animals than plants.

1.2 Scientific Method – The Formal Practice of Science

Let's focus here on the essentials of the scientific method originally inspired by Robert Boyle, and then on how science is practiced today. *Scientific method* is one or another standardized protocol for observing, asking questions about, and investigating natural

phenomena. Simply put, it says look/listen, infer, and test your inference. According to the Oxford English Dictionary, all scientific practice relies on the *systematic observation, measurement, and experiment, and the formulation, testing and modification of hypotheses*. Here is the scientific method as you might read it a typical science textbook:

- ***Read*** the science of others and ***Observe*** natural phenomena on your own.
- Infer and state an ***hypothesis*** (explanation) based on logic and reason.
- Hypotheses are declarative sentences that sound like fact but aren't! Good hypotheses are testable, easily turned into *if/then (predictive) statements*, or just as readily into *yes-or-no* questions.
- ***Design an experiment*** to test the hypothesis: results must be measurable evidence for or against the hypothesis.
- ***Perform that experiment*** and then observe, measure, collect data and test for statistical validity (where applicable). Then, repeat the experiment.
- Consider how your data supports or does not support your hypothesis and then ***integrate your experimental results*** with earlier hypotheses and prior knowledge.
- Finally, publish (i.e., make public) your experiments, results and conclusions. In this way, shared data and experimental methods can be repeated and evaluated by other scientists.

We'll return to the scientific method and how it is practiced shortly.

So, what are scientific *theories* and *laws* and how do they fit into the scientific method? Contrary to what many people think, a ***scientific theory*** *is not a guess*. Rather, a theory is a statement well supported by experimental evidence and widely accepted by the scientific community. In common parlance, theories might be thought of as 'fact', but scientists recognize that they are still subject to testing and modification, and may even be overturned. One of the most enduring and tested theories in biology is of course Darwin's ***Theory of Evolution***. While some of Darwin's notions have been modified over time, they did not topple the theory. The modifications have only strengthened our understanding that species diversity is the result of natural selection. For more recent commentary on the evolutionary underpinnings of science, check out Dobzhansky T (1973, *Nothing in biology makes sense except in the light of evolution*. Am. Biol. Teach. 35:125-129) and Gould, S.J. (2002, *The Structure of Evolutionary Theory*. Boston, Harvard University Press). You can check out some of Darwin's *own* work at On the Origin of Species by C Darwin.

A ***scientific Law*** is thought of as universal and even closer to 'fact' than a theory! Scientific laws are most common in math and physics. In life sciences, we refer to Mendel's *Law of Segregation* and *Law of Independent Assortment* as much in his honor as for their universal and enduring explanation of genetic inheritance in living things. But

Laws are not facts*!* Like Theories, Laws are always subject to experimental test. Astrophysicists are actively testing universally accepted laws of physics. Strictly speaking, Mendel's *Law of Independent Assortment* should not even be called a law. Indeed, it is not factual as he stated it! Check the Mendelian Genetics section of an introductory textbook to see how chromosomal crossing over violates this law.

To sum up, in describing how we do science, the Wikipedia entry states that *the goal of a scientific inquiry is to obtain knowledge in the form of testable explanations (hypotheses) that can predict the results of future experiments. This allows scientists to gain an understanding of reality, and later use that understanding to intervene in its causal mechanisms (such as to cure disease).* The better an *hypothesis* is at making predictions, the more useful it is. In the last analysis, think of hypotheses as *educated guesses* and think of theories and/or laws not as proofs of anything, but as one or more experimentally supported hypothesis that everyone agrees should serve as *guideposts* to help us evaluate new observations and hypotheses.

In other words, hypotheses are the bread and butter of the scientific enterprise. Good ones are testable and should predict either/or results of well-designed experiments. Those results (observations, experimental data) should support or nullify the hypotheses being tested. In either case, scientific data generates conclusions that inevitably lead to new hypotheses whose predictive value will also be tested. If you get the impression that scientific discovery is a cyclic process, that's the point! Exploring scientific questions reveals more questions than answers!

A word about well-designed experiments. **Erwin Schrödinger (**winner of the **Nobel Prize** in physics in 1933) once proposed a *thought experiment*. Schrödinger wanted his audience to understand the requirements of scientific investigation, but gained greater fame (and notoriety) far beyond the world of theoretical physics. Perhaps you have heard of his cat! Considered a founding father of quantum physics, he recognized that adherence to scientific method is not strict and that we can (and should) occasionally violate adherence to the dictates of scientific method.

In the now popular story of ***Schrödinger's Cat***, Schrödinger asked that if you sealed a cat in a box with a toxic substance, how could you know if the cat was alive or dead unless you open the box. Wearing his philosopher's hat (yes, he had one!), he postulated that until you open the box, the cat is both "dead and alive". That is, until the box was opened, the cat was in a sense, neither dead nor alive, but both! Often presented as little more than an amusing puzzle, Schrödinger was in fact illustrating that there were two alternate hypotheses: (1) *the cat exposed to toxin survived*, ***or*** (2) *the cat exposed to toxin died*. Note that either hypothesis is a declarative sentence, and that either could be tested. Just open the box!

In a twist however, Schrödinger added that by opening the box, the investigator would become a factor in the experiment. For example, let's say (for the sake of argument) that you find a dead cat in the box. Is it possible that instead of dying from a poison, the cat was scared to death by your act of opening the door! Or that the toxin made the cat more likely to die of fright but was not lethal by itself? How then to determine whether it was the toxin or your action that killed the cat? This made the puzzle even more beguiling, and to the many laypersons, his greatest scientific contribution! But to a scientist, the solution to the puzzle just means that a scientist must take all possible outcomes of the experiment into account, including the actions of the experimenter, ensuring sound experimental design with all necessary controls. The bottom line, and often the reason that scientific manuscripts suffer negative peer review, is the absence or inadequacy of control experiments. See more about ***Schrödinger's cat*** at https://www.youtube.com/watch?v=IOYyCHGWJq4.

1.2.1 The Method as It Is Really Practiced!

If you become a scientist, you may find that adherence to the 'rules' of scientific method are honored as much in the breach as in their rigorous observance. An understanding of those rules, or more appropriately principles of scientific method guides prudent investigators to balance personal bias against the leaps of intuition that successful science requires. Deviations from protocol are allowed! I think that we would all acknowledge that the actual practice of science by would be considered a success by almost any measure. *Science is a way of knowing* the world around us through constant test, confirmation, and rejection that ultimately reveals new knowledge, integrating that knowledge into our worldview.

An element often missing but integral to any scientific method is that *doing science is collaborative*. Less than a century ago, many scientists worked alone. Again, Gregor Mendel is an example, and his work was not appreciated until decades after he published it. In this day and age, most publications have two or more coauthors who contribute to a study. But the inherent collaborative nature of science extends beyond just the investigators in a study. In fact, when a paper (or a research grant for that matter) is submitted for consideration, other scientists are recruited to evaluate the quality of hypotheses, lines of experimentation, experimental design and soundness of its conclusions a submitted paper reports. This *peer review* of fellow scientists is part and parcel of good scientific investigation.

1.2.2 Logic and the Origins of the Scientific Method

The scientist, defined as a both observer and investigator of natural phenomena, is only a few centuries old. Long before that, philosophers developed formal rules of *deductive* and *inferential logic* to try and understand nature, humanity's relationship to

nature, and the relationship of humans to each other. We owe to those *philosophers* the logical underpinnings of science. They came up with systems of *deductive* and *inductive logic* so integral to the scientific method. The scientific method grew from those beginnings, along with increasing empirical observation and experimentation.

We recognize these origins when we award the Ph.D. (*Doctor of Philosophy),* our highest academic degree! We are about to learn about the life of cells, their structure and function, and their classification, or grouping based on those structures and functions. Everything we know about life comes from applying the principles of scientific method to our intuition. For a bemused take on how scientists think, check out *The Pleasure of Finding Things Out: The Best Short Works of Richard Feynman (*1999, New York, Harper Collins).

1.1 Domains of Life

We believe with good reason that all life on earth evolved from a common ancestral cell that existed soon after the origins of life on our planet. At one time, all life was divided into two groups: the true bacteria and everything else! Now we group life into one of three ***domains***:

- ***Prokaryotes*** are among the first descendants of that common ancestral cell. They lack nuclei (*pro* meaning *before* and *karyon* meaning *kernel*, or *nucleus*). They include *bacteria* and *cyanobacteria* (blue-green algae).
- ***Eukaryotes*** include all higher life forms, characterized by cells with true nuclei (*Eu*, true; *karyon*, nucleus).
- ***Archaebacteria***, (meaning "old" bacteria) include many ***extremophile*** bacteria ('lovers' of life at extreme temperatures, salinity, etc.). Originally classified as ancient prokaryotes, *Archaebacteria* were shown by 1990 to be separate from prokaryotes and eukaryotes, a third domain of life.

The archaea are found in such inhospitable environments as boiling hot springs or arctic ice, although some also live in conditions that are more temperate. Carl Woese compared the DNA sequences of genes for ribosomal RNAs in normal bacteria and extremophiles. Based on sequence similarities and differences, he concluded that the latter are in fact a domain separate from the rest of the bacteria as well as from eukaryotes. For a review, see (Woese, C. 2004; *A new biology for a new century*. Microbiol. Mol. Biol. Rev. 68:173-186) The three domains of life (**Archaea**, **Eubacteria** and ***Eukarya***) quickly supplanted the older division of living things into Five Kingdoms, the *Monera (prokaryotes), Protista, Fungi, Plants, and Animals (all eukaryotes!)*. In a final surprise, the sequences of archaebacterial genes clearly indicate a common ancestry of archaea and eukarya. The evolution of the three domains is illustrated below (Fig.1.1).

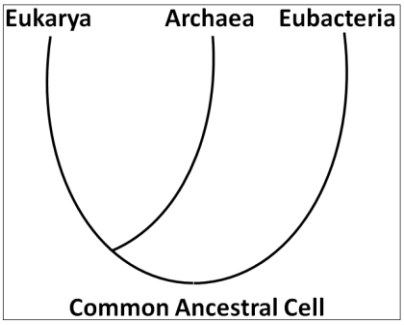


Fig. 1.1: Evolution of three domains showing a closer relationship between archaebacteria and Eukaryotes.

From this branching, Archaea are *not* true bacteria! They share genes and proteins as well as metabolic pathways found in eukaryotes but *not* in bacteria, supporting their close evolutionary relationship to eukaryotes. That they also contain genes and proteins as well as metabolic pathways unique to the group is further testimony to their domain status. Understanding that all living organisms belong to one of three domains has dramatically changed our understanding of evolution.

At this point you may be asking, "What about viruses?" Where do they belong in a tree of life? You may already know that viruses require live cellular hosts to reproduce, but that they are not themselves alive. In fact much about the place of viruses in evolution is an open question that we will consider in a later chapter. For now, let's look at how we come to know about viruses and some of their peculiarities.

1.3.1. Viruses: Dead or Alive; Big and Small - A History of Surprises

Viruses that infect bacteria are called ***bacteriophage*** (phage meaning eaters, hence *bacteria eaters*). Eukaryotic viruses include many that cause diseases in plants and animals. In humans, the corona viruses that cause influenza, the common cold, *SARS* and *COVID-19* are ***retroviruses***, with an RNA genome. Familiar retroviral diseases also include *HIV* (AIDS), Ebola, Zika, yellow fever and some cancers. On the other hand, Small pox, Hepatitis B, Herpes, chicken pox/shingles, adenovirus and more are caused by **DNA viruses**.

Viruses were not identified as agents of disease until late in the 19th century, and we have learned much in the ensuing century. In 1892, Dmitri Ivanofsky, a Russian botanist, was studying plant diseases. One that damaged tobacco (and was thus of agricultural significance) was the ***mosaic disease*** (Fig.1.2, below).

Fig. 1.2: Tobacco mosaic virus symptoms on a tobacco leaf.

Ivanofsky showed that extracts of infected tobacco leaves were themselves infectious. The assumption was that the extracts would contain infectious bacteria. But his extracts remained infectious even after passing them through a *Chamberland-Pasteur* filter, one with a pore size so small that bacteria would not pass into the filtrate. Thus the infectious agent(s) were not bacterial. Since the infectious material was not cellular and depended on a host for reproduction with no independent life of its own, they were soon given the name ***virus***, a term that originally just meant *toxin*, or *poison*. The virus that Ivanofsky studied is now called *Tobacco Mosaic Virus*, or TMV.

Invisible by light microscopy, viruses are sub-microscopic non-cellular bits of life-chemistry that only become reproductive (come alive) when they parasitize a host cell. Because many viruses cause disease in humans, we have learned much about how they are similar and how they differ. In other chapters, we'll learn how viruses have even become tools for the study of cell and molecular biology. Here we'll take a look at one of the more recent surprises from *virology*, the study of viruses.

As eventually seen in the electron microscope, viruses (called virions or viral particles) are typically 150 nm or less in diameter. And that is how we have thought of viruses for over a century! But in 2002, a *particle* inside an amoeba, originally believed to be a bacterium, was also shown by electron microscopy to be a virus…, albeit a ***giant virus***! Since this discovery, several more *giant*, or ***Megavirales*** were discovered. ***Megavirales*** fall into two groups, *pandoraviruses* and *mimiviruses*. At 1000nm (1 μm) *Megavirus chilensis* (a *pandoravirus*) may be the largest. Compare a few giant viruses to a bacterium (*E. coli*) and the AIDS virus Fig. 1.3 below.

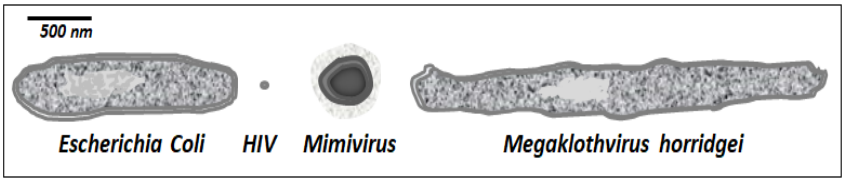


Fig. 1.3: Transmission electron micrographs of giant viruses, the AIDS (HIV) virus and the bacterium *E. coli*. *K. casanovai* is at least the same size and *M. horridgei* is twice the size of the bacterium. All the giant viruses, even the mimivirus, dwarf HIV, a typical eukaryotic virus.

Consider that a typical virus contains a relatively small genome, encoding an average of 10 genes. In contrast, the *M. chilensis* genome contains 2.5×10^6 base pairs that encode up to 1,100 proteins. Nevertheless, it still requires host cell proteins to infect and replicate. What's more surprising is that 75% of the putative coding genes in the recently sequenced 1.2×10^6 base-pair *mimivirus* genome ***had no counterparts*** in other viruses or cellular organisms! Equally surprising, some of the remaining 25%, of mimiviruses genes encode proteins homologous to those used for translation in prokaryotes and eukaryotes. If all, including the giant viruses, only use host cell enzymes and ribosomal machinery to synthesize proteins, what are these genes doing in a mimivirus genome?

Think of the surprises here as questions - the big ones concern where and when ***Megavirales*** (giant viruses) evolved:

- What are those genes with no cellular counterparts all about?
- What were the selective advantages of large size and large genomes?
- Were giant viruses once large free-living cells that invaded other cells, eventually becoming parasites and eventually losing most but not all of their genes? Or were they originally small viruses that incorporated host cell genes, resulting in increased genome size and coding capacity?

Clearly, viruses cause disease. Most were identified precisely because they are harmful to life. 2020 began with a novel viral epidemic in Wuhan, China. The disease, *COVID-19*, is caused by the ***SARS-CoV-2*** retrovirus. In a few short months *COVID-19* became a pandemic, one we are confronting as this is being written. It is interesting that so far, only a few viruses resident in human have been shown to be beneficial. This is in marked contrast to bacteria, some clearly harmful to humans. But many are beneficial, even necessary symbionts in our gut *microbiome*. The same is undoubtedly true of other living things, especially animals.

Let's now turn our attention to cells, entities that we define as living, with *all* of the *properties of life…,* starting with *eubacteria*.

1.3.3 The Prokaryotes (Eubacteria = *Bacteria* and C*yanobacteria*)

Prokaryotic cells lack a nucleus and other eukaryotic organelles such as mitochondria, chloroplasts, endoplasmic reticulum, and assorted eukaryotic vesicles and internal membranes. Bacteria do contain *bacterial microcompartments* (BMCs), but these are made up entirely of protein and are ***not*** surrounded by a phospholipid membrane. These function for example in CO_2 fixation to sequester metabolites toxic to the cells. Click Bacterial Organelles for more information. Bacteria are typically unicellular, although a few (like some cyanobacteria) live colonial lives at least some of the time. Transmission and scanning electron micrographs and an illustration of rod-shaped bacteria are shown below (Fig. 1.4).

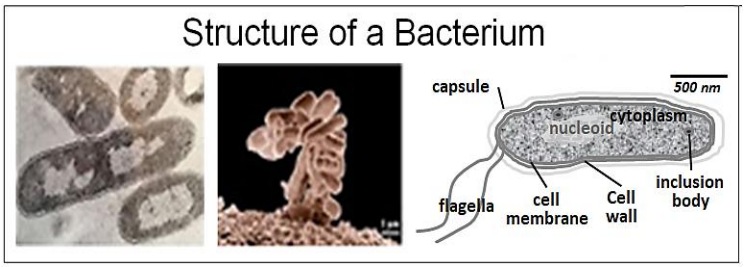


Fig. 1.4: Transmission and scanning electron micrographs of the gram-negative *E. coli* bacterium (left and middle), with its basic structure illustrated at the right).

1.3.2.a Bacterial Reproduction

Without the compartments afforded by the internal membrane systems common to eukaryotic cells, intracellular chemistries, from reproduction and gene expression (DNA replication, transcription, translation) to all the metabolic biochemistry of life happen in the cytoplasm of the cell. Bacterial DNA is a circular double helix that duplicates as the cell grows. While not enclosed in a nucleus, bacterial DNA is concentrated in a region of the cell called the ***nucleoid***. When not crowded at high density, bacteria replicate their DNA throughout the life of the cell, dividing by ***binary fission***. The result is the equal partition of duplicated bacterial *"chromosomes"* into new cells. The bacterial chromosome is essentially naked DNA, unassociated with proteins.

1.3.2.b Cell Motility and the Possibility of a Cytoskeleton

Movement of bacteria is typically by *chemotaxis*, a response to environmental chemicals. Some may respond to other stimuli such as light (*phototaxy*). They can move to or away from nutrients, noxious/toxic substances, light, etc., and

achieve motility in several ways. For example, many move using flagella made up largely of the protein ***flagellin***. Flagellin is absent from eukaryotic cell. On the other hand, the cytoplasm of eukaryotic cells is organized within a complex cytoskeleton of rods and tubes made of *actin* and *tubulin* proteins. Prokaryotes were long thought to lack these or similar cytoskeletal components. However, two bacterial genes that encode proteins homologous to eukaryotic actin and tubulin were recently discovered. The *MreB* protein forms a *cortical ring* in bacteria undergoing *binary fission*, similar to the actin cortical ring that pinches dividing eukaryotic cells during *cytokinesis* (the actual division of a single cell into two smaller daughter cells). This is modeled below (Fig.1.5) in the cross-section (right) near the middle of a dividing bacterium (left).

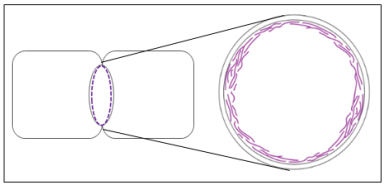


Fig. 1.5: Illustrated cross section of a dividing bacterium showing location of MreB cortical ring protein (purple).

The *FtsZ* gene encodes a homolog of tubulin proteins. It seems that together with flagellin, the MreB and FtsZ proteins may be part of a primitive prokaryotic *cytoskeleton* involved in cell structure and motility.

1.3.2.c Some Bacteria Have Internal Membranes

While bacteria lack organelles (the membrane-bound structures of eukaryotic cells), internal membranes in some bacteria form as inward extensions, or *invaginations* of plasma membrane. Some of these capture energy from sunlight (photosynthesis) or from inorganic molecules (*chemolithotrophy*). *Carboxysomes* (Fig. 1.6) are membrane bound photosynthetic vesicles in which CO_2 is fixed (reduced) in cyanobacteria. Photosynthetic bacteria have less elaborate internal membrane systems.

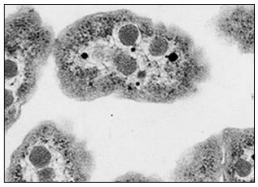


Fig. 1.6: Carboxysomes in a cyanobacterium, as seen by transmission electron microscopy.

1.3.2.d Bacterial Ribosomes Do the Same Thing as Eukaryotic Ribosomes… and Look Like Them!

Ribosomes are the protein-synthesizing machines of life. *Ribosomes* of prokaryotes are smaller than those of eukaryotes but are able to translate eukaryotic messenger RNA (mRNA) *in vitro*. Underlying this common basic function is the fact that the ribosomal RNAs of all species share base sequence and structural similarities indicating a long and conserved evolutionary relationship. Recall the similarities between RNA sequences that revealed a closer relationship of archaea to eukarya than prokarya.

Clearly, the prokarya (*eubacteria*) are a diverse group of organisms, occupying almost every wet, dry, hot or cold nook and cranny of our planet. Despite this diversity, all prokaryotic cells share many structural and functional metabolic properties with each other… and with the archaea and eukaryotes! As we have seen with ribosomes, shared structural and functional properties support the common ancestry of all life. Finally, we not only share common ancestry with prokaryotes, we even share living arrangements with them. Our gut bacteria represent up to 10X more cells than our own! Read more at The NIH Human Microbiome Project. Also check out the following link for A Relationship Between Microbiomes, Diet and Disease.

1.3.3 The Archaebacteria (Archaea)

Allessandro Volta, a physicist who gave his name to the 'volt' (electrical potential energy), discovered methane producing bacteria (*methanogens*) way back in 1776! He found them living in the extreme environment at the bottom of Lago Maggiore, a lake shared by Italy and Switzerland. These unusual bacteria are *chemoautotrophs* that get energy from H_2 and CO_2 and also generate methane gas in the process. It was not until the 1960s that Thomas Brock (from the University of Wisconsin-Madison) discovered *thermophilic* bacteria living at temperatures approaching 100^{o}C in Yellowstone National Park in Wyoming.

Organisms living in any extreme environment were soon nicknamed *extremophiles*. One of the thermophilic bacteria, now called *Thermus aquaticus*, became the source of *Taq* polymerase, the heat-stable DNA polymerase that made the *polymerase chain reaction* (PCR) a household name in labs around the world! Extremophile and "normal" bacteria are similar in size and shape(s) and lack nuclei. This initially suggested that most extremophiles were prokaryotes. But as Carl Woese demonstrated, it is the archaea and eukarya that share a more recent common ancestry! While some bacteria and eukaryotes can live in extreme environments, the archaea include the most diverse extremophiles. Here are some of them:

- *Acidophiles*: grow at acidic (low) pH.
- *Alkaliphiles*: grow at high pH.
- *Halophiles*: require high [salt], e.g., *Halobacterium salinarium* (Fig.1.7 below).
- *Methanogens*: produce methane.
- *Barophiles*: grow best at high hydrostatic pressure.
- *Psychrophiles*: grow best at temperature 15 °C or lower.
- *Xerophiles*: growth at very low water activity (drought or near drought conditions).
- *Thermophiles* and *hyperthermophiles*: organisms that live at high temperatures. *Pyrolobus fumarii*, a hyperthermophile, lives at 113°C! *Thermus aquaticus* (Fig.1.8 below)) normally lives at 70ºC; it is noted for its role in developing the polymerase chain reaction.
- *Toxicolerants*: grow in the presence of high levels of damaging elements (e.g., pools of benzene, nuclear waste).

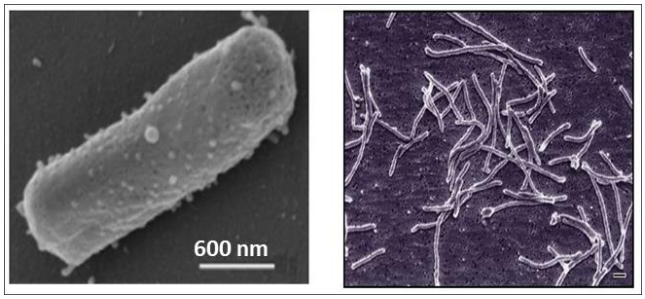


Fig. 1.7: Left: Scanning electron micrograph of *Halobacterium salinarium*, a salt-loving bacterium.
Fig. 1.8: Right: Scanning electron micrograph of 'heat-loving' *Thermus aquaticus* bacteria.

Archaea were originally seen as oddities of life, thriving in unfriendly environments. They also include organisms living in less extreme environments, including soils, marshes and even in the human colon. They are also abundant in the oceans where they are a major part of plankton, participating in the carbon and nitrogen cycles. In the guts of cows, humans and other mammals, methanogens facilitate digestion, generating methane gas in the process. In fact, cows have even been cited as a major cause of global warming because of their prodigious methane emissions! On the plus side, methanogenic Archaea are being exploited to create biogas and to treat sewage. Other extremophiles are the source of enzymes that function at high temperatures or in organic solvents. As already noted, some of these have become part of the biotechnology toolbox.

1.3.4 The Eukaryotes

The volume of a typical eukaryotic cell is some 1000 times that of a typical bacterial cell. Imagine a bacterium as a 100 square foot room (the size of a small bedroom, or a large walk-in closet!) with one door. Now imagine a room 1000 times as big. That is, imagine a 100,000 square foot 'room'. You would expect many smaller rooms inside such a large space, each with its own door(s). The eukaryotic cell is a lot like that large space, with lots of interior "rooms" (i.e., organelles) with their own entryways and exits. In fact, eukaryotic life would not even be possible without a division of labor of eukaryotic cells among different *organelles* (the equivalence to the small rooms in our metaphor).

The smaller prokaryotic "room" has a much larger plasma membrane *surface area-to-volume ratio* than a typical eukaryotic cell, enabling required environmental chemicals to enter and quickly diffuse throughout the cytoplasm of the bacterial cell. Chemical communication between parts of a small cell is therefore rapid. In contrast, the communication over a larger expanse of cytoplasm inside a eukaryotic cell requires the coordinated activities of subcellular components and compartments. Such communication can be relatively slow. In fact, eukaryotic cells have lower rates of metabolism, growth and reproduction than do prokaryotic cells. The existence of large cells required an evolution of divided labors supported by *compartmentalization*.

Fungi, more closely related to animal than plant cells, are a curious beast for a number of reasons! For one thing, the organization of fungi and fungal cells is somewhat less defined than animal cells. Structures between cells called *septa* separate fungal *hyphae*, allow passage of cytoplasm and even organelles between cells. Some primitive fungi have few or no septa, in effect creating *coenocytes*, which are single giant cell with multiple nuclei. Fungal cells are surrounded by a wall, whose principal component is ***chitin***. Chitin is the same material that makes up the exoskeleton of *arthropods* (which includes insects and lobsters!).

We end this look at the domains of life by noting that, while eukaryotes are a tiny minority of all living species, "their collective worldwide biomass is estimated to be equal to that of prokaryotes" (Wikipedia). And we already noted that the bacteria living commensally with us humans represent 10 times as many cells as our own human cells! Clearly, each of us (and probably most animals and even plants) owes as much of its existence to its microbiome as it does to its human cells. For now, keeping in mind that plants and animal cells share many internal structures and organelles that perform the same or similar functions, let's look at them and briefly describe their functions. Typical animal and plant cells with organelles and other structures are illustrated below (Fig.1.9, Fig.1.10).

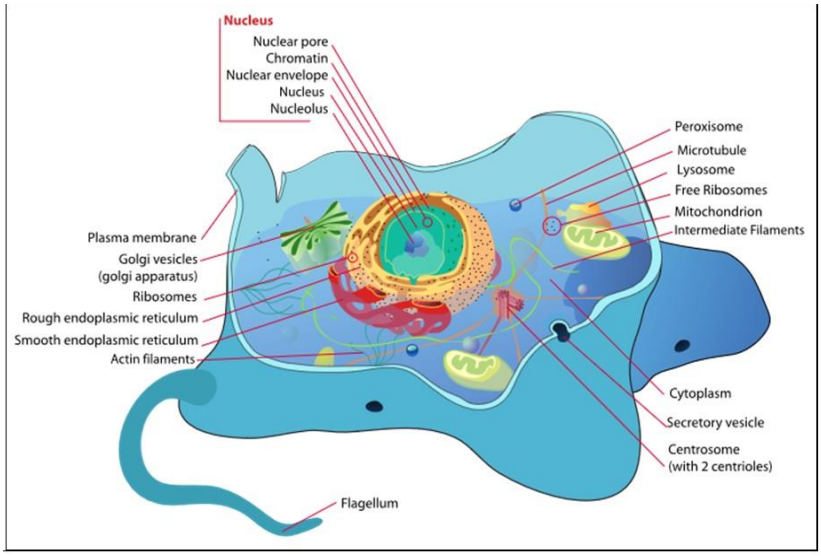


Fig. 1.9: Illustration of the structural components of a typical animal cell.

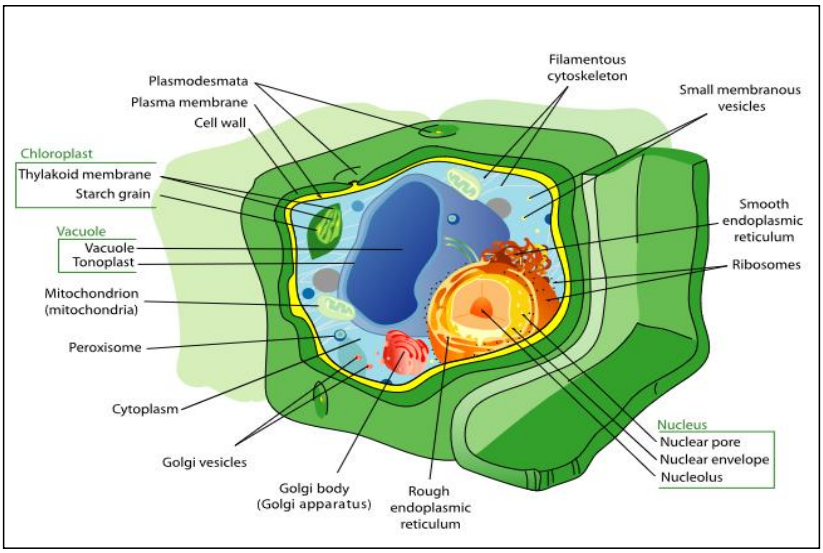


Fig. 1.10: Illustration of the structural components of a typical plant cell.

1.4 Tour of the Eukaryotic Cell

Here we take a closer look at the division of labors among the organelles and structures within eukaryotic cells. We'll look at cells and their compartments in a microscope and see how the organelles and other structures were isolated from cells and identified not only by microscopy, but by biochemical and molecular analysis of their isolates.

1.4.1 The Nucleus

The nucleus separates the genetic blueprint (DNA) from the cell cytoplasm. The nucleus breaks down during mitosis and meiosis as chromosomes form and cells divide. The genetic material spends most of its time as chromatin during ***interphase***, the time between cell divisions. This is when the status of genes and therefore of proteins produced in the cell, are regulated. mRNA, rRNA and tRNA are transcribed from genes, processed in the nucleus, and exported to the cytoplasm through ***nuclear pores***. Some RNAs remain in the nucleus, often participating in regulating gene activity. The dividing cells of all organisms must make and partition copies of their genetic material equally into new daughter cells. Let's look at the structural organization of the nucleus and then at its role in cell and whole organism genetics.

1.4.1.a Structure of the Interphase Nucleus

A typical electron microscope and cartoon of a nucleus, the largest eukaryotic organelle in a cell, are shown in Fig. 1.11 (below).

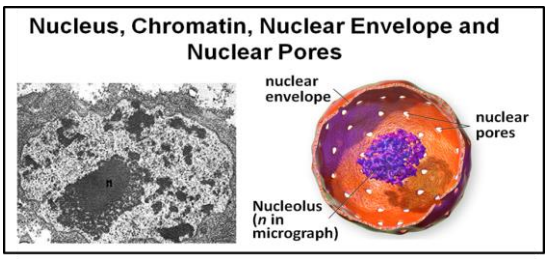


Fig. 1.11: LEFT - Transmission electron micrograph of an insect cell nucleus indicates the nucleolus(**n**)); RIGHT – Illustration of a nucleus showing chromatin (purple) and pores in the nuclear envelope (orange).

This cross-section of an interphase nucleus reveals its double membrane, or ***nuclear envelope***. The outer membrane of the nuclear envelope is continuous with the *RER* (rough endoplasmic reticulum). Thus, the lumen of the RER is

continuous with the space separating the nuclear envelope membranes. The electron micrograph also shows a prominent ***nucleolus*** (labeled **n**) and a darkly granular RER surrounding the nucleus.

Zoom in on the micrograph; you may see the double membrane of the nuclear envelope. You can also make out ribosomes (small granules) bound to both the RER and the outer nuclear membrane. Nuclear envelope ***pores*** (illustrated in the cartoon at the right) allow large molecules and even particles to move in and out of the nucleus across both membranes. We learn what some of this nuclear pore traffic is all about in later chapters.

104-2 The nucleus

The nucleus is *not* an unorganized space surrounded by the nuclear envelope, as might appear in transmission electron micrographs. The nucleolus is the largest of several inclusions that seem to segregate nuclear functions. Over 100 years ago ***Santiago Ramón y Cajal*** reported other structures in the nuclei of neurons, including what came to be known as ***Cajal bodies*** *(CBs)*. His elegant hand-drawn illustrations of nuclear bodies (made long before the advent of photomicrography) can be seen at Cajal's Nuclear Bodies and Cajal's Beautiful Brain Cells. ***Cajal*** and ***Camillo Golgi*** shared the Nobel Prize in Physiology or Medicine 1906 for their studies of nerve cell structure. In the electron microscope, ***Cajal bodies*** *(CBs)* look like coils of tangled thread, and were thus called ***coiled bodies*** (conveniently, also CBs). Other nuclear bodies since identified include ***Gems***, ***PML bodies***, nuclear speckles (or ***splicing speckles***), ***histone locus bodies*** (*HLBs*)**,** and more! Different nuclear bodies are associated with specific proteins. Fig. 1.12 illustrates the results of immunofluorescence localization of nuclear body proteins.

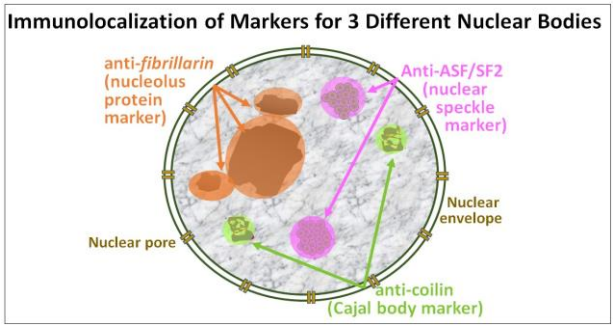


Fig. 1.12: Simulated fluorescence micrograph showing immunolocalization of antibodies against fibrillarin, coilin and ASF/SF2 protein to different *nuclear bodies*.

Nucleoli contain ***fibrillarin*** proteins and stain red because they have been treated with red-fluorescence-tagged ***antifibrillarin*** antibodies. CBs contain the protein ***coilin***. They fluoresce pink because the nuclei were treated with fluorescence-tagged ***anticoilin*** antibodies. Green-fluorescent antibodies to the **ASF/SF2** protein localize to nuclear speckles. As part of, or included in a nuclear matrix, nuclear bodies organize and regulate different aspects of nuclear activity and molecular function. The different nuclear bodies perform specific functions and interact with each other and with proteins DNA and RNA to do so. We will revisit some nuclear bodies in their working context in later chapters.

1.4.1.b Every Cell (i.e., Every Nucleus) of an Organism Contains the Same Genes

We read earlier that bacteria are busy doubling and partitioning their naked DNA chromosomes at the same time as they grow and divide by binary fission. In eukaryotic cells, a ***cell cycle*** divides life into discrete consecutive events. During most of the cell cycle, cells are in interphase and DNA is wrapped up in proteins in a structure called ***chromatin***. It is not merely the DNA, but chromatin that must be duplicated when cells reproduce. Duplication of DNA also involves rearranging, or disturbing the chromatin proteins resting on the DNA. This occurs before cell division (***mitosis*** and ***cytokinesis***). As the time of cell division nears, chromatin associates with even more proteins, condensing to form *chromosomes*, while the nuclear envelope dissolves. You may recall that every somatic cell of a eukaryotic organism contains paired *homologous chromosomes*, and therefore two copies of every gene an organism owns. On the other hand, sperm and eggs contain one of each pair of chromosomes, and thus one copy of each gene. Whether by mitosis or meiosis, cytokinesis separates duplicated chromosomes to *daughter* cells. In the fluorescence micrograph of a cell in the *metaphase* stage of *mitosis* (Fig.1.13), the chromosomes (blue) are just about to be pulled apart by microtubules of the spindle apparatus (green).

The Mitotic Spindle

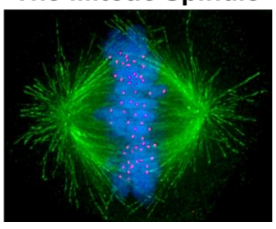


Fig. 1.13: Fluorescence micrograph of a mitotic spindle treated with antibodies to chromosomal proteins (blue) and spindle fiber proteins (green).

As the chromosomes separate and daughter cells form, nuclei reappear and chromosomes de-condense. These events mark the major visible difference between cell division in bacteria and eukaryotes. Cytokinesis begins near the end of mitosis. *Sexual reproduction*, a key characteristic of eukaryotes, involves ***meiosis*** rather than mitosis. The mechanism of *meiosis*, the division of *germ cells* leading to production of sperm and eggs, is similar to mitosis except that the ultimate daughter cells have just one each of the parental chromosomes, eventually to become the gametes (eggs or sperm). Google *meiosis* and/or *mitosis* to remind yourself about the differences between the two processes, meiosis and mitosis.

A key take-home message here is that every cell in a multicellular organism, whether egg, sperm or somatic, contains the same genome (genes) in its nucleus. This was understood since mitosis and meiosis were first described in the late 19th century. However, it was finally demonstrated in 1962, when John Gurdon and Shinya Yamanaka transplanted nuclei from the intestinal cells the frog *Xenopus laevis* into enucleated eggs (eggs from which its own nucleus had been removed). These 'eggs' grew and developed into normal tadpoles, proving that no genes are lost during development, but just expressed differentially.

We will revisit animal cloning later in this book. But for now, it's sufficient to know that Molly the cloned frog was followed in 1996 by Dolly, the first cloned sheep, and then other animals, all cloned from enucleated eggs transplanted with differentiated cell nuclei. Click Cloning Cuarteterra for the *60 Minutes* story of the cloning of *Cuarteterra*, a champion polo mare whose clones are also champions! For their first animal cloning experiments, Gurdon and Yamanaka shared the 2012 Nobel Prize form Physiology or medicine.

1.4.2 Ribosomes

On the other end of the size spectrum, ribosomes are evolutionarily conserved protein synthesizing machines in all cells. They consist of a large and a small subunit, each made up of multiple proteins and one or more molecules of ribosomal RNA (rRNA). Ribosomes bind to messenger RNA (mRNA) molecules and then move along the mRNA as they translate 3-base code words (codons) to link amino acids into polypeptides. Multiple ribosomes can move along the same mRNA, becoming a polyribosome, simultaneously translating the same polypeptide encoded by the mRNA. The granular appearance of cytoplasm in electron micrographs is largely due to the ubiquitous distribution of ribosomal subunits and polysomes in cells. Fig. 1.14 shows a ***polyribosome*** 'string' of ribosomes, or ***polysome*** for short.

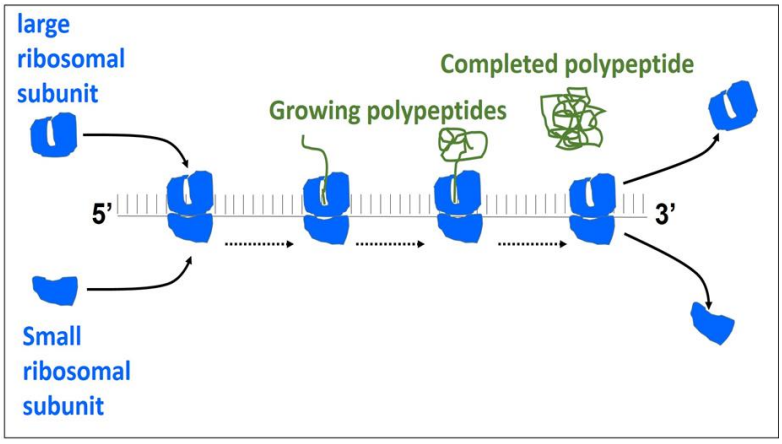


Fig. 1.14: To form a polysome, ribosomes (blue) assemble at the left on an mRNA molecule. As they move along the mRNA from left to right, they translate the message into a polypeptide (green), shown growing and emerging from ribosomes in the polysome.

In the illustration, ribosomes assemble at the left of the messenger RNA (mRNA) to form the polysome. When they reach the end of the message, the ribosomes disassemble from the RNA and release the finished polypeptide. In electron micrographs of leaf cells from the dry, quiescent desiccation-tolerant dessert plant *Selaginella lepidophylla* (Fig.1.15), you can make out randomly distributed ribosomes and ribosomal subunits (arrows, below left). In cells from a fully hydrated plant, you can see ***polysomes*** as more organized strings of ribosomes (arrows, below right).

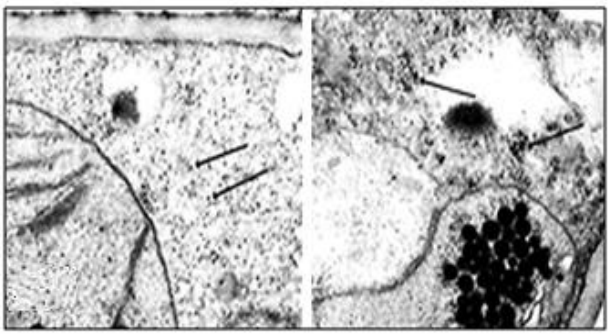


Fig. 1.15: Transmission electron micrographs of cells from desiccated and fully hydrated *Selaginella lepidophylla* plants. Free ribosomes or ribosomal subunits in the desiccated cells (LEFT) appear to have organized to form polysomes in the hydrated plant cells (RIGHT).

Eukaryotic and prokaryotic ribosomes differ in the number of RNAs and proteins in their large and small subunits, and thus in their overall size. Isolated ribosomes and their subunits can be separated based on differences in mass. Fig.1.16 shows the difference in ribosomal subunit 'size', protein and ribosomal RNA (rRNA) composition.

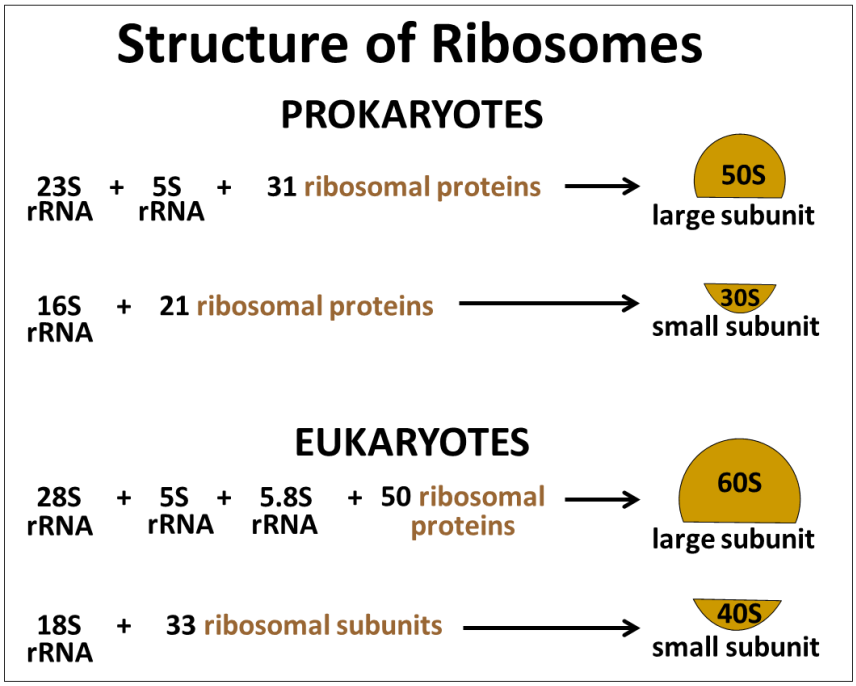


Fig. 1.16: Key differences between prokaryotic and eukaryotic ribosomes: Eukaryotic ribosomes and their subunits are larger and contain more proteins and larger ribosomal RNAs (rRNAs) than those of bacteria. The components were separated by sucrose density gradient centrifugation in which particles and macromolecules (like RNA) move through a sugar gradient at rates dependent on their mass (in effect, their size).

The position of ribosomal subunits in the gradient is represented by an ***S value,*** after *Svedborg*, who first used sucrose density gradients to separate macromolecules and particles by mass. Note that ribosomal RNAs themselves also separate on sucrose density gradients by size, hence their different S values.

101 Ribosomes & Polysomes

1.4.3 Internal membranes and the Endomembrane System

Microscopists of the 19th century saw many of sub-cellular structures using the art of histology, staining cells to increase the visual contrast between cell parts. One of these, ***Camillo Golgi***, an early neurobiologist, developed a silver (black) stain that first detected a network of vesicles we now call ***Golgi bodies*** or ***Golgi vesicles*** in nerve cells. For his studies of membranes now named after him, ***Camillo Golgi*** shared the 1906 Nobel prize for Medicine or Physiology with ***Santiago Ramón y Cajal***.

Many ***vesicles*** and ***vacuoles*** in cells, including Golgi vesicles, are part of the ***endomembrane system*****.** Proteins synthesized on the ribosomes of the **RER (*rough endoplasmic reticulum*)** can enter the interior space (*lumen*) or can become part of the RER membrane itself. The synthesis of ***RER*****,** ***SER*** *(**smooth endoplasmic reticulum**)***, ******Golgi bodies*****,** ***lysosomes*****,** ***microbodies*** and other vesicular membranes (as well as their protein content) all begin in the RER. The RER and protein contents bud into *transport vesicles* that fuse with *Golgi Vesicles* (**G** in the electron micrograph in Fig. 1.17).

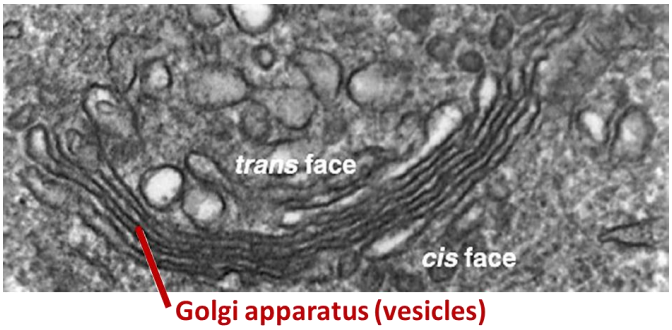


Fig. 1.17: Transmission electron micrograph of an insect cell showing Golgi bodies (G).

In moving through the endomembrane system, *packaged proteins* undergo stepwise modifications (*maturation*) before becoming biologically active (Fig.1.18, below). Some proteins made in the endomembrane system are secreted by ***exocytosis***. Others end up in organelles such as ***lysosomes*** that contain hydrolytic enzymes. These enzymes are activated when the lysosomes fuse with other organelles destined for degradation. For example, *food vacuoles* form when a plasma membrane *invaginates,* engulfing food particles. They then fuse with lysosomes to digest the engulfed nutrients. Still other proteins synthesized by ribosomes on the RER are incorporated into the RER membranes, destined to become part of lysosomes, peroxisomes and even the plasma membrane itself.

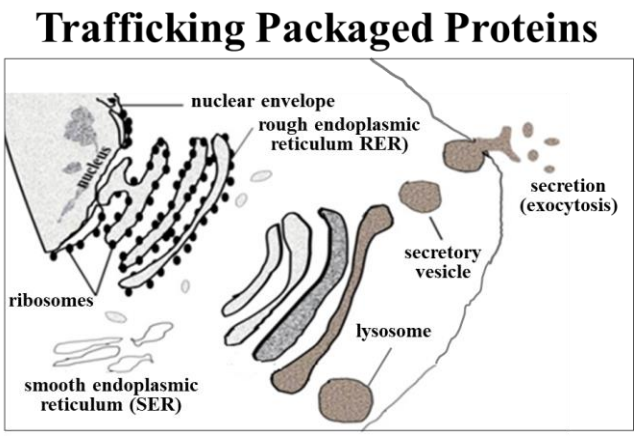


Fig. 1.18: Illustration of 'packaged' protein traffic through a cell, from the RER to organelles (e.g., lysosomes) or to the plasma membrane for exocytosis (i.e., secretion). RER and Golgi vesicles are major sites for the modification (i.e., maturation) of packaged proteins.

100-2 The RER-Rough Endoplasmic Reticulum

102 Golgi Vesicles & the Endomembrane System

Autophagosomes are small vesicles that surround and eventually encapsulate tired organelles (for example, worn out mitochondria), eventually merging with lysosomes whose enzymes degrade their contents. In 2016, Yoshinori Ohsumi earned the Nobel Prize in Physiology and Medicine for nearly 30 years of research unraveling the cell and molecular biology of autophagy. ***Microbodies*** are a class of vesicles smaller than lysosomes but formed by a similar process. Among them are peroxisomes that break down toxic peroxides formed as a by-product of cellular biochemistry. Some vesicles emerging from the RER lose their ribosomes to become part of the SER, which has several different functions (e.g., alcohol detoxification in liver cells).

103-2 Smooth Endoplasmic Reticulum

Other organelles include the ***contractile vacuoles*** of freshwater protozoa that expel excess water that enters cells by osmosis. Some protozoa have *extrusomes*, vacuoles that release chemicals or structures that deter predators or enable prey capture. A large aqueous central vacuole dominates the volume of many higher plant cells. When filled with water, they will push all other structures against the plasma

membrane. In a properly watered plant, this water-filled vacuole exerts osmotic pressure that among other things, keeps plant leaves from wilting and stems upright.

1.4.4 Mitochondria and Plastids

Nearly all eukaryotic cells contain *mitochondria,* shown in Fig.1.19.

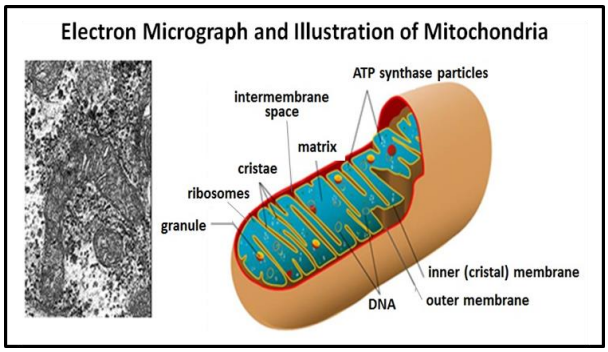


Fig. 1.19: Transmission electron micrograph (LEFT) and drawing of a mitochondrion (RIGHT).

A double membrane surrounds the mitochondrion. Each contains and replicates its own DNA, which contains genes encoding some mitochondrial proteins. Note that the surface area of the inner mitochondrial membrane is increased by being folded into ***cristae***, which are sites of ***cellular respiration*** (aerobic nutrient oxidation).

Earlier, we speculated that some eukaryotic organelles could have originated within bacteria. But mitochondria most likely evolved from a complete aerobic bacterium (or proto-bacterium) that was engulfed by a primitive eukaryotic cell. The bacterium escaped destruction, becoming an ***endosymbiont*** in the host cell cytoplasm. Lynn Margulis proposed the ***Endosymbiotic Theory*** in 1967 (Margulis, L. [*Sagan, L*], *1967. On the origin of mitosing cells.* Journal of Theoretical Biology **14** (3): 225–274). Read her paper at Margulis-Endosymbiosis. She proposed that chloroplasts (one among several different ***plastids***) also started as *endosymbionts*. Both mitochondria and the plastids of plants and some algae have their own DNA, supporting their origins as bacteria and cyanobacteria engulfed by primitive eukaryotic cells. Living at first in symbiosis with the rest of the cell, they would eventually evolve into the organelles that we are familiar with.

105-2 Endosymbiosis-Mitochondria & Chloroplasts

A handful of protozoa were discovered that lack mitochondria and other organelles. This had suggested they might share ancestry with those primitive eukaryotes that acquired mitochondria by endosymbiosis. However, since such cells contain other organelles such as *hydrogenosomes* and *mitosomes*, it is thought more likely that these species *once had, but then lost mitochondria*. Therefore, descendants of ancient eukaryotic cells missing mitochondria probably no longer exist…, if they ever existed at all! More evidence for the *Endosymbiotic Theory* is discussed elsewhere.

Chloroplasts and *cyanobacteria* contain chlorophyll and use a similar photosynthetic mechanism to make glucose. Typical chloroplasts are shown (Fig. 1.20, below). The one on the right is shows a few starch granules.

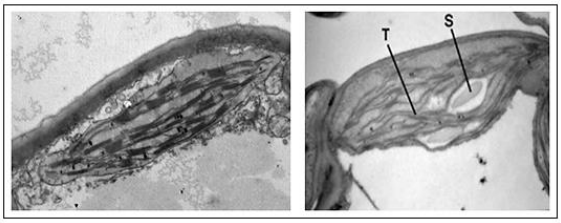


Fig. 1.20: Transmission electron micrograph of chloroplast that could have begun photosynthesizing (LEFT), and one that has photosynthesized long enough to accumulated starch granules (RIGHT). S, starch granule; T, thylakoids.

The *leucoplast* in Fig. 1.21 below is a plastid, actually a chloroplast filled with starch granules after a day's photosynthetic work.

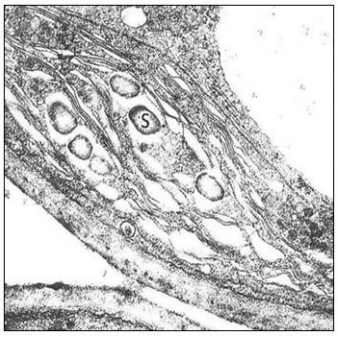


Fig. 1.21: Transmission electron micrograph of a leucoplast, a chloroplast that has become filled with starch granules (S).

1.4.5 Cytoskeletal structures

We have come to understand that the cytoplasm of a eukaryotic cell is highly structured, permeated by rods and tubules. The three main components of this ***cytoskeleton*** are ***microfilaments***, ***intermediate filaments*** and ***microtubules.*** The main cytoskeletal components are shown in Fig.1.22.

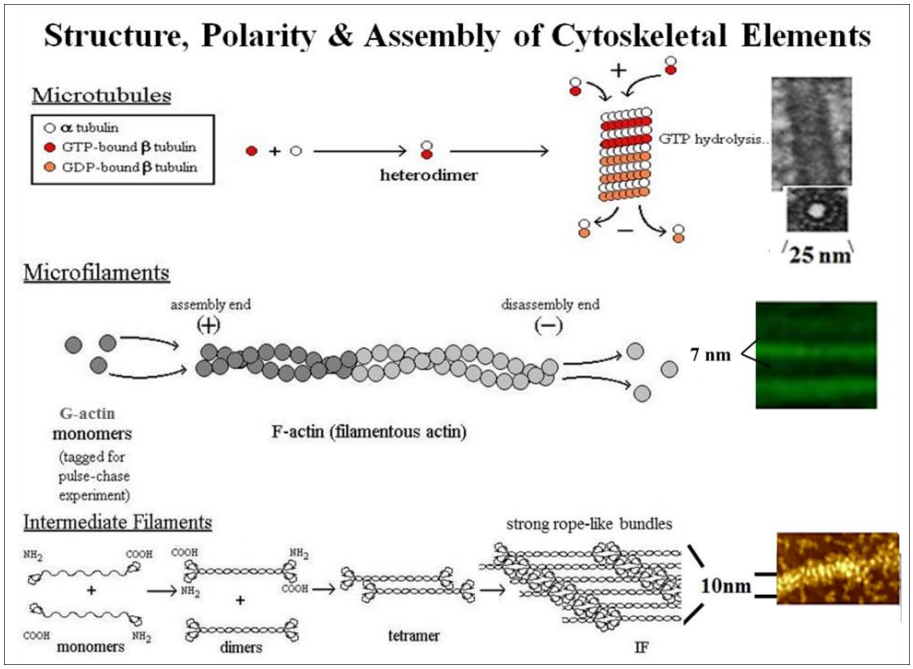


Fig. 1.22: Illustration of assembly, structure and polarity of microtubules, microfilaments and intermediate filaments alongside fluorescence micrographs made using fluorescent antibodies against isolated microtubule, microfilament and intermediate filament proteins (TOP, MIDDLE and BOTTOM, respectively).

Microtubules are composed of α– and β-*tubulin* protein monomers. Monomeric *actin* proteins make up microfilaments. Intermediate filament proteins are related to *keratin*, a protein found in hair, fingernails, bird feathers, etc. Cytoskeletal rods and tubules not only determine *cell shape*, but also play a role in *cell motility*. This includes the movement of cells from place to place and the movement of structures within cells. We have already noted that a prokaryotic cytoskeleton is composed in part of proteins homologous to actins and tubulins. As in a eukaryotic cytoskeleton, these bacterial

proteins may play a role in maintaining or changing cell shape. On the other hand, flagellum-powered movement in bacteria relies on flagellin, a protein not found in eukaryotic cells.

A bacterial flagellum is actually a rigid hook-like structure attached to a molecular motor in the cell membrane that spins to propel the bacterium through a liquid medium. In contrast, eukaryotic *microtubules* slide past one another causing a more flexible flagellum to undulate in wave-like motions. The motion of a eukaryotic cilium is also based on sliding microtubules, but in this case causing the cilia to beat rather than undulate. Cilia are involved not only in motility, but also in feeding and sensation. Microtubules in eukaryotic flagella and cilia arise from a *basal body* (similar to *kinetosomes* or *centrioles*) such as the one in Fig. 1.23 below.

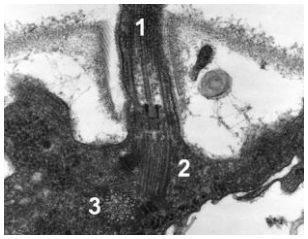


Fig. 1.23: Transmission electron micrograph showing a flagellum (#1) emerging from a basal body (2). Number 3 is another basal body, this time in cross section.

Aligned in a flagellum or cilium, microtubules form an ***axoneme*** surrounded by plasma membrane. In electron micrographs of cross sections, a ciliary or flagellar *axoneme* is typically organized as a ring of nine paired microtubules (called *doublets*) around two *singlet* microtubules. This ***9+2*** arrangement of microtubules is illustrated in Fig. 1.24.

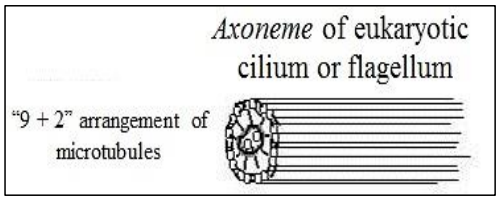


Fig. 1.24: The characteristic "9+2" arrangement of microtubules seen in cross-sections of eukaryotic cilia and flagella is maintained the *axoneme*, a structure remaining after removing the plasma membrane from isolated cilia or flagella.

Centrioles are themselves comprised of a ring of microtubules. In animal cells they participate in spindle fiber formation during mitosis and are the point from which microtubules radiate thorough the cell to help form and maintain its shape. These

structures do not involve axonemes. The spindle apparatus in plant cells, which typically lack centrioles, form from an amorphous structure called the *MTOC*, or *MicroTubule Organizing Center*, which serves the same purpose in mitosis and meiosis as centrioles do in animal cells.

106-2 Filaments & Tubules of the Cytoskeleton

Elsewhere, we describe how microfilaments and microtubules interact with motor proteins (*dynein*, *kinesin*, *myosin, etc.*) to generate force that results in the sliding of filaments and tubules to allow cellular movement. You will see that motor proteins can also carry cargo molecules from one place to another in a cell.

1.5 How We Know the Functions of Cellular Organelles and Structures: Cell Fractionation

We can see and describe cell parts in the light or electron microscope, but we could not definitively know their function until it became possible to release them from cells and separate them from one another. This was possible with the advent of the ultracentrifuge and differential centrifugation (the separation of ribosomes and ribosomal components was mentioned earlier). A *cell fractionation* scheme is illustrated below in Fig 1.25.

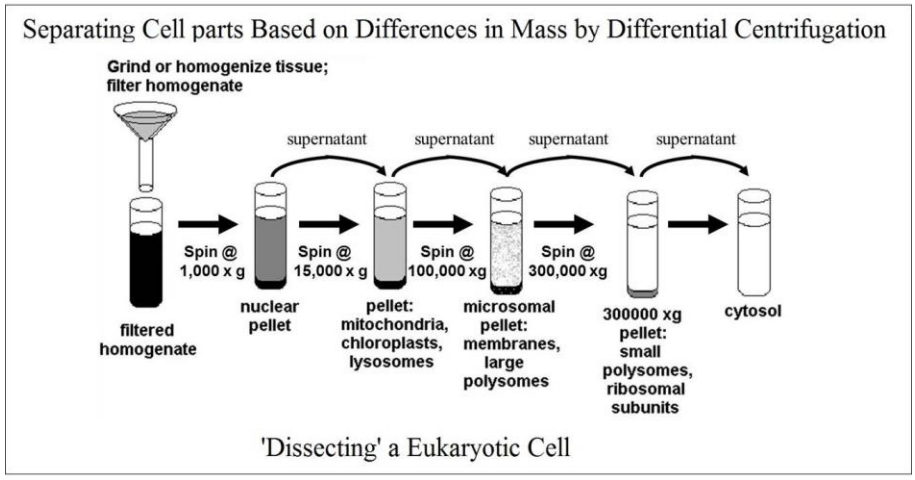


Fig. 1.25: Cell fractionation is an isolation of sub-cellular components (organelles, ribosomes, etc.) and a soluble fraction (cytosol) from disrupted cells. Serial centrifugation at progressively higher G-forces in an ultracentrifuge separates the components.

Under centrifugal force generated by a spinning centrifuge, subcellular structures separate by differences in mass. Structures that are more massive reach the bottom of the centrifuge tube before less massive ones. .

107-2 Dissecting the Cell-a Cell Fractionation Scheme

Cell fractionation separates cells into their constituent parts. The first step of a cell fractionation is to break open the cells and release their contents. This can be done by physical means such as grinding in a mortar and pestle, tissue grinder or similar device, exposure to ultrasound or high pressure, or exposure to enzymes or other chemicals that can selectively degrade the plasma membrane. The next step is to isolate the subcellular organelles and particles from the cytoplasm (i.e., cytosol) by differential centrifugation. The centrifugation of broken cells at progressively higher centrifugal force separates particulate cell components based on their mass. At the end of this process, a researcher will have isolated mitochondria, chloroplasts, nuclei, ribosomes etc. After re-suspension, each pellet can be re-suspended and prepared for microscopy. Electron micrographs of several isolated subcellular fractions are shown in Fig. 1.25, below.

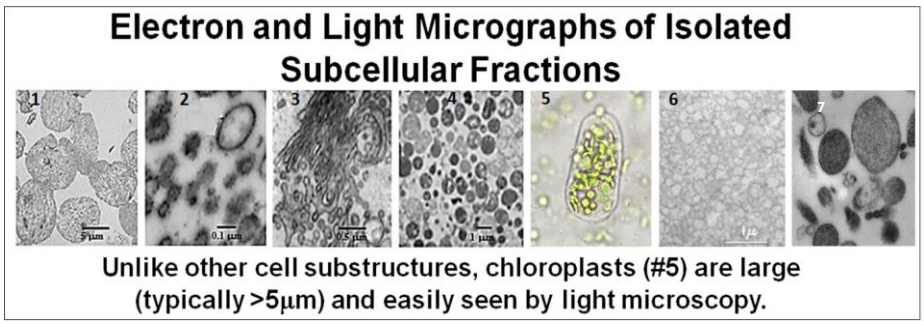


Fig. 1.26: Organelles isolated by cell fractionation from eukaryotic cells.

108-2 Isolated Nuclei

109-2 Isolated RER

110-2 Isolated Golgi Vesicles

111-2 Lysosomes & Peroxisomes

112-2 Isolated Mitochondria

113-2 Isolated Chloroplasts

114-2 Isolated Membranes Form Vesicles

These structures can be tentatively identified by microscopy based on their dimensions and appearance. Molecular analyses and biochemical tests on the cell fractions then help to confirm these identities. Can you tell what organelles have been purified in each of these fractions based on the electron micrographs alone? Consider the structures on the left as an example. These were found in a low speed centrifugal pellet, implying that they are large structures. They look a bit like nuclei, which are also the largest structures in a eukaryotic cell…, and indeed that's what they are!

Biochemical analysis of the isolated cell fractions can reveal what different organelles and cellular substructures do. The physical separation of subcellular structures and their biochemical-molecular analysis have revealed their basic functions. Such experiments continue to reveal previously un-noticed structures and functions in cells. What biochemical tests might you do to confirm the identities of the structures shown?

All of cell and molecular biology is devoted to understanding how prokaryotic and eukaryotic cells (and organisms) use their common structural and biochemical inheritance to meet very different survival strategies. As you progress in your studies, watch for experiments in which cell parts are separated and reassembled (i.e., reconstituted). *Reconstitution* is a recurring experimental theme in the functional analysis of cell parts. Look for it as you continue your studies. Also look another theme, namely how evolution accounts for the common biochemistry and genetics of life…, *and* its structural diversity!

1.6 The Origins, Evolution, Speciation, Diversity and Unity of Life

The question of how life began has been with us since the beginnings or recorded history. It is now accepted that there was a time, however brief or long, when the earth was a lifeless (prebiotic) planet. Life's ***origins*** on earth date to some 3.7-4.1 billion years ago under conditions that favored the formation of the first cell, the first entity with all of the properties of life.

But couldn't those same conditions have spawned multiple cells independently, each with all of the properties of life? If so, from which of these did life, as we know it today, descend? Whether there were one or more different "first cells", evolution (a property of life) could only begin with 'that or those' cells.

115 Properties of Life

The fact that there is no evidence of cells of independent origin may reflect that they never existed. Alternatively, we can propose that the cell we call our ancestor was evolutionarily successful at the expense of other early life forms, which thus became

extinct. In any event, whatever this successful ancestor may have looked like, its descendants would have evolved quite different biochemical and physiological solutions to achieving and maintaining life's properties. One of these descendants evolved the solutions we see in force in all cells and organisms alive today, including a common (*universal*) genetic code to store life's information, as well as a common mechanism for retrieving the encoded information. Francis Crick called is commonality the "Central Dogma" of biology. That ancestral cell is called our ***Last Universal Common Ancestor***, or ***LUCA***.

116 The Universal Genetic Code **117 Origins of Life**

118 Life Origins vs Evolution

Elsewhere we consider in more detail how we think about the origins of life. For the moment, our focus is on evolution, the property of life that is the basis of speciation and life's diversity. Charles Darwin's theory of evolution was an explanation of the *structural* diversity of species. A naturalist, Darwin lived at a time of ferment where scientific discovery was challenging religion. But by 1839, Charles Darwin had published his ***Narrative of the Surveying Voyages of His Majesty's Ships Adventure and Beagle***, the first of many reports of his careful observations of nature, with the seeds of what was to become his theory of ***natural selection***. He published his more fully formed theory of evolution by natural selection in 1859 in ***The Origin of Species***. There he finally acknowledged his evidence-based belief that that new species arise when beneficial traits are selected from random genetic differences in individuals in a population, while less fit individuals are culled from the population. If natural selection acting on individuals, the emergence of new species (evolution) results from the persistence and spread of selected, heritable changes through successive generations in a population. In this way, evolution results in *an increase in diversity and complexity* at all levels of biological organization, from species to individual organisms and all the way down to biomolecules. Darwin recognized the discord his theory would generate between science and biblical accounts of purposeful creation. He addressed the issue with great tact in introducing *The Origin of Species*: "*Although much remains obscure, and will long remain obscure, I can entertain no doubt, after the most deliberate study and dispassionate judgement of which I am capable, that the view which most naturalists entertain, and which I formerly entertained–namely, that each species has been independently created–is erroneous."*

According to creationists, the exquisite eyes could only have formed by the intelligent design of a creator. But see the article in National Geographic by E. Yong (Feb., 2016, with photography by D. Littschwager). Over time science favored Darwin. With the rediscovery of Mendel's genetic experiments at the turn of the 20th century, it became

increasingly clear that it is an organism's genes that are inherited, are passed down the generations, and are the basis of an organism's traits. It also became clear that Mendel had found the genetic basis for Darwin's theory and the evolution of eyes can be explained. Over time, science and religion have found ways to co-exist but as we know, the controversy persists.

Repeated speciation occurs with the continual divergence of life forms from an ancestral cell through natural selection and evolution. Our shared cellular structures, nucleic acid, protein and metabolic chemistries (the 'unity' of life) supports our common ancestry with all life. These shared features date back to our LUCA! Take as an example the fact that most living things even share some early *behaviors*. Our ***biological clock*** is an adaptation to our planet's 24-hour daily cycles of light and dark that have been around since the origins of life; all organisms studied so far seem to have one!. The discovery of the genetic and molecular underpinnings of ***circadian rhythms*** (those daily cycles) earned Jeffrey C. Hall, Michael Rosbash and Michael W. Young the 2017 Nobel Prize in Medicine or Physiology (click Molecular Studies of Circadian Rhythms wins Nobel Prize to learn more)!

The molecular relationships common to all living things largely confirm what we have learned from the species represented in the fossil record. Morphological, biochemical and genetic traits that are shared across species are defined as ***homologous*** and can be used to reconstruct evolutionary histories. The biodiversity that scientists (in particular, environmentalists) try to protect is the result of millions of years of speciation and extinction. Biodiversity needs protection from the unwanted acceleration of evolution arising from human activity, including blatant extinctions (think passenger pigeon), and near extinctions (think American bison by the late 1800s). Think also of the consequences of the introduction of invasive aquatic and terrestrial species and the effects of climate change.

Let's look at the biochemical and genetic unity among livings things. We've already considered what happens when cells get larger when we tried to explain how larger cells divide their labors among smaller intracellular structures and organelles. When eukaryotic cells evolved further into multicellular organisms, it became necessary for the different cells to communicate with each other and to respond to environmental cues. Some cells evolved mechanisms to "talk" directly to adjacent cells and others evolved to transmit electrical (neural) signals to other cells and tissues. Still other cells produced hormones to communicate with cells far away, to which they had no physical attachment.

As species diversified to live in very different habitats, they also evolved very different nutritional requirements, along with more extensive and elaborate biochemical pathways to digest their nutrients and capture their chemical energy. Nevertheless, through billions

of years of evolution and astonishing diversification, the underlying genetics and biochemistry of living things on this planet is remarkably unchanged. Early in the 20th century, Albert Kluyver first recognized that cells and organisms vary in form appearance in spite of an essential biochemical unity of all organisms (see Albert Kluyver in Wikipedia). This unity amidst the diversity is a life paradox that we examine in this course.

1.6.1 Random Acts of Genetic Variation, the Basis of Natural Selection

DNA contains the genetic instructions for the structure and function of cells and organisms. When and where a cell or organism's genetic instructions are used (i.e., to make RNA and proteins) are regulated. Genetic variation results from random mutations. Genetic diversity arising from mutations is in turn, the basis of natural selection during evolution.

119 The Random Basis of Evolution

1.6.2 The Genome: An Organism's Complete Genetic Instructions

We've seen that every cell of an organism carries the DNA that includes genes and other kinds DNA sequences. The genome of an organism is the *entirety* of its genetic material (DNA, or for some viruses, RNA). The genome of a common experimental strain of *E. coli* was sequenced by 1997 (Blattner FR et al. 1997*The complete genome sequence of Escherichia coli K-12.* Science 277:1452-1474). Sequencing of the human genome was completed by 2001, well ahead of the predicted schedule (Venter JC 2001*The sequence of the human genome*. Science 291:1304-1351). As we have seen in the re-classification of life from five kingdoms into three domains, nucleic acid sequence comparisons can tell us a great deal about evolution. We now know that evolution depends not only on gene sequences, but also, on a much grander scale, on the structure of genomes. Genome sequencing has confirmed not only genetic variation between species, but also considerable variation between individuals of the same species. The genetic variation within species is the raw material of evolution. It is clear from genomic studies that genomes have been shaped and modeled (or remodeled) in evolution. We'll consider genome remodeling in more detail elsewhere.

1.6.3 Genomic 'Fossils' Can Confirm Evolutionary Relationships.

It had been known for some time that gene and protein sequencing could reveal evolutionary relationships and even familial relationships. Read about an early demonstration of such relationships based on amino acid sequence comparisons across evolutionary time in Zuckerkandl E and Pauling L. (1965) *Molecules as documents of evolutionary theory.* J. Theor. Biol. 8:357-366. It is now possible to

extract DNA from fossil bones and teeth, allowing comparisons of extant and extinct species. DNA has been extracted from the fossil remains of humans, other hominids, and many animals. DNA sequencing reveals our relationship to animals (from bugs to frogs to mice to chimps…) and to Neanderthals and our other hominid ancestors. Unfortunately, DNA from organisms much older than 10,000 years is typically so damaged or simply absent, that relationship building beyond that time is impossible.

Using what we know from gene sequences of species alive today, investigators recently *'constructed'* a genetic phylogeny suggesting the sequences of genes of some of our long-gone progenitors, including bacteria (click here to learn more: Deciphering Genomic Fossils). The comparison of these *'reconstructed'* ancestral DNA sequences suggests when photosynthetic organisms diversified and when our oxygenic planet became a reality.

Closer to home, many remains of ancestral humans have been discovered in the Americas. These promise to unlock mysteries of human settlement of the continents, but not without controversy. Indian tribal cultures treat their ancestors as sacred and argue against sampling such remains for DNA Analysis. In one example a well preserved 'mummified' body discovered in the Nevada desert in the 1940s. Tests of hair and clothing fragments revealed that this *Spirit Cave mummy* was over 10,000 years old. The *Fallon Paiute-Shoshone* tribe that lives near the burial site asserted a cultural relationship to the body and requested the right to its return to the tribe in compliance with the *Native American Graves Protection and Repatriation Act*. Anthropologists counter-asserted a need for further study of the body to learn more about its origins and about native American origins in general. The dispute ended only after 20 years. By the time DNA tests were allowed, the results established that the remains *was* indeed that of an ancestor to the tribe, and the *Spirit Cave mummy* was reburied with tribal rites at the beginning of 2018. To read more, see Resolving American Indian Ancestry - a 60-Year Old Controversy or The-worlds-oldest-natural-mummy-unlocks-secrets-ice-age-tribes-americas

120-2 Genomic Fossils-Molecular Evolution

1.7 Microscopy Reveals Life's Diversity of Structure and Form

Broadly speaking, there are two kinds of microscopy. In *Light Microscopy*, the specimen on the slide is viewed through optical glass lenses. In *Electron Microscopy*, the viewer is looking at an image on a screen created by electrons passing through or reflected from the specimen. For a sampling of light and electron micrographs, check out this Gallery of Micrographs. Here we compare and contrast different microscopic techniques…

1.7.1 Light Microscopy

Historically one form or other of light microscopy has revealed much of what we know of cellular diversity. Check out the Drawings of Mitosis for a reminder of how eukaryotic cells divide and then check out The Optical Microscope for descriptions of different variations of light microscopy (e.g., *bright-field, dark field*, *phase-contrast*, *fluorescence*, etc.). Limits of *magnification* and *resolution* of 1200X and 2 μm, (respectively) are common to all forms of light microscopy. The main variations of light microscopy are briefly described below.

- *Bright-Field microscopy* is the most common kind of light microscopy, in which the specimen is illuminated from below. Contrast between regions of the specimen comes from the difference between light absorbed by the sample and light passing through it. Live specimens lack contrast in conventional bright-field microscopy because differences in refractive index between components of the specimen (e.g., organelles and cytoplasm in cells) diffuse the resolution of the magnified image. This is why *Bright-Field microscopy* is best suited to fixed and stained specimens.

- In *Dark-field* illumination, light passing through the center of the specimen is blocked and the light passing through the periphery of the beam is diffracted ("scattered") by the sample. The result is enhanced contrast for certain kinds of specimens, including live, unfixed and unstained ones.

- In *Polarized light microscopy*, light is polarized before passing through the specimen, allowing the investigator to achieve the highest contrast by rotating the plane of polarized light passing through the sample. Samples can be unfixed, unstained or even live.

- *Phase-Contrast* or *Interference microscopy* enhances contrast between parts of a specimen with higher refractive indices (e.g., cell organelles) and lower refractive indices (e.g., cytoplasm). *Phase–Contrast* microscopy optics shift the phase of the light entering the specimen from below by a half a wavelength to capture small

- differences in refractive index and thereby increase contrast. Phase–Contrast microscopy is a most cost-effective tool for examining live, unfixed and unstained specimens.

- In a *fluorescence microscope*, short wavelength, high-energy (usually UV) light is passed through a specimen that has been treated with a fluorescing chemical covalently attached to other molecules (e.g., antibodies) that fluoresces when struck by the light source. This fluorescent *tag* was chosen to recognize and bind

specific molecules or structures in a cell. Thus, in *fluorescence microscopy*, the visible color of fluorescence marks the location of the target molecule/structure in the cell.

- *Confocal microscopy* is a variant of fluorescence microscopy that enables imaging through thick samples and sections. The result is often 3D-like, with much greater depth of focus than other light microscope methods. Click at Gallery of Confocal Microscopy Images to see a variety of confocal micrographs and related images; look mainly at the specimens.

- *Lattice Light-Sheet Microscopy* is a 100-year old variant of light microscopy that allows us to follow subcellular structures and macromolecules moving about in living cells. There has been renewed interest in this form of light microscopy. Read more about this technique at Lattice Light Sheet Microscopy**.**

1.7.2 Electron Microscopy

Unlike light (optical) microscopy, electron microscopy generates an image by passing electrons through, or reflecting electrons from a specimen, and capturing the electron image on a screen. Transmission Electron Microscopy (TEM) can achieve much higher magnification (up to 10^6X) and resolution (2.0 nm) than any form of optical microscopy! *Scanning Electron Microscopy* (SEM) can magnify up to 10^5X with a resolution of 3.0-20.0 nm. TEM, together with biochemical and molecular biological studies, continues to reveal how different cell components work with each other. The higher voltage in *High Voltage Electron microscopy* is an adaptation that allows TEM through thicker sections than regular (low voltage) TEM. The result is micrographs with greater resolution, depth and contrast. SEM allows us to examine the surfaces of tissues, small organisms like insects, and even of cells and organelles. Check this link to Scanning Electron Microscopy for a description of scanning EM, and look at the gallery of SEM images at the end of the entry.

121-2 Electron Microscopy

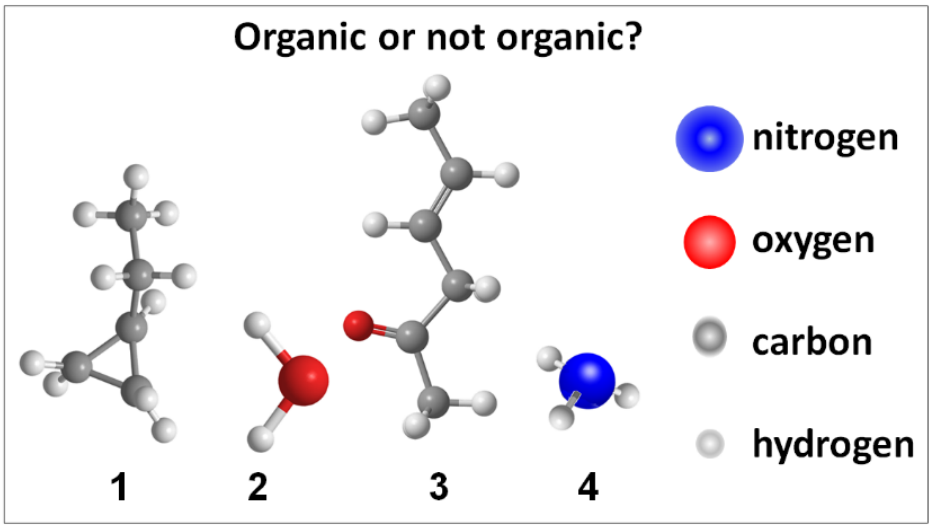
Some iText & VOP Key words and Terms

Actin	**Eukaryotes**	**Nuclear envelope**
Archaea	**Eukaryotic flagella**	**Nuclear pores**
Bacterial cell walls	**Evolution**	**Nucleoid**
Bacterial Flagella	**Exocytosis**	**nucleolus**

Binary fission	Extinction	Nucleus
Cell fractionation	Hypothesis	Optical microscopy
Cell theory	Inference	Plant cell walls
Chloroplasts	Intermediate filaments	Plasmid
chromatin	keratin	Progenote
Chromosomes	Kingdoms	Prokaryotes
Cilia	LUCA	Properties of life
Confocal microscopy	Lysosomes	Rough endoplasmic reticulum
Cytoplasm	Meiosis	Scanning electron microscopy
Cytoskeleton	Microbodies	Scientific method
Cytosol	Microfilaments	Secretion vesicles
Deduction	Microtubules	Smooth endoplasmic reticulum
Differential centrifugation	Mitochondria	Speciation
Diversity	Mitosis	Theory
Domains of life	Motor proteins	Tonoplast
Dynein	Mutation	Transmission electron microscopy
Endomembrane system	Natural selection	Tubulins

Chapter 2: Basic Chemistry, Organic Chemistry and Biochemistry

Atoms and Elements (protons, neutrons, electrons, atomic models); Chemical Bonds (covalent, polar covalent, ionic, H-bonds); Water properties, chemistry, pH; Organic molecules, monomers and polymers; biochemistry, chemical groups, condensation and hydrolysis, macromolecules (polysaccharides, polypeptides, proteins, DNA, RNA), lipids



Do you know? Did you guess correctly?

2.1 Introduction

In this chapter, we start with a review *basic chemistry* from ***atomic structure*** to ***molecular bonds*** to the structure and properties of ***water***, followed by a review of key principles of *organic chemistry* - the chemistry of carbon-based molecules. You may find it useful to have your old general chemistry textbook handy or check out the excellent introduction to general chemistry by Linus Pauling (1988, *General Chemistry* New York, Springer-Verlag). We'll see how the ***polar covalent bonds*** define the ***structure*** and explain virtually all of the ***properties of water***. These range from the energy required to melt a gram of ice to vaporize a gram of water to its surface tension to its ability to hold heat…, not to mention its ability to dissolve a wide variety of ***solutes*** from salts to proteins and other macromolecules. We will distinguish water's ***hydrophilic*** interactions

with solutes from its ***hydrophobic*** interactions with fatty molecules. Then we review some basic biochemistry. Well-known biological molecules include monomers (sugars, amino acids, nucleotides, lipids…) and polymers (polysaccharides, proteins, nucleic acids, fats…).

Biochemical reactions that link *glucose* monomers into polymers on the one hand, and break the polymers down on the other, are essential reactions for life… on earth and probably anywhere else! On our planet, photosynthetic organisms link glucose monomers into starch, a polysaccharide. Amylose is a simple starch, a large **homopolymer** of repeating glucose monomers. Likewise, polypeptides are **heteropolymers** of 20 different *amino acids*. DNA and RNA nucleic acids are also heteropolymers, made using only four different *nucleotides*.

Digestive enzymes in your gut catalyze the ***hydrolysis***, or breakdown of the plant or animal polymers we ate back down to monomers. Hydrolysis adds a water molecule across the bonds linking the monomers in the polymer, breaking the linkages. Our cells then take up the monomers. Once in our cells, ***condensation*** (***dehydration synthesis***) ***reactions*** remove water molecules from participating monomers to grow new polymers that are more useful to *us*. Strictly speaking, ***triglycerides*** (fats) and ***phospholipids*** are not macromolecules, but they are also broken down by hydrolysis and synthesized in condensation reactions. Triglycerides are energy-rich molecules, while phospholipids (chemical relatives of triglycerides) are the basis of cellular membrane structure. Weak interactions between macromolecules e.g., ***hydrogen bonds*** (H-bonds), **electrostatic interactions**, ***Van der Waals forces***, etc., hold many cellular structures and molecules together. While individually these bonds are weak, millions of them keep the two complementary DNA strands tightly in a stable double helix, and thousands of them can hold a polypeptide into its folded three-dimensional shape. We will see this theme of strength in numbers repeated in other molecular and cellular structures. Monomers also serve other purposes related to energy metabolism, cell signaling etc. Depending on your chemistry background, you may find "Googling" these subjects interesting and useful. The short VOPs in this chapter might also help as a guide to understanding the basic chemistry and biochemistry presented here.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast the definitions of *atom*, *element* and *molecule.*
2. list differences between atoms, elements and molecules and between *energy* and *position*-based atomic models.
3. describe sub-atomic particle behavior when they absorb and release energy.
4. state the difference between atomic *shells* and *orbitals.*

5. state how *kinetic* and *potential* energy apply to atoms and molecules.
6. explain the behavior of atoms or molecules that fluoresce when excited by high-energy radiation…, and those that do not.
7. distinguish *polar* and *non-polar* covalent bonds and their physical-chemical properties.
8. predict the behavior of electrons in compounds held together by *ionic interactions.*
9. explain how the *properties of water* account for the solubility of macromolecules and salts and the role of H-bonds in supporting those properties.
10. consider why some salts are not soluble in water in terms of water's properties.
11. describe how molecular linkages form during polymer metabolism and place hydrolytic and dehydration synthetic reactions in a *metabolic context.*
12. distinguish between chemical "bonds" and "linkages" in polymers.
13. categorize different chemical bonds based on their strengths.

2.2 Atoms and Basic Chemistry

The difference between ***elements*** and ***atoms*** is often confused in casual conversation. Both terms describe ***matter***, substances with ***mass***. Different elements are different kinds of matter distinguished by different physical and chemical properties. In turn, the atom is the fundamental unit of matter…, that is, of an element. The number of *positively charged* ***protons*** and *neutral* ***neutrons*** in an atomic nucleus account for most of the mass of an atom. Each negatively charged ***electron*** that orbits a nucleus is about 1/2000th of the mass of a proton or neutron. Thus, they do not add much to the mass of an atom. Electrons stay in atomic orbits because of electromagnetic forces, i.e., their attraction to the positively charged nuclei. Nuclear size (mass) and the cloud of electrons around its nucleus define structure of an atom. And that structure dictates the different properties of the elements. Let's take a closer look at the elements, their atoms and how their atomic properties account for the formation of molecules and molecular structure.

2.2.1 Overview of Elements and Atoms

Recall that atoms are chemically most stable when they are electrically uncharged, with an equal number of protons and electrons. ***Isotopes*** of the same element are atoms with the same number of protons and electrons, but a different number of neutrons. Therefore, isotopes are also chemically stable, but they may not be physically stable. For example, the most abundant isotope of hydrogen contains one proton, one electron and *no neutrons*. The nucleus of the ***deuterium*** isotope of hydrogen contains one neutron and that of ***tritium*** contains two neutrons. Both

isotopes can be found in water molecules. Deuterium is stable. In contrast, the tritium atom is radioactive, subject to nuclear decay over time. Whether physically stable or not, all isotopes of an element share the same chemical and electromagnetic properties and behave the same way in chemical reactions. The electromagnetic forces that keep electrons orbiting their nuclei allow the formation of chemical bonds in molecules. We model atoms to illustrate the average physical location of electrons (the ***orbital model***) on one hand, and their potential energy levels (the ***Bohr***, or ***shell model***) on the other. Look at these atomic models for the element helium illustrated in Fig. 2.1, below.

Up to two electrons move in a space defined as an ***orbital***. In addition to occupying different areas around the nucleus, electrons exist at different ***energy levels***, moving with different ***kinetic energy***. Electrons can also absorb or lose energy, jumping or falling from one energy level to another.

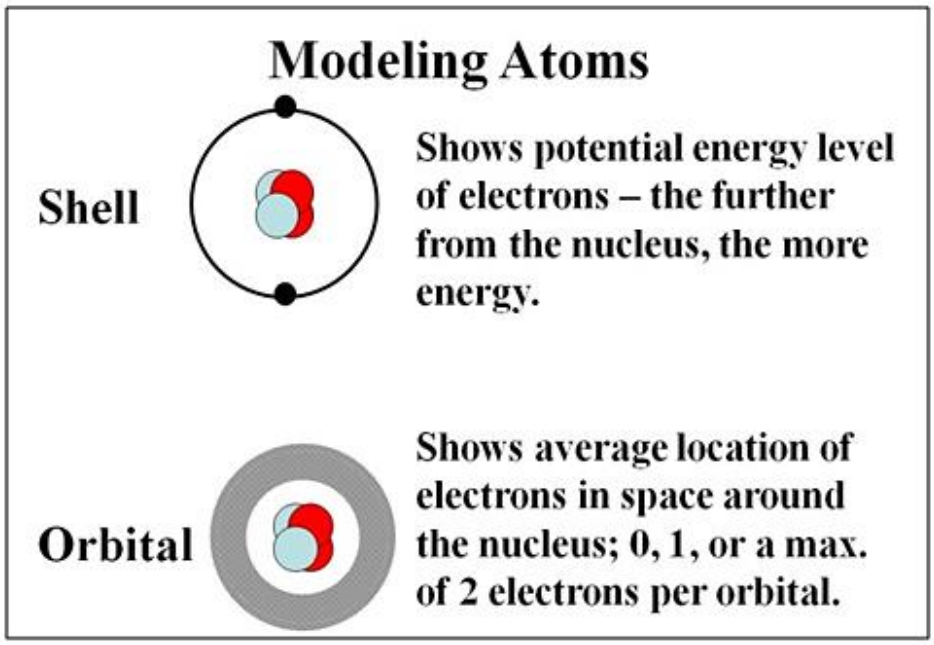


Fig. 2.1: The shell (Bohr) and orbital models of atoms respectively emphasize the energy of electrons and space occupied by the electrons moving around the atomic nucleus.

A unique ***atomic number*** (number of protons) and ***atomic mass*** (usually measured in ***Daltons***, or *Da*) characterize different elements. For example, the atomic number of carbon (C) is 6, the number of protons in its nucleus. Its mass is 12 Da (that of 6 protons plus 6 neutrons, at 1 Da each). Remember that the mass of all the electrons in a C atom is negligible! Find the C atom and look at some of the other atoms of elements in the partial periodic table below (Fig. 2.2, below). Superscripted atomic numbers and subscripted atomic mass numbers uniquely define each element. This partial periodic table shows the elements essential for all life in greater or lesser amounts, as well as some that may also be essential in humans.

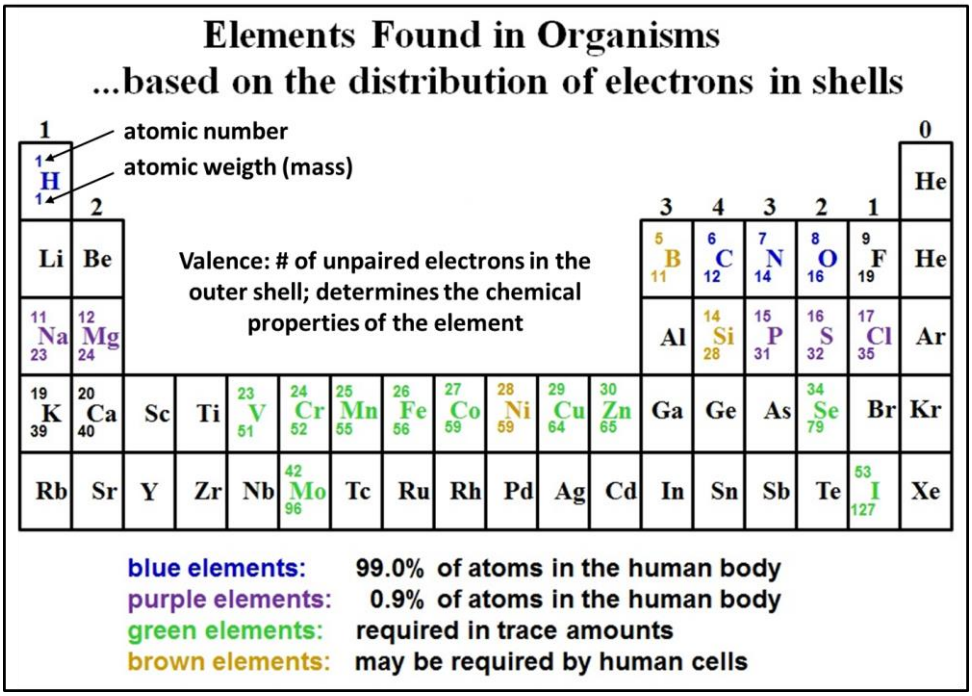


Fig. 2.2: The *Periodic Table of the Elements*, emphasizing those found in living things (blue, purple, green or brown).

122-2 Atoms & Elements

2.2.2 Electron Configuration – Shells and Subshells

The *Bohr* model of the atom reveals how electrons can absorb and release energy. The shells indicate the energy levels of electrons. Electrons can absorb different kinds of energy including electrical energy, radiation, and light (actually just a form of radiation – weaker than some and stronger than others).

Beaming ultraviolet (UV) light at atoms can excite electrons. If an electron in an atom absorbs a full ***quantum*** (a ***photon***) of UV radiant energy, it will be boosted from the ***ground state*** (the shell it normally occupies) into a higher shell, an ***excited state***. Excited electrons move at greater speed around the nucleus, with more ***kinetic energy*** than it did at *ground*. Excited electrons also have more ***potential energy*** than ground state electrons. This is because they are unstable, releasing some of the energy gained during excitation as they return to *ground*, i.e., their starting energy level, or shell (Fig. 2.3.)

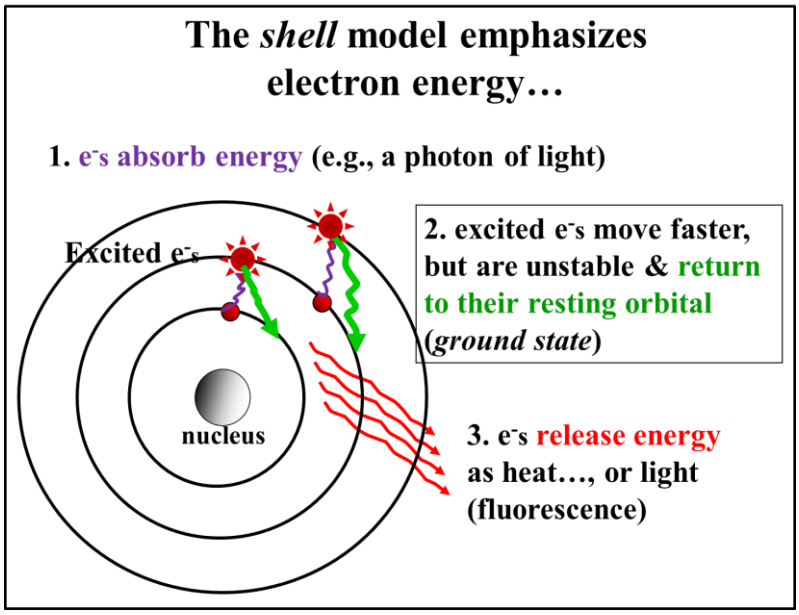


Fig. 2.3: *Excited* electrons gain their energy by absorbing light. Absorbance of one *photon* causes an electron to 'jump' to the next shell further from the atomic nucleus. Excited electrons have *potential energy* that is released as the electron returns to *ground state*, releasing heat or light (*fluorescence*).

Electrons falling back to ground typically release excitation energy as heat. Atoms whose excited electrons release their energy as light ***fluoresce***; they are ***fluorescent***. A fluorescent light is an example of this phenomenon. Electrical energy excites electrons out of atomic orbitals in the molecules coating the interior surface of the bulb. As all those excited electrons return to ground state, they *fluoresce*, releasing light. These atoms can be repeatedly excited by electricity. As we shall see, biologists and chemists have turned fluorescence into tools of biochemistry, molecular biology and microscopy. The ground state is also called the ***resting state***, but electrons at ground are by no means resting! They just move with less kinetic energy excited electrons.

123 Electron Energy & Fluorescence

2.3 Chemical bonds

Atoms form bonds to make molecules, and there are three main classes of chemical bonds. There are two kinds of *covalent bonds*; both are *strong* bonds. They involve *unequal or equal* sharing of a pair of electrons. Unequal sharing of electrons results in ***polar covalent bonds***. Equal sharing forms ***non-polar covalent bonds***. ***Ionic bonds*** are created by electrostatic interactions between elements that gain or lose electrons. Individually, ionic bonds are weaker than covalent bonds. ***Hydrogen bonds* (*H-bonds*)** are in a class by themselves! These electrostatic interactions account for the physical and chemical properties of water. They are also involved in interactions between and within other molecules. Note that while atoms can share, gain or lose electrons in chemical reactions, they will neither gain nor lose protons or neutrons. Let's look more closely at chemical bonds and how even the "weak" bonds are essential to life.

2.3.1 Covalent Bonds

Electrons are shared in covalent bonds. Hydrogen gas (H_2) is a molecule, not an atom! The two H atoms in the H_2 molecule share their electrons equally. Likewise, the carbon atom in methane (CH_4) shares electrons equally with four hydrogen atoms. A single pair of electrons in H_2 forms the covalent bond between two H atoms in the hydrogen molecule. In methane, the carbon (C) atom has four electrons in its outer shell that it can share. Each H atom has a single electron to share. If the C atom shares each of its four electrons with the electron in each of the four H atoms, there will be four paired electrons (8 electrons in all) moving in filled orbitals around the nucleus of the C atom some of the time, and one pair moving around each of the H atomic nuclei some of the time. Thus, the outer shell of the C atom and each of the H atoms are filled at least some of the time. This stabilizes the molecule. Remember

that atoms are most stable when their outer shells are filled and when each electron orbital is filled (i.e., with a pair of electrons). The equal sharing of electrons in ***non-polar covalent bonds*** in H_2 and CH_4 is shown in Fig. 2.4.

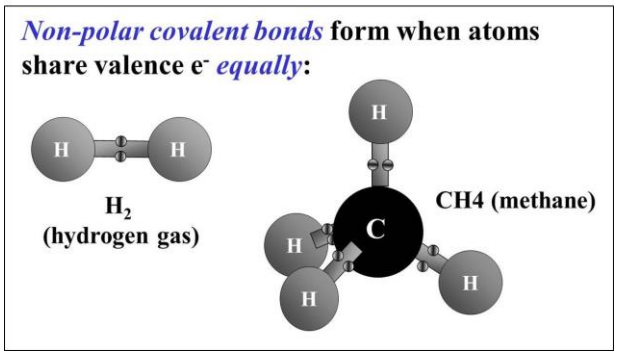


Fig. 2.4: Non-polar covalent bonds *vs.* polar covalent bonds.

Polar covalent bonds form when electrons in a molecule are shared unequally. This happens if the atomic nuclei in a molecule are very different in size, as is the case with water (Fig. 2.5).

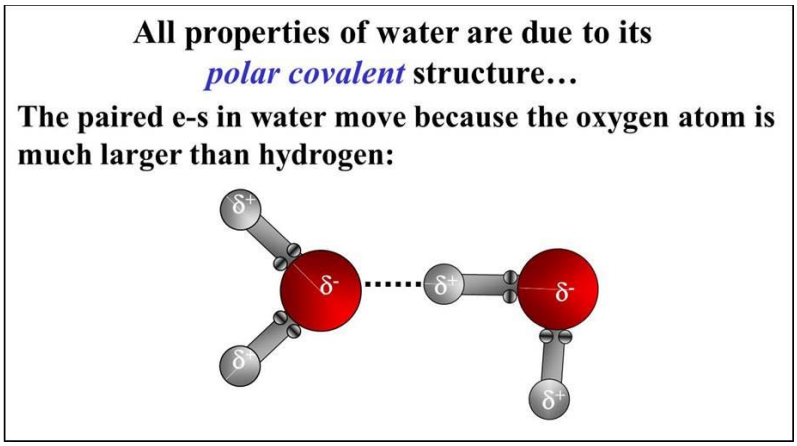


Fig. 2.5: Electrons on the H atoms of water molecules are drawn close to the large, positively charged nucleus of the O atom. Thus, the H atoms 'lose' electrons and acquire a partial positive charge, while the oxygen atoms 'gain' those electrons and have a partial negative charge. The resulting polar covalent water molecules attract other water molecules.

The larger nucleus of the oxygen atom in H_2O attracts electrons more strongly than does either of the two H atoms. As a result, the shared electrons spend more of their time orbiting the O atom, such that the O atom carries a *partial negative charge* while each of the H atoms carry a *partial positive charge*. The Greek letter delta (δ) indicates partial charges in polar covalent bonds. In the two illustrations above, compare the position of the paired electrons in water with those illustrated for hydrogen gas or methane. Water's polar covalent bonds allow it to attract and interact with other polar covalent molecules, including other water molecules. The polar covalent nature of water also goes a long way to explaining its physical and chemical properties, and why water is essential to life on this planet!

124 Covalent Bonds

Both polar and non-polar covalent bonds play major roles in the structure of macromolecules, as in the protein hormone *insulin* (Fig.2.6, below).

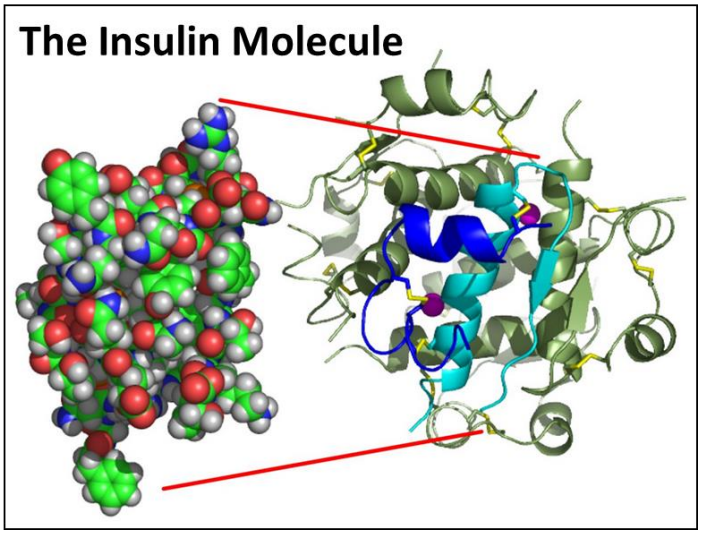


Fig. 2.6: Computer-generated space-filling (LEFT) and 'ribbon' models (RIGHT) of insulin structure.

The X-ray image of a space-filling model of the *hexameric* form of stored insulin (above left) emphasizes its tertiary structure in great detail. The ribbon diagram (above right) highlights regions of internal secondary structure. When secreted from

Islets of Langerhans cells of the *pancreas*, active insulin is a dimer of A and B polypeptides (blue and cyan in the ribbon diagram, respectively). The subunit structure and the interactions holding the subunits together result from many electrostatic interactions (including H-bonds) and other weak interactions. The disulfide bonds (bridges) seen as yellow 'Vs' in the ribbon diagram stabilize the associated A and B monomers. We will look at protein structure in more detail in an upcoming chapter.

2.3.2 Ionic Bonds

Atoms that gain or lose electrons to achieve a filled outer shell acquire a negative or a positive charge (respectively) to form **ions**. Despite being electrically charged, ions are stable because their outer electron shells are filled. Common table salt (Fig. 2.7, below) is a good example.

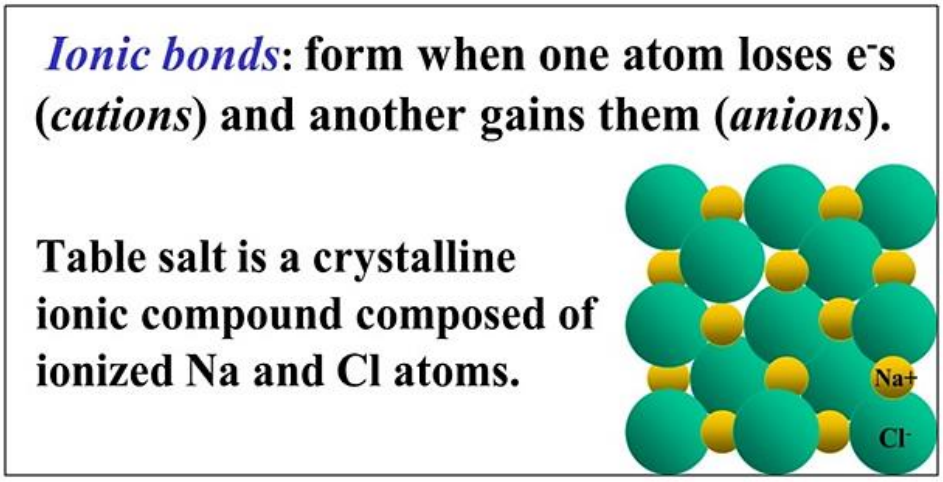


Fig. 2.7: Ionic bonds in table salt (NaCl) crystals; Opposite charges on Na^+ and Cl^- hold the ions together in a regular crystalline array.

Na (sodium) atoms can donate a single electron to Cl (chlorine) atoms, generating Na^+ (sodium) and Cl^- (chloride) ions. The oppositely charged ions then come together forming an ***ionic bond***, an *electrostatic interaction* of opposite charges that holds the Na^+ and Cl^- ions together in crystal salt. Look up the Bohr models of these two elements and see how ionization of each leaves filled outer shells (energy levels) in the ions.

2.3.3 Hydrogen Bonds

The hydrogen bond is a subcategory of electrostatic interaction formed by the attraction of oppositely charges. As noted above, water molecules attract one another (***cohere***) because of strong electrostatic interactions that form the H-bonds. Water's polar covalent structure enables it to attract positively and negatively charged groups of molecules, making it a good solvent. Solutes (water-soluble molecules) or poplar (charged) molecular surfaces that attracted to water are ***hydrophilic***. Lipids like fats and oils are not polar molecules and therefore do not dissolve in water; they are ***hydrophobic*** (from *hydro, water; phobic, fearing)*. Next, we'll take a closer look at the chemistry and properties of water.

2.4 Water Chemistry

Soluble salts like NaCl dissolve because the Cl^- and Na^+ ions attract the partial positive and negative charges (respectively) of water molecules more strongly than other water molecules. As a result, the salt ionizes; the ions separate from each other. The ionization of NaCl dissolving in water is shown below (Fig. 2.8).

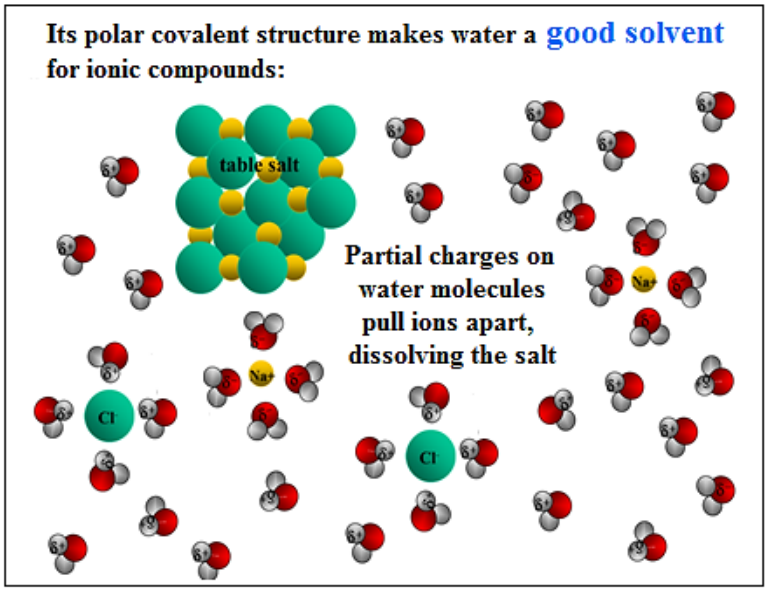


Fig. 2.8: Table salt (NaCl) dissolving in water. Water's solvent properties result from its polar covalent structure, allowing interactions with Na^+ and Cl^- in the crystal.

Water is also a good solvent for macromolecules (proteins, nucleic acids) with exposed polar chemical groups on their surface that attract water molecules (Fig. 2.9).

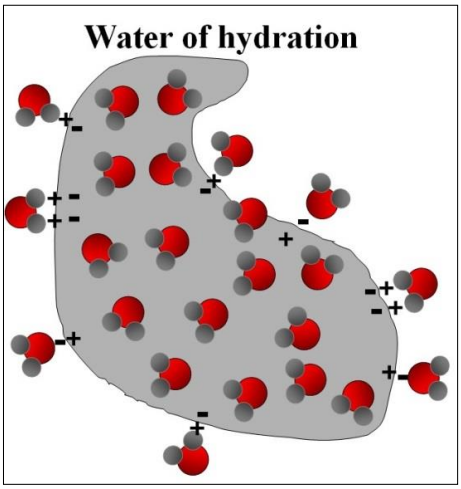


Fig. 2.9: Charged groups on the macromolecule (e.g., protein) attract the partial charges on water molecules, hydrating the molecule.

125-2 Water-Hydrogen & Ionic Bonds

In addition to its being a good solvent, we recognize the following properties of water (all of which result from its polar nature and H-bonding abilities):

- ***Cohesion***: the ability of water molecules to stick together via H-bonds.
- ***High Surface tension***: water's high cohesion means that it can be hard to break the surface; think the water strider, an insect that 'walks on water'.
- ***Adhesion***: this results from water's electrostatic interactions with ions and the partial charges on polar covalent molecules or functional groups. Adhesion explains water's solvent properties and (at least in part) capillary action where water molecules 'crawl' along hydrophilic surfaces, often against the force of gravity.
- ***High specific heat***: The cohesion of water molecules is so strong that it takes a lot of energy to separate the molecules and make them move faster. In other words, it takes a lot of energy to heat water. Specifically, it takes 1 *Kcal*, (1 *Calorie*, with a capital C) to heat a gram of water 1^{o}C. Incidentally, high specific heat also explains why water "holds its heat", staying hotter longer that the pot it's in!).

- ***High heat of vaporization***: It takes even more energy per gram of water to turn it into water vapor!

One last property of water: it ionizes weakly to form H^+ and OH^- ions… or more correctly, H_3O^+ and OH^- ions. You can think of this as happening in the following two reactions:

1. $H_2O + H_2O \rightleftharpoons 2H^+ + 2OH^-$
2. $2H^+ + 2OH^- \rightleftharpoons H_3O^+ + OH^-$

Acid molecules added to water will dissociate and release protons. This drives reaction 2 to form more H_3O^+ ions in the solution, in turn driving reaction 1 forward. A pH meter measures the relative acidity or concentration of protons in a solution. Acidic solutions have a pH below 7.0 (*neutrality*). Bases ionizing in water release hydroxyl (OH^-) ions. The increase in OH^- ions removes protons from the solution, driving both reactions in reverse and raising the pH of the solution. To summarize acid-base chemistry, when dissolved in water, acids release H^+ while bases accept H^+. Since the pH of a solution is the negative logarithm of the hydrogen ion concentration, a solution

- at pH 7.0 is neutral.
- below a pH of 7.0 is acidic.
- above a pH of 7.0 is basic.

Check a basic chemistry textbook to be reminded of the relationship between pH and the $[H^+]$ in a solution!

2.5 Some Basic Biochemistry: Carbon, Monomers, Polymers and the Synthesis and Degradation of Macromolecules

Like evolution, the origin of life involved some prebiotic 'natural selection' of chemicals in the environment. As with evolution, this *chemical selection* would favor expanding increasing biochemical possibilities and diversity. In simple terms, atoms that could interact with a maximal number of other atoms to form the largest number of stable molecules would have been most likely to accumulate in the environment. The tetravalent C atom met these criteria for chemical selection, proving ideal for building an organic chemistry set.

At the same time, water turned out to be the perfect place to launch prebiotic *chemical selection* experiments. Water persists as the life's universal solvent, which explains why evidence of water in places beyond our earth (other planets in our solar system, the moons of other planets, other planets ion other solar systems) gets us all excited!

2.5.1 Isomerism in Organic Molecules and the Diversity of Shape

The ***carbon skeleton*** is a perfect platform of organic molecule diversity. The differences in arrangement of atoms and functional chemical groups around C atom result in ***isomerism***. ***Isomers*** of an organic molecule have the same chemical formula but different shapes (and so, potentially different chemical properties and biochemical function). The larger the carbon skeleton of an organic molecule, the greater the diversity of molecular shapes available for chemical selection. Look at the examples of ***structural isomers*** and ***geometric isomers*** in Fig. 2.10.

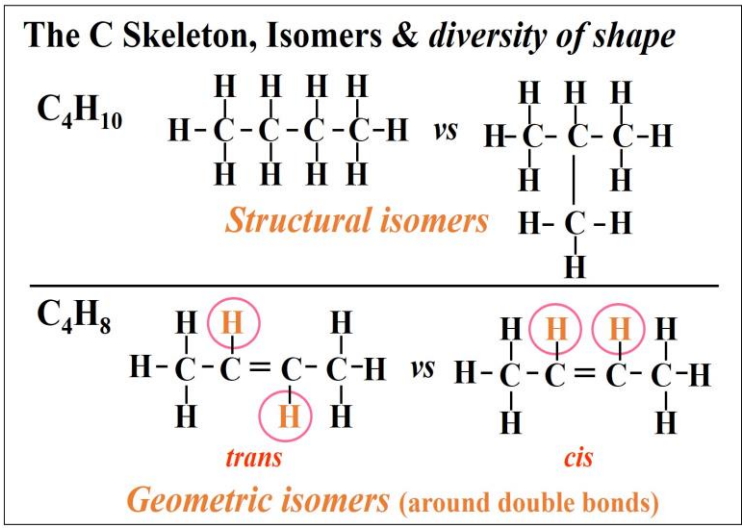


Fig. 2.10: Structural and geometric *isomers* of hydrocarbons create molecules with the same chemical formula but different shapes. Illustration by G. Bergtrom

It is easy to see that the structural isomers of C_4H_{10} (Fig. 2.10, left and right) have different shapes. You cannot convert one structural isomer to the other without breaking covalent bonds. In the geometric isomers of C_4H_8 in the lower panel, the H atoms on the double-bonded C atoms can be on the same (*cis*) or opposite (*trans*) side of the *planar* double bond. Geometric isomers too, cannot be interconverted without breaking chemical bonds. ***Optical isomers*** are yet a third kind of isomer. They exist around ***optically active*** (***asymmetric*** or ***chiral***) carbons. A chiral C is one that is covalently linked to 4 different atoms and/or molecular groups. The principle of chirality is illustrated below in Fig. 2.11.

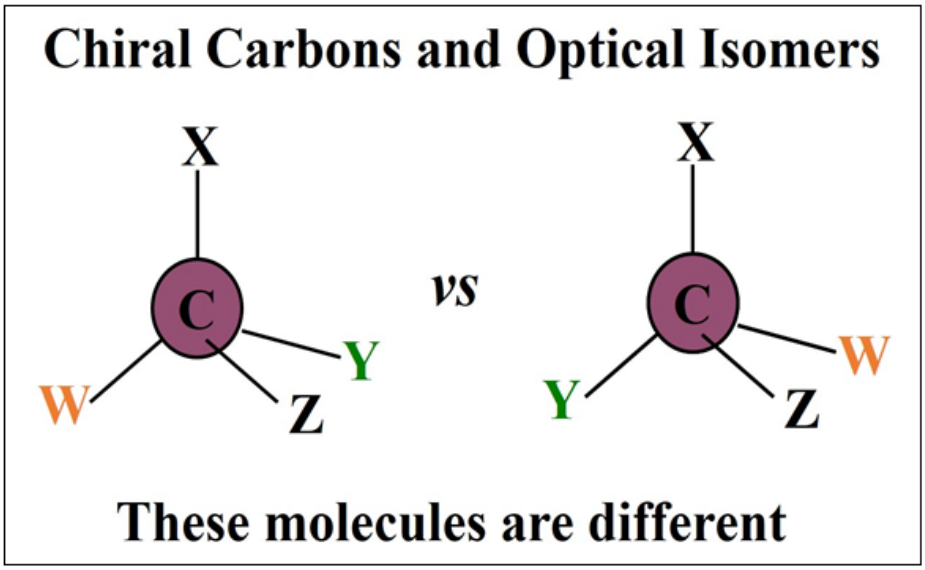


Fig. 2.11: The two different generic molecules (*enantiomers*) are mirror images of one another. They form from alternate arrangements of the same molecular groups around a *chiral* carbon.

Optical isomers (also called ***enantiomers***) also differ in *shape*, and just like structural and geometric isomers, they can't be converted from one to the other without breaking and re-making covalent bonds. Enantiomers are defined as *optically active* because they bend, or rotate light in opposite directions in a *polarimeter*. Light passing through a solution of one optical isomer is rotated in one direction while light passing through the other isomer is rotated in the opposite direction. These directions are referred to as ***l,*** or *levo* (meaning left) and ***d*** or *dextro* (meaning right). If a molecule has more than one chiral C (glucose for example has four chiral carbons), its behavior in a polarimeter will be based on the sum of optical activities of all the chiral carbons. The common isomer of glucose in our diet is *d-glucose*.

Glucose enantiomers are also referred to as ***D*** and ***L*** respectively, a convention based on the configuration of the four different atoms or groups around the *last optically active carbon* in a molecule (^{5}**C** in glucose). For glucose, *d* and *l* in fact correspond to *D* and *L* respectively. As we will see for some molecules, the upper-case designation of a chiral molecule does not always indicate how it bends light in a polarimeter, while the lower-case *d* and *l* always do! The d- and l- isomers of glucose are illustrated in Fig. 2.12 (below), with chiral carbons in red.

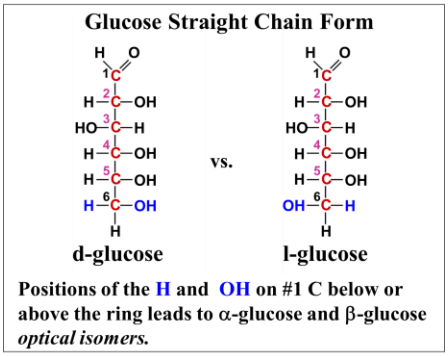


Fig. 2.12: Two straight chain forms of glucose (d-glucose and l-glucose) are enantiomers (optical isomers), differing in the arrangement of the H atom and the OH group around C5.

Remember that the shape and chemical properties of a molecule dictate its function. Isomerism in organic (carbon-based) molecules would have increased the diversity of molecular shapes available for chemical selection. Early selection of isomers (specific optical isomers in particular) during chemical evolution contributed greatly to chemical functions and reactions we recognize in cells, even before there was life on earth. That all life uses the same isomers of glucose in energy reactions and of amino acids to build proteins confirms the prebiotic selection of those isomers!

2.5.2 Monomers to Polymers and Back: Dehydration Synthesis and Hydrolysis

All living things build and break down polymers (macromolecules) by ***dehydration synthesis*** (***condensation*** reactions) and ***hydrolysis***, respectively. *Dehydration synthesis* and *hydrolysis* reactions are essentially the reverse of each other (Fig. 2.13 below).

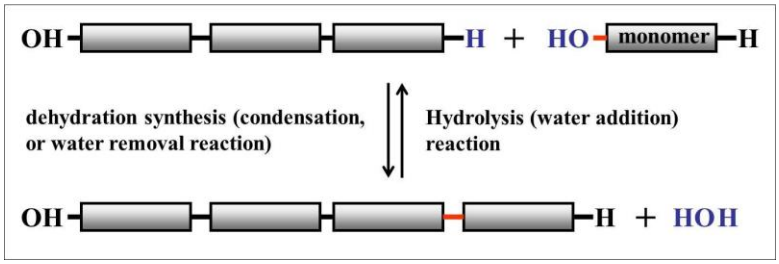


Fig. 2.13: A generic monomer is linked to a growing polymer by water removal (*dehydration synthesis*); Water addition across the linkage between monomers (*hydrolysis*) breaks the polymer

Condensation reactions build macromolecules by removing a water molecule from interacting monomers. The 'bond' that forms in a condensation reaction *is not a single bond*, but a ***linkage*** involving several bonds! The linkages form when an OH from one monomer and an H group from the other are removed and combine to form a water molecule.

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Repeated condensation reactions such as one between two amino acids (Fig. 2.14) form the ***peptide linkages*** that build polypeptides during translation.

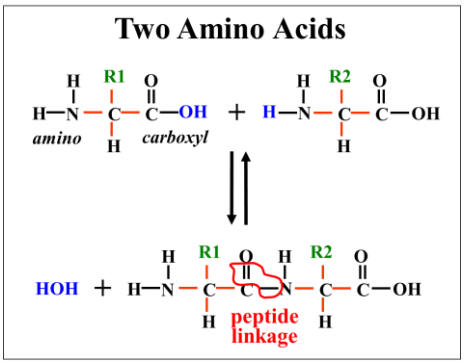


Fig. 2.14: *Dehydration synthesis* forms peptide linkage (circled); *hydrolysis* is the reverse reaction.

Cells perform repeated condensation reactions to build diverse polymers, including polysaccharides and polynucleotides (the RNA and DNA nucleic acids). Consider the polymerization of glucose monomers into storage or structural polysaccharides for example. Cells use only ***(d)glucose*** to make polysaccharides. Here's how this works:

- Straight-chain (***d)glucose*** with four chiral carbons becomes cyclic when dissolved in water where the cyclic molecule acquires a fifth chiral carbon, namely, the #1 carbon.).
- Since the #1 carbon is now optically active, the ***(d)glucose*** can form a new pair of enantiomers in solution, called ***α(d)glucose*** and ***β(d)glucose***.
- Having selected *d-glucose* for most cellular energy metabolism, life then exploited the additional chiral carbon in cyclic *d-glucose* to make the different polysaccharide polymers we now find in plants and animals.

In solution, 90% or glucose monomers are in the ring form, as show in Fig. 2.15 below.

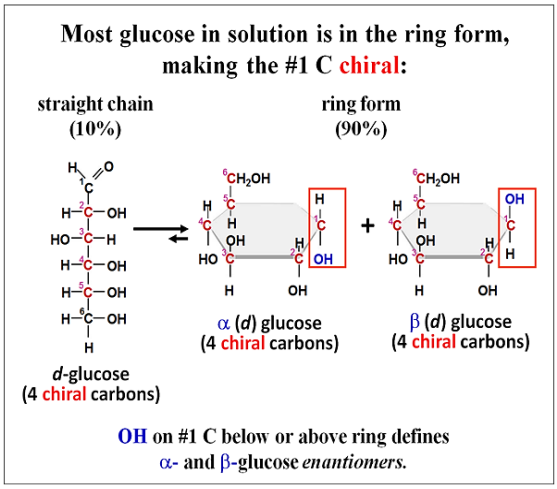


Fig. 2.15: When straight chain glucose forms a cyclic molecule in solution in water, C_1 becomes optically active (chiral), creating a racemic mixture of α(d)glucose and β(d)glucose enantiomers.

The condensation reactions shown below link glucose monomers, forming storage and structural polysaccharides (Fig. 2.16).

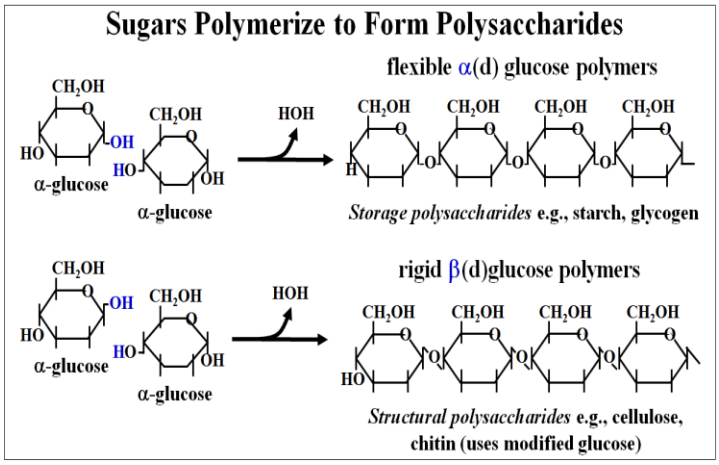


Fig. 2.16: Polysaccharide sugar polymers form by dehydration synthesis: α(d)glucose monomers polymerize to form energy-storage molecules (e.g., starch, glycogen), while β-d-glucose monomers polymerize to form structural polysaccharides (starch, cellulose).

The -OH (hydroxyl) groups on the #1 C of ***α(d)glucose*** are *below* the glucose rings. The condensation reaction removes a water molecule, linking the sugars by an ***α1,4 glycoside linkage*** in the dimer, connecting them by their # 1 and # 4 carbons. Other *linkages* are possible. For example, diverse *α-glycoside* linkages characterize *branched* ***storage polysaccharides*** like *glycogen* in animals and the *starches* in plants. On the other hand, when ***β(d)glucose*** enantiomers polymerize, they form rigid ***structural polysaccharides*** such as those of cellulose in plant cell walls. A modified β-glucose called *N-acetyl glucosamine* (not shown) polymerizes to form *chitin*, the principal component of fungal cell walls and of the tough exoskeleton of arthropods (insects, crustaceans). In another chapter, we'll revisit the linkage of amino acids during *translation* to build a polypeptide; only ***L*** amino acids are used to make proteins! We'll also look at the details of *replication* and *transcription* that cells use to catalyze condensation reactions that synthesize DNA and RNA from nucleotide monomers. To summarize:

- Linkages in the biopolymers are broken down and re-formed daily in our lives! Digestion (the breakdown) begins after a meal. The hydrolysis of glycoside linkages begins in your mouth. Further polysaccharide digestion and the breakdown of peptide (among other) linkages continues in your stomach and small intestines. Then our cells use condensation reactions to complete the job of turning carrot- and cow-derived monomers into you or me!
- Prebiotic ***chemical evolution*** has selected only one of the optical isomers (enantiomers) of glucose, amino acid and other monomers with which to build polymers. This is so even though some of the different isomers are available and even used by cells for different purposes. Flexible ***α(d)glucose*** polysaccharides were selected as storage to be used for energy. Storage polysaccharides include plant *starches* and animal *glycogen*. Likewise, the rigid inflexibility of ***β(d)glucose*** polymers was selected precisely because it reinforced cell structure and stability. Since all organisms store carbohydrate energy in ***α(d)glucose*** polymers and since ***β(d)glucose*** polymers are almost universally used to strengthen cell structure, these selections must have occurred early in the history of life.

127-2 Carbohydrates-Sugars & Polysaccharides

128-2 Lipids-Triglycerides-Phospholipids

129-2 Proteins-Amino Acids & Polypeptides

130-2 DNA & RNA-Nucleotides & Nucleic Acids

2.5.3 A Tale of Chirality Gone Awry

To conclude this chapter and to emphasize the significance of chirality to life, here is what can happen if the wrong isomer ends up in the wrong place at the wrong time…

Consider the story of *Thalidomide*, a tragic example of what happens when we are unaware of enantiomeric possibilities. Introduced in 1957, Thalidomide was sold as an over-the-counter anti-nausea drug for patients undergoing cancer therapies and as a very effective morning sickness remedy for pregnant women. However, by the early 1960s, the birth of about 10,000 infants with severely deformed limbs was connected to the drug. These deformities characterized the roughly half of these infants that survived. Once the connection was made, the response was of course, to pull Thalidomide off the market.

Thalidomide is a *teratogen*. Teratogens are substances or conditions (drugs, chemicals, radiation, illness during pregnancy, etc.) that cause deformities during embryogenesis and fetal development. The chemical bases of Thalidomide's effects are based on its *enantiomeric* (*chiral*) structure in which an amine-containing ring can exist in front of, or behind the rest of the molecule. The structure of Thalidomide is shown in Fig. 2.17 below.

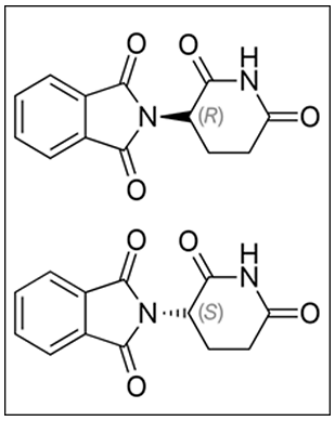


Fig. 2.17: Two enantiomers (optical isomers) of Thalidomide (**R** and **S**) form in water.

The two enantiomers are referred to as 'S' and 'R'. Of these, the S isomer is the teratogen. While synthesis of pure R is possible, when used in treatment, R and S easily interconvert, creating a ***racemic mixture***. In the mother, S is transferred to the embryo or fetus, with its terrible consequences.

Remarkably, there were relatively few cases of Thalidomide-induced birth deformities in the United States, largely because of the efforts of Frances Oldham Kelsey, the person in charge of FDA review of the drug. The German pharmaceutical company *Chemie Grünenthal* (developer Thalidomide) and an American pharmaceutical company had applied for FDA approval for U.S. distribution of the drug. But Dr. Oldham Kelsey refused approval on multiple occasions, arguing that the safety of Thalidomide had not been demonstrated. This was even before it was shown to cause birth deformities! In 1962 President Kennedy presented Dr. Oldham Kelsey the *President's Award for Distinguished Federal Civilian Service* for not allowing thalidomide to be approved for sale in the US without sufficient safety testing…, potentially saving thousands of lives.

Of course, we already knew that cells synthesized polymers from specific optical isomers of their precursor monomers. So the sad Thalidomide story resulted from the untested effects of an unexpected optical isomer. Many countries quickly tightened their pre-approval drug testing regulations because of this tragedy.

In a more hopeful twist of the tale, Thalidomide has turned out to be effective in treating cancer, leprosy, rheumatoid arthritis and other autoimmune diseases. Such therapeutic benefits may be due to its *anti-inflammatory effects*. The effects of thalidomide on tumor growth seem to be due to its inhibition of *angiogenesis* (development of blood vessels) in the tumors. Ironically, blockade of angiogenesis might also have contributed to the failure of proper limb growth during pregnancy.

To conclude, when all is normal, the shapes of molecules have been uniquely selected for the specificity of reactions essential to life.

131-2 Shape & the Specificity of Molecular interactions

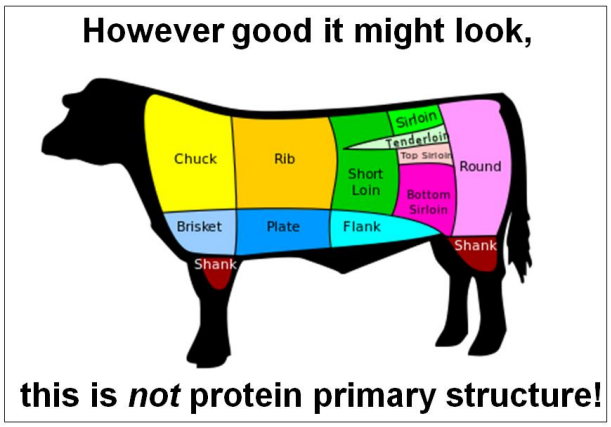
Some iText & VOP Key Words and Terms

acids and bases	**geometric isomers**	**polar covalent bonds**
adhesion	**glycogen**	**polymers**
α-glucose	**glycoside linkage**	**polynucleotides**
amino acids	**heat of vaporization**	**polypeptides**
angiogenesis	**hydrogen bonds**	**polysaccharides**
atom	**hydrolysis**	**potential energy**
atomic mass	**hydrophilic**	**properties of water**

β-glucose	hydrophobic	protons
Bohr model	ionic bonds	quantum
carbohydrates	ionization	racemic mixture
cellulose	isomers	RNA
chirality	isotopes	salts
chiral carbon	kinetic energy	scanning tunneling microscope
chitin	lipids	sharing electrons
cohesion	macromolecules	solutes
condensation reaction	molecule	specific heat
dehydration synthesis	monomers	starches
digestion	neutrons	structural isomers
DNA	nucleotides	surface tension
electron shell	optical isomers	teratogen(ic)
electrons	orbitals	Thalidomide
electrostatic interaction	partial charge	triglycerides
element	peptide linkage	valence
enantiomers	pH	Van der Waals forces
ester linkage	phosphate ester linkage	water ions
excitation	phosphodiester linkage	water of hydration
fats	phospholipids	
fluorescence	photon	

Chapter 3: Details of Protein Structure

Protein Structure and Configuration: Primary, Secondary, Tertiary, Quaternary; Protein Folding, Domains and Motifs, Studying Proteins



3.1 Introduction

The protein is the workhorse of cells, responsible for just about all aspects of life. Comprised of one or more *polypeptides*, they:

- are the ***catalysts*** that make biochemical reactions possible.
- are ***membrane components*** that selectively let substances in and out of the cell.
- allow ***cell-cell communication*** and cell's ***response to environmental change***.
- form the internal structure of cells (***cytoskeleton***) and nuclei (***nucleoskeleton***).
- enable the ***motility*** of cells and of things (particles, organelles…) inside cells.
- are responsible for other cell functions too numerous to summarize here!

We owe much of what we know about biomolecular structure to the development of X-ray crystallography. In fact, an early determination of the structure of insulin (as well as penicillin and vitamin B12) using X-ray crystallography earned Dorothy Hodgkin's the 1964 Nobel Prize in Chemistry. In this chapter, we look at the different levels ('orders') of protein structure…, in fact what it takes to be a functional protein.

The ***primary structure*** (***1º structure***) of a polypeptide is its amino acid sequence. Interactions between amino acids near each other in the sequence cause the polypeptide to fold into ***secondary (2º) structures,*** including the α-helix and the β-pleated sheet (or

just β-sheet) conformations. ***Tertiary (3º) structures*** form when non-covalent interactions occur between amino acid side-chains at some distance from one another in the primary sequence, causing a polypeptide to further fold into a more complex 3-dimensional shape. Other proteins (called *chaperones*!) facilitate the accurate folding of a polypeptide into its correct, bioactive, 3-dimensional conformation. **Quaternary (4º) structure** refers to proteins made up of two or more polypeptides. Refer to the four levels, or orders of structure on the next page (Fig. 3.1) as we explore how each level affects the shape and biological/biochemical function of the protein.

In addition to higher-order protein structures dictated by non-covalent interactions, we'll see covalent bonds between specific amino acids (e.g., cysteines) that end up near each other after folding. These function to stabilize tertiary and quaternary structures. Many proteins also bind metal ions (e.g., Mg^{++}, Mn^{++}) or small organic molecules (e.g., heme) before they become biologically active. Finally, we look beyond these orders of structure at ***domains*** and ***motifs*** of proteins that have evolved to perform one or another specific chemical function. Fig. 3.1 illustrates the four *orders* (*levels*) of protein structure.

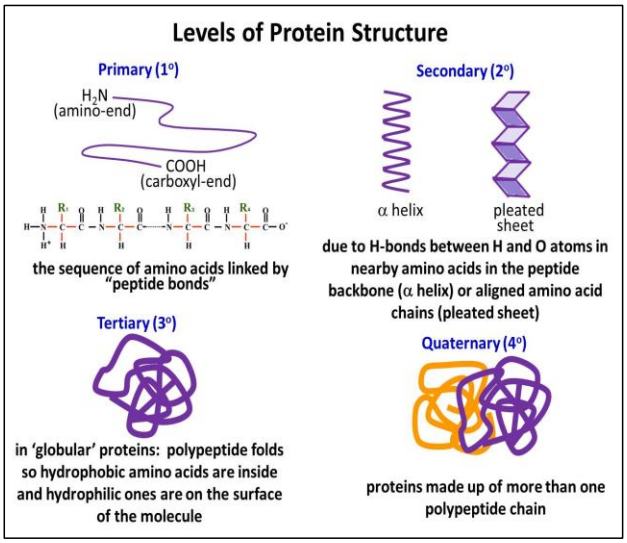


Fig. 3.1: The four orders (levels) of protein structure. Primary, secondary and tertiary structures describe polypeptides; quaternary structure applies to proteins composed of 2 or more polypeptides.

Clearly, in trying to understand molecular (especially macromolecular) function, a recurring theme emerges: the function of a protein depends on its ***conformation***. In turn, protein conformation is based on the location and physical and chemical properties of

critical ***functional groups***, usually amino acid side chains. Watch for this theme as we look at enzyme catalysis, the movement of molecules in and out of cells, the response of cells to their environment, the ability of cells and organelles to move, how DNA replicates, how gene transcription and protein synthesis are regulated… and just about everything a cell does! We will conclude this chapter with a look at some techniques for studying protein structure.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. define and distinguish between the orders of protein structure.
2. differentiate between β-sheet, α-helix and 'random coil' structures based on the atomic interactions involved on each.
3. trace the path to the formation of a polypeptide; define its primary structure and how it might be determined by its amino acid sequence.
4. describe how globular proteins arise from the hydrophobic and hydrophilic interactions that drive protein folding and how changes in protein shape can cause disease.
5. formulate an hypothesis (or look one up) to explain why the amino acid glycine is a disruptor of alpha helical polypeptide structure.
6. compare and contrast motif and domain structure of proteins and polypeptides, and their contribution to protein function.
7. describe different techniques for studying proteins and the physical/chemical differences between proteins that make each technique possible.

3.2 Levels (Orders) of Protein Structure

The three levels of polypeptide structure are primary, secondary and tertiary structure. Quaternary structure refers to associations of two or more polypeptides, creating higher order protein structures. Superimposed on these basic levels are other features of protein structure. These are created by the specific amino acid configurations in the mature, biologically active protein. Let's begin with a look at primary structure.

3.2.1 Protein Primary Structure; L Amino Acids and the C-N-C-N-… Polypeptide Backbone

The ***primary structure*** of a protein simply refers to the amino acid sequence of its polypeptide chain(s). Cells use only 20 amino acids to make polypeptides and proteins, although they do use a few additional amino acids for other purposes. ***Peptide linkages*** between amino acids in polypeptides form in *condensation reactions* during protein synthesis (i.e., ***translation***). The linkages involve multiple covalent bonds. They break and rearrange between the *carboxyl* and *amino* groups of amino acids during linkage formation. The 20 amino acids found in proteins are shown below in Fig. 3.2.

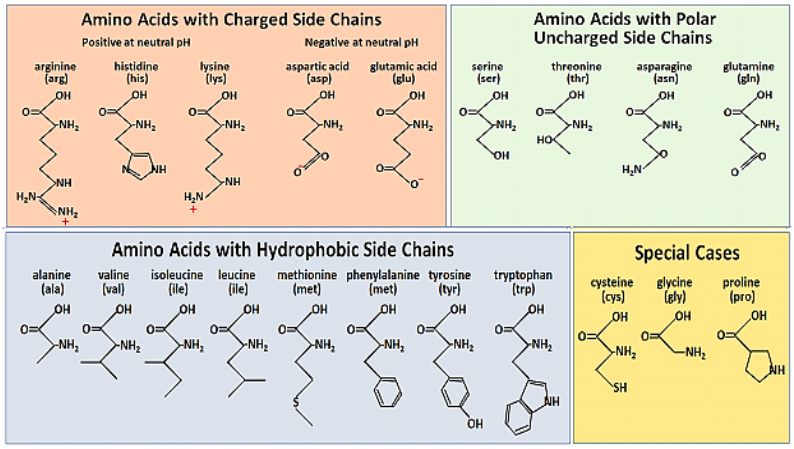


Fig. 3.2: Chemical characteristics of the 20 amino acids found in the proteins of cells.

Except for glycine, the ***α-carbon*** in the 19 other amino acids is bound to four different groups, making them *chiral* or *optically active*.

3.2.1.a Formation of Peptide Bonds Leads to Polypeptide Primary Structure

Recall that chiral carbons allow for mirror image ***D*** and ***L*** (or ***d*** and ***l***) optical isomers. Remember, *only* the lower-case ***d*** and ***l*** actually define the optical properties of isomers. Just to make life interesting, ***L*** amino acids are actually dextrorotary in a polarimeter, making them ***d-amino acids***! While both ***d*** and ***l*** amino acid enantiomers exist in cells, only ***d-*** (i.e., ***L-***) amino acids (along with glycine) are used by cells to build polypeptides and proteins. A partial polypeptide is shown in Fig. 3.3 below.

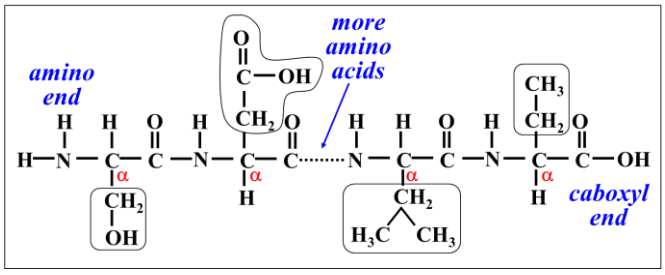


Fig. 3.3: Partial polypeptide; amino and carboxyl ends of a polypeptide define its polarity, with positively charged amino and negatively charged carboxyl ends at physiological pH.

The result of translation in a cell is a polypeptide chain. We say that polypeptides have polarity because they have a 'free' ***carboxyl end*** and a free **amino end**. Amino acid side chains (circled above) end up alternating on opposite sides of a ***C-N-C-N-… polypeptide backbone*** because of the covalent bond angles along the backbone. You could prove this to yourself by assembling a short polypeptide with a molecular modeling kit, the kind you might have used in a chemistry class! The C-N-C-N-…backbone is the underlying basis of higher orders, or levels of protein structure (see below).

132-2 Amino Acid Sequence & Protein Primary Structure

3.2.1.b. Determining Protein Primary Structure - Polypeptide Sequencing

Frederick Sanger demonstrated the first practical protein sequencing method when he reported the amino acid sequence of the two polypeptides of *Bovine* (cow) *insulin*. His technique involves stepwise hydrolysis (an *Edman Degradation*) of polypeptide fragments in which each hydrolysis leaves behind a polypeptide fragment shortened by one amino acid that can be identified. Sanger received a Nobel Prize in 1958 for this feat. By convention, the display and counting of amino acids always starts at the amino end (N-terminus), the end with a free NH_2-group.

Primary structure is dictated directly by the gene encoding the protein. After transcription of a gene, a ribosome *translates* the resulting mRNA into a polypeptide.

For some time now, the sequencing of DNA has replaced most direct protein sequencing. The method of DNA sequencing, colloquially referred to as the *Sanger dideoxy* method, quickly became widespread and was eventually automated, enabling rapid gene (and even whole genome) sequencing. Now, instead of directly sequencing polypeptides, we can infer amino acid sequences from gene sequences isolated by cloning or revealed after complete genome sequencing projects. This is the same Sanger who first sequenced proteins, and yes…, he won a second Nobel Prize for the DNA sequencing work in 1980!

The different physical and chemical properties of each amino acid result from the side chains on its α-carbons. The unique physical and chemical properties of polypeptides and proteins are determined by their unique combination of amino acid side chains and their interactions within and between polypeptides. In this way, primary structure reflects the genetic underpinnings of polypeptide and protein function. The higher order structures that account for the functional *motifs* and *domains* of a mature protein derive from its primary structure.

Christian Anfinsen won a half-share of the 1972 Nobel Prize in Chemistry for demonstrating that this was the case for the ribonuclease enzyme (Stanford Moore and William H. Stein earned their share of the prize for relating the structure of the active site of the enzyme to its catalytic function). See 1972 Nobel Prize in Chemistry for more.

3.2.2 Protein Secondary Structure

Secondary structure refers to highly regular local structures within a polypeptide (e.g., ***α helix***) and either within or between polypeptides (***β-pleated sheets***). Secondary structure conformations occur due to the spontaneous formation of hydrogen bonds between amino groups and oxygens along the polypeptide backbone, as shown in the two left panels in Fig. 3.4. Note that amino acid side chains play no significant role in secondary structure.

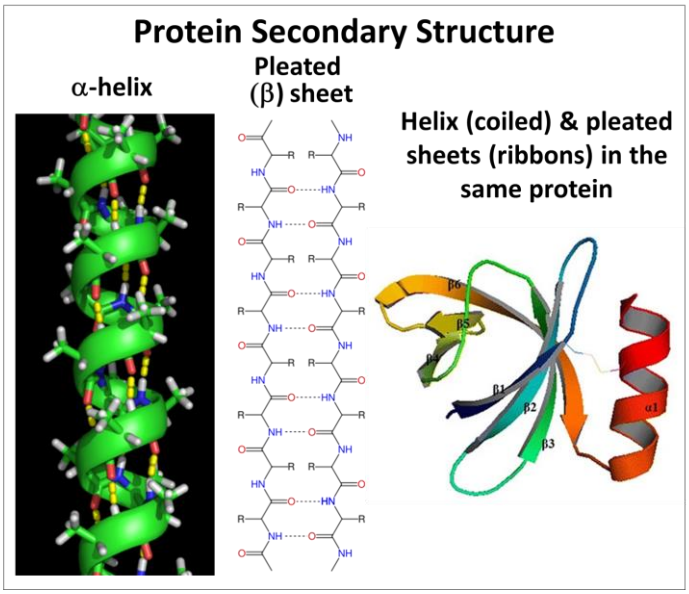


Fig. 3.4: In the secondary structure of a polypeptide, more organized α-helical and β-pleated sheet structures are separated by less organized, random coil stretches of amino acids.

Linus Pauling and coworkers suggested these two types of secondary structure in 1951. A little Linus Pauling history is would be relevant here! By 1932, Pauling had developed his *Electronegativity Scale* of the elements that could predict the strength of

atomic bonds in molecules. He contributed much to our understanding of atomic orbitals and later to the structure of biological molecules. He earned the 1954 Nobel Prize in Chemistry for this work.

He and his colleagues later discovered that sickle cell anemia was due to an abnormal hemoglobin. Then they went on to predict the alpha helical and pleated sheet secondary structure of proteins. While he did not earn a second Nobel for these novel studies of molecular genetics, he did win the 1962 Nobel Peace prize for convincing almost 10,000 scientists to petition the United Nations to vote to ban atmospheric nuclear bomb tests. A more detailed review of his extraordinary life (e.g., at Linus Pauling-Short Biography) is worth a read! Coincidentally, Max. F. Perutz and John C. Kendrew earned the 1962 Nobel Prize in Chemistry for their X-Ray crystallographic studies of the 3-dimensional structure of hemoglobin. 1962 was a good year for Nobel prizes for protein studies!

133-2 Protein Secondary Structure

The α helix or β sheets are a most stable arrangement of H-bonds in the chain(s), and both are typically found in the same protein (at the right in Fig. 3.4). These regions of ordered secondary structure in a polypeptide can be separated by varying lengths of less structured peptide called ***random coils***. All three of these elements of secondary structure can occur in a single polypeptide or protein that has folded into its tertiary structure, as shown at the right in the illustration. The pleated sheets are shown as ribbons with arrowheads representing *N-to-C* or *C-to-N* polarity of the sheets. As you can see, a pair of peptide regions forming a pleated sheet may do so either in the parallel or antiparallel directions (look at the arrowheads of the ribbons), which will depend on other influences dictating protein folding to form tertiary structure. Some polypeptides never go beyond their secondary structure, remaining fibrous and insoluble. Keratin is perhaps the best-known example of a ***fibrous protein***, making up hair, fingernails, bird feathers, and even filaments of the cytoskeleton. However, most polypeptides and proteins do fold and assume tertiary structure, becoming soluble ***globular proteins***.

3.2.3 Protein Tertiary Structure

Polypeptides acquire their *tertiary structure* when *hydrophobic* and non-polar side chains spontaneously come together to exclude water, aided by the formation of *salt bridges* and H-bonds between polar side chains that find themselves inside the globular polypeptide. In this way, α helices or β sheets are folded and incorporated into globular shapes. The forces that cooperate to form and stabilize 3-dimensional polypeptide and protein structures are illustrated below in Fig. 3.5.

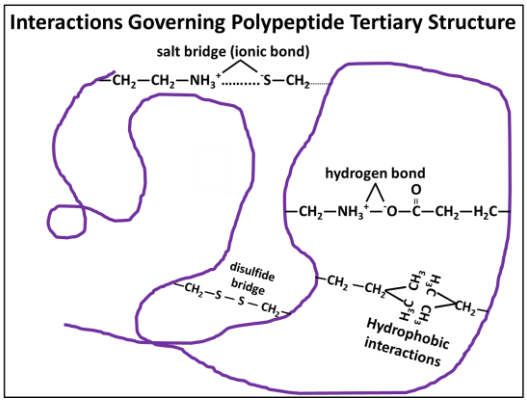


Fig. 3.5: Tertiary structure is created by non-covalaent *hydrophobic* amino acid interactions as well as *H-bonding* in the interior of a polypeptide, leaving charged (hydrophilic) amino acid side chains to interact with water on the exterior of a typical "globular" protein. Stable covalent disulfide bonds between cysteine amino acids help stabilize tertiary structures.

Polar (*hydrophilic*) side chains that can find no other side-chain partners are typically found on the outer surface of the 'globule', where they interact with water and thus dissolve the protein (recall *water of hydration*). Based on non-covalent bonds, tertiary structures are nonetheless strong simply because of the large numbers of otherwise weak interactions that form them. Nonetheless, covalent disulfide bonds between cysteine amino acids in the polypeptide (shown above) can further stabilize tertiary structure. Disulfide bonds (bridges) form when cysteines far apart in the primary structure of the molecule end up near each other in a folded polypeptide. Then the **SH** (***sulfhydryl***) groups in the cysteine side chains are oxidized, forming the disulfide **(–S-S-**) bonds (See Fig. 3.6, below).

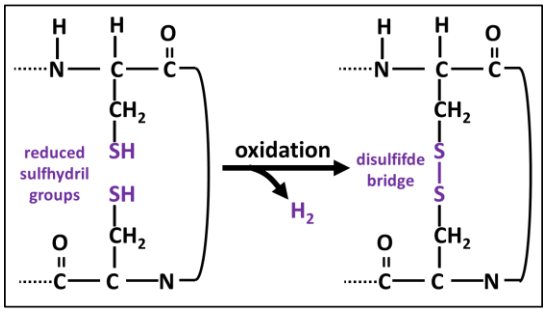


Fig. 3.6: Disulfide bridges form in oxidation reactions between SH (sulfhydryl) groups on cysteine amino acids ('residues') in a polypeptide.

To better understand how disulfide bridges can support the 3-dimensional structure of a protein, just imagine its physical and chemical environment. Changing the temperature or salt concentration surrounding a protein might disrupt non-covalent bonds involved in the 3D shape of the active protein. Unaffected by these changes, disulfide bridges limit the disruption and enable the protein to re-fold correctly and quickly when conditions return to normal (think *homeostasis*!).

134-2 Protein tertiary structure

135-2 Disulfide Bridges Stabilize 3º Structure

3.3 Changes in Protein Shape Can Cause Disease

While the conformation of a protein determines its biological function, an allosteric (shape) change can moderate or disrupt its function. Under normal circumstances, cells use changes in protein shape to regulate metabolism. Such *allosteric regulation* is well documented in familiar biochemical pathways such as glycolysis and is discussed in more detail elsewhere. Less well understood is how (or why) conformational change in some proteins cells can have devastating effects.

3.3.1 Sickle Cell Anemia

Mutant globin genes can cause hemoglobin disorders characterized by inefficient oxygen delivery by blood. In the 1940s, the British biochemist J.B.S. Haldane made a correlation between southern African regions with high incidences hemoglobin disorders and malaria, suggesting that heterozygous individuals (i.e., those that had only one copy of a mutant hemoglobin gene), were somehow protected from malaria. *Sickle Cell Anemia* is a well-known example of a hemoglobin disorder, caused by a single base change in the gene for human β-hemoglobin (one of the polypeptides in hemoglobin). Since red blood cells are rich in hemoglobin, the abnormal shape of the β-hemoglobin can cause the cells themselves to become sickle-shape. Sickled cells disrupt capillary flow and oxygen delivery, causing the symptoms of anemia. While Sickle Cell Anemia originated in Africa, it probably spread to the United States as a consequence (or even a cause) of the slave trade. This is in part because Europeans exploiting their African colonies' natural resources were dying of malaria while the African natives seemed unaffected. Having brought malarial mosquitos to the new world as stowaways in the first place, the Europeans figured that African slaves would survive the illness in the Americas where they could not.

Individuals heterozygous for the have *sickle cell trait* and are generally unaffected because at least some of their hemoglobin is normal. Homozygous individuals that can make only the sickle cell β-hemoglobin variant suffer more frequent and severe

suffer episodes of the disease. Stressors that can trigger sickling include infection or dehydration. Compare normal red blood cells to a sickle cell in Fig. 3.7 (below).

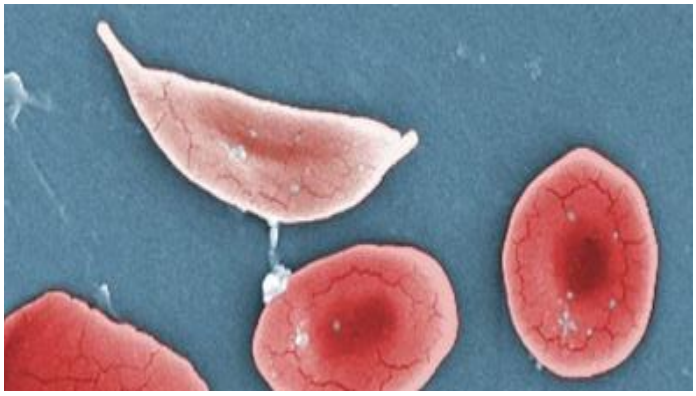


Fig. 3.7: Light micrograph of a sickled erythrocyte (red blood cell).

The sickle cell gene affects perhaps more than 100 million people worldwide, including 8-10% of African Americans. For more demographic information, see Sickle Cell Trait Demographics Article and Sickle Cell Data from the CDC. In Africa, heterozygotes with sickle cell trait are protected from malaria, confirming Haldane's hypothesis. But patients homozygous for the β-hemoglobin mutation derive little benefit from its anti-malarial effects.

In the meantime, despite a 33% reduction in cases of malaria in recent years, this disease caused by a mosquito-borne parasite still threatens half of the people on the planet, causing over a half-million deaths per year. There are treatments other than mosquito nets and killing mosquitos, but at this time, there is still no preventive vaccine.

3.3.2. The Role of Misshapen and Mis-Folded Proteins in Alzheimer's Disease

Prion proteins, when first discovered, seemed to behave as infectious agents that could reproduce without DNA or other nucleic acid. As you can imagine, this highly unorthodox and novel hereditary mechanism generated its share of controversy. Read about research on the cellular PrP^c prion protein at en.wikipedia.org/wiki/Prion. Of course, prions turn out *not* to be reproductive agents of infection after all. Recent studies of prions have revealed several normal prion protein functions including roles in memory formation in mice and in sporulation in yeast (Check out *Prion Proteins May Play a Role in Memory Formation*).

A mutant version of the prion protein (*PrP^{Sc}*) is able to mis-fold and take on an abnormal shape. The deformed *PrP^{Sc}* can then induce abnormal folding even other prions, of other normal PrP^{c} molecules. These events, illustrated below in Fig. 3.8, result in the formation of so-called *amyloid plaques*.

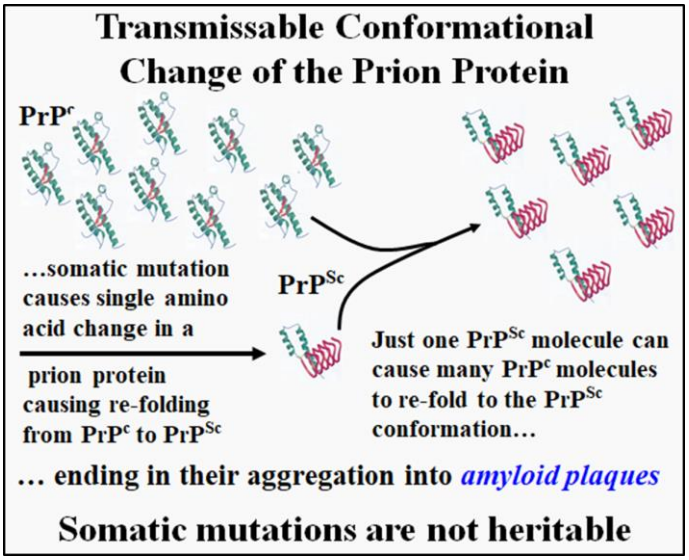


Fig. 3.8: Mutations in a normal gene for a prion protein *(PrP^{c})* may produce some abnormally folded prion proteins (*PrP^{Sc}*). The misfolded *PrP^{Sc}* molecules interact with other (even normal) prions to mis-fold, precipitating *PrP^{Sc}* proteins into aggregated amyloid plaques.

Abnormally folded prions (*PrP^{Sc}*) have been associated Alzheimer's Disease. Alzheimer's Disease affects about 5.5 million Americans. *PrP^{Sc}* is also associated with *Mad Cow disease* and *Creutzfeldt-Jakob-Disease* (mad cow disease in humans), as well as *Scrapie* in sheep, among others. We are beginning to understand that the role of prion proteins in ***Alzheimer's Disease*** is less causal than indirect.

3.3.2.a. The *Amyloid Beta* (*A_β*) peptide

Post-mortem brains of patients that suffered Alzheimer's disease show characteristic *extracellular* ***amyloid plaques*** to be composed largely of the ***amyloid beta*** (***A_β***) peptide. Enzymatic cleavage of the ***amyloid precursor protein*** (**APP**) generates extracellular 39-43 amino acid ***A_β*** peptides. Normally,

excess A_β peptides are themselves digested. Unregulated A_β peptide formation however, leads to the formation of *beta amyloid plaques seen in Alzheimer's disease* (Fig. 3.9, below).

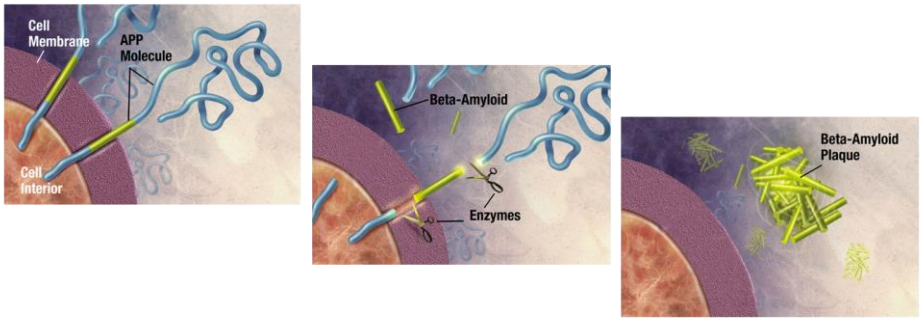


Fig. 3.9: Steps in the formation of β-amyloid plaques. Amyloid precursor proteins (*APP*) are embedded in cell membranes (upper left). Enzymes digest APP, releasing β-amyloid protein fragments (middle panel). Unregulated accumulation of β-amyloid protein results in the formation of extracellular amyloid plaques.

The scissors in the illustration represent two enzymes that digest the APP. Prion proteins are not a proximal cause of Alzheimer's Disease, but may have a role in initiating events that lead to it. Normal prion protein (***PrP^c***) is itself a membrane receptor and is thought to bind A_β peptides, effectively preventing their aggregation into plaques. An experimental reduction of *PrP^c* was shown to increase the extracellular A_β peptides. Presumably prion protein aggregation induced by the mutant PrP protein (***PrPsc***) prevents prion proteins from binding to A_β peptide, leading to its accumulation and ultimately to amyloid plaque formation and neurodegeneration.

3.3.2.b. The Tau protein

A protein called ***tau*** is also associated with Alzheimer's Disease. Misshapen tau accumulating in ***neurofibrillary tangles*** in hippocampus brain neurons may be a more immediate cause of the neuronal dysfunction associated with the disease. In normal neurons, ***MAP-T*** (**M*icrotubule-Associated Protein Tau***) is phosphorylated and then binds to and stabilizes microtubules. But when neuronal *tau* becomes *hyper-phosphorylated,* its conformation changes. No longer stabilized, the microtubules disassemble and the deformed tau proteins

form ***neurofibrillary tangles.*** Immunostaining of *hippocampal* neurons with antibodies to tau protein localizes the ***neurofibrillary tangles*** as seen in the micrograph in Fig. 3.10.

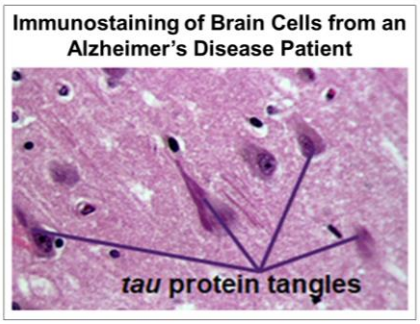


Fig. 3.10: Non-fluorescent immunostaining of *tau* proteins of an Alzheimers brain reveals *tau tangles*.

The formation of neurofibrillary tau protein tangles in a diseased neuron is compared to normal neurons in Fig. 3.11, below.

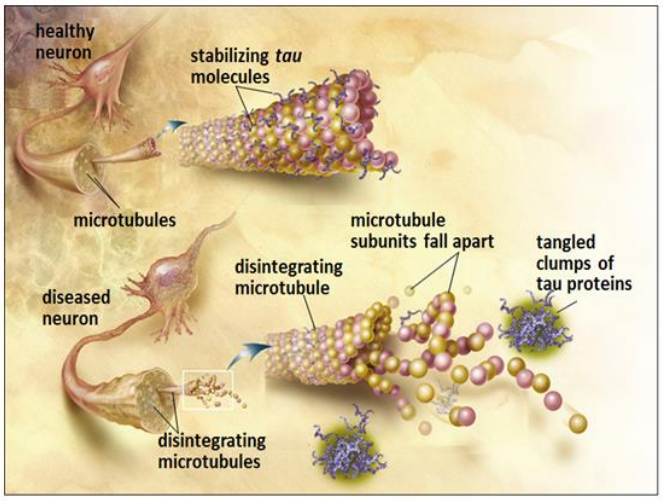


Fig. 3.11: Formation of tau tangles occurs in diseased neurons that can't maintain normal microtubule structur. In the absence of stabilizing tau molecules, microtubules degenerate and tau proteins clump.

The tangled clumps of tau proteins in this illustration are what stain deep purple in the micrograph of immunostained neurons in the light micrograph (above).

There is no cure for Alzheimer's disease, although treatments with *cholinesterase inhibitors* seem to slow its advancement. For example, the drug *Aricept* inhibits acetylcholine breakdown by *acetylcholinesterase*, thereby enhancing cholinergic neurotransmission, which may in turn prolong brain neural function. Unfortunately, there is as yet no treatment to restore lost memories and the significant cognitive decline associated with Alzheimer's disease. Perhaps more promising in this respect is the recent detection in the blood and serum of proteins or peptides associated with Alzheimer's Disease. Amyloid beta (***A_β***) and/or ***tau*** protein fragments that escape into the blood stream can be detected 6-8 or more years before Alzheimer's symptoms appear. A ***neurofilament light chain*** (***NfL***) seen in a familial form of Alzheimer's Disease (among other neuropathies) is detectable at 16 years before the symptoms! The ability to detect of these Alzheimer's marker proteins or derived peptides so far in advance of symptoms raises hopes for early monitoring of at-risk individuals and for new therapies for Alzheimer's disease. For brief reviews, see Alzheimer's Disease: The Early Detection of Circulating Ab Peptides and Alzheimer's Disease: The Early Detection of Circulating tau Peptides. For a recent report on tracking the ***NfL*** protein, see Alzheimer's Disease: The Early Detection of Circulating Neurofilament Light Chain Protein

3.3.2.c. Some Relatives of Alzheimer's Disease

Some of the same protein abnormalities seen in Alzheimer's disease also characterize other neurodegenerative diseases as well as traumatic brain damage.

An abnormal accumulation of tau protein is diagnostic of ***CTE*** (*Chronic Traumatic Encephalopathy*). In the early 20th century, disoriented boxers staggering about after a fight were called 'punch drunk', suffering from *dementia pugilistica*. We now know they suffered from *CTE*, as do other athletes exposed to repetitive mild-to-severe brain trauma, such as football players. Immunostaining of whole brains and brain tissue from autopsied *CTE* patients with antibodies to tau protein show accumulations of abnormal tau proteins and tau neurofibrillary tangles very much like those found in of Alzheimer's patients. Many National Football League and other football players have been diagnosed post-mortem with *CTE*, and many still living show signs of degenerative cognition and behavior consistent with *CTE* (see a List_of_NFL_players_with_chronic_traumatic_encephalopathy to see how many!

Parkinson's Disease is yet another example of a neurodegenerative disease that results when a single protein changes shape in brain cells. Though they are not characterized as plaques, aggregates can form in brain cells when the protein

alpha-synuclein undergoes anomalous conformational change. The change results in ***MSA*** *(Multiple System Atrophy)* or Parkinson's Disease. Click https://www.sciencedirect.com/science/article/pii/S0925443911002250 to read details of this recent research. Much of the high-resolution electron microscopy that can reveal protein structure and capture the conformational changes we now recognize comes from the work of Jacques Dubochet, Joachim Frank and Richard Henderson who received the 2017 Chemistry Nobel Prize for Chemistry for developing and refining cryo-electron microscopy for biomolecular imaging (see 2017 Nobel Prize for Chemistry for more).

3.4 Protein Quaternary Structure, Prosthetic Groups & Chemical Modifications

Quaternary structure describes a protein composed of two or more polypeptides. Like tertiary structure, such multimeric proteins are formed by non-covalent interactions and may be stabilized disulfide bonds. Specifically, a *dimer* contains two, a *trimer* three, a *tetramer* four polypeptides… and so on. Multimers made up of identical subunits are referred to with a prefix of "homo-" (e.g. a homotetramer). Those made up of different subunits are *heteromers*. The vertebrate hemoglobin molecule is a *heterotetramer*, with two α- and two β- globins (Fig. 3.12).

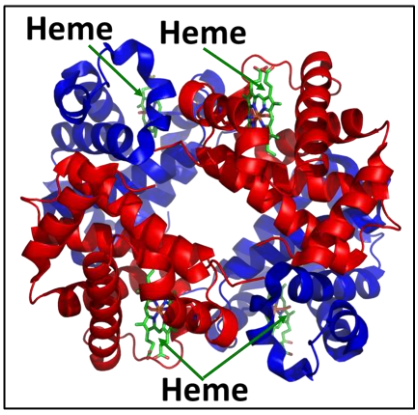


Fig. 3.12: The vertebrate hemoglobin molecule, consisting of 4 globin subunits (two α and two β polypeptides). Each globin is associated with a heme group bound to iron.

Prosthetic groups are organic molecules associated with proteins. Hemoglobins exemplify the role of prosthetic groups in protein function. To be biologically active, globin polypeptides must associate with *heme*, a cyclic organic molecule with iron at its center. The iron is what reversibly binds oxygen. All kinds of organisms, from bacteria to plants

and animals and even in some anaerobic organisms contain some kind of hemoglobin with a heme-like prosthetic group. Other proteins must be bound to different metal ions (magnesium, manganese, cobalt…) to be biologically active.

136-2 Protein Quaternary Structure & Prosthetic Groups

Chemical modifications are post-translational enzyme catalyzed events required to make a protein fully functional or to regulate its activity. Many polypeptides are structurally modified by *glycosylation* (e.g., to make membrane *glycoproteins*). Others are *phosphorylated* at one or more specific amino acids in the chain, to regulate their biological activity. These modifications account for and enhance the molecular and functional diversity of proteins within and across species.

3.5. Domains, Motifs, and Folds in Protein Structure

The structures of two different proteins in Fig. 3.13 share a common *PH* (Pleckstrin Homology) *domain* (maroon).

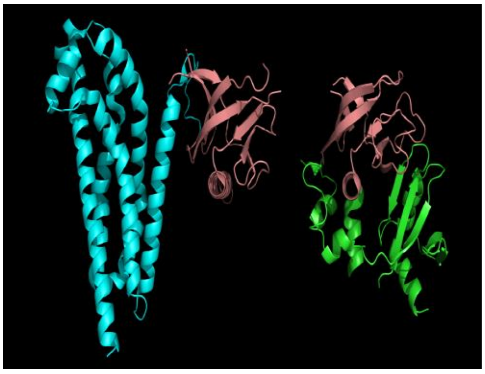


Fig. 3.13: The *Pleckstrin Homology* (PH) domains shown here are an example of common domains (maroon colored sequences) in two different proteins. Because they have a PH domain, both proteins can interact with cell signaling factors with roles in intercellular communication.

These two and many other proteins have this domain, allowing them to bind a molecule of *phosphatidyl-inositol triphosphate* that is generated as part of a common cell-signaling pathway. The implication of this common *domain* is that a cell can have signaling pathways that allowing it to respond to different signals that lead to the same response, albeit under different conditions and probably at different times. Proteins are typically described as consisting of several distinct sub-structures, discussed here.

A ***domain*** is an element of the protein's overall structure that is stable and often *folds* independently of the rest of the protein chain. Like the PH domain above, many domains are not unique to the protein products of one gene, but instead appear in a variety of proteins. Proteins sharing *more than a few* common domains are encoded by members of evolutionarily related genes comprising ***gene families***. Genes for proteins that share only one or a few domains may belong to ***gene superfamilies***. Superfamily members can have one function in common, but the rest of their sequences are otherwise unrelated. Domain names often derive from their prominent biological function in the protein they belong to (e.g., *the calcium-binding domain* of *calmodulin*), or from their discoverers (the PH domain!). The domain swapping that gives rise to gene families and superfamilies are natural genetic events. Because protein domains can also be "swapped" on purpose by genetic engineering, we can make *chimeric proteins* with novel functions.

137-2 Protein Domain Structure &Function

Protein ***motifs*** are small regions of protein three-dimensional structure or amino acid sequence shared among different proteins. They are recognizable regions of protein structure that may (or may not) be defined by a unique chemical or biological function.

Supersecondary structure refers to a combination of secondary structure elements, such as *beta-alpha-beta* units or the *helix-turn-helix motif*. They may be also referred to as structural motifs. "Google" *Supersecondary structure* for examples.

A ***protein fold*** refers to a general aspect of protein architecture, like *helix bundle*, *beta-barrel*, *Rossman fold* or other "folds" provided in the Structural Classification of Proteins database. Click Protein Folds to read more about these structures.

3.6. Proteins, Genes and Evolution: How Many Proteins are We?

If evolution did not have to select totally new proteins for each new cellular function, then how many genes does it take to make an organism? The number of genes in an organism that encode proteins may be far fewer than the number of proteins they actually make. Estimates have suggested that it takes from 19,000 to 25,000 coding genes to make and operate a human and all its proteins (check out Pertea and Salzberg at Estimating the number of genes in the human genome and Abascal F. et al. at Loose ends: almost one in five human genes still have unresolved coding status. Nevertheless, our cells (and those of eukaryotes generally) may express as many as 100,000 different proteins. How is this possible? Are there more efficient ways to evolve new and useful cellular tasks than evolving new genes?

As we already noted, the use of the same 20 amino acids to make proteins in all living things speaks to their early (even pre-biotic) selection and to the common ancestry of all living things. Complex conserved domain structures shared among otherwise different proteins imply that evolution of protein function has occurred as much by recombinatorial exchange of DNA segments encoding these substructures, as by an accumulation of base substitutions in otherwise redundant genes. Likewise, motifs and folds might also be shared in this way. Protein number can exceed gene number in eukaryotes, in part because cells can produce different RNA variants from the same genes by *alternate splicing*; this creates mRNAs that code different combinations of substructures from same gene! Alternate splicing is discussed in detail in a later chapter. The conservation of amino acid sequences across species (e.g., histones, globins, etc.) is testimony to the common ancestry of eukaryotes. Along with the synthesis of alternate versions of an RNA, an ongoing repurposing of useful regions of protein structure may prove a strategy for producing new proteins without adding new genes to a genome.

3.7. Directed Evolution: Getting Cells to Make New Proteins… for our use and pleasure

Investigators have long speculated that proteins with useful functions could be adapted to human use. Enzyme additives in laundry detergents or spot-removers are already used to digest organic stains. Such enzymes must be extracted from a suitable biological source. But what if we could engineer even better versions of an enzyme? At the molecular level, protein evolution is the natural selection of gene sequences that encode functional polypeptides. This implies that variant 'mutant' versions of a gene encoding related polypeptides already exist, from which nature can select one. Thus, changing environmental circumstances might favor one variant protein over another. In nature, mutations that could create polypeptide variants are entirely random. In other words, we humans might wait a very long time before a *better version* of a protein, say an enzyme, would be available for human use. Can we speed up the selective process to more rapidly evolve better, more useful proteins? Yes we can!

Many industries (fuel, pharmaceutical…) have used molecular techniques to create new proteins. It is possible to clone a desired gene, make targeted mutations in the gene and then express them in suitable cells. The expressed proteins can then be screened for e.g., mutant enzymes with improved or even novel useful activities. This is a far cry from older techniques irradiating or otherwise mutagenizing cells or organisms that would then be screened for mutants that looked interesting! Since we can now target mutations to a single specific base within a gene, one that for example avoids a drastic change in protein folding, it is possible to study the functional effects of subtle conformational changes in a protein. The technique, called ***directed evolution***, was originally pioneered by Frances Arnold, who engineered enzymes that could make renewable fuels (e.g., isobutanol),

environmentally friendly pharmaceuticals and enzymes that function hundreds of times faster and/or that function at a broader temperature range than their naturally occurring counterparts. For these achievements, Frances Arnold was awarded the Nobel Prize for Chemistry in 2018!

3.8. View Animated 3D Protein Images in the NCBI Database

We can't see them with our own eyes, but viewed by *X-Ray diffraction*, proteins exhibit exquisite diversity. You can get an X-Ray eye's view of protein structures at *National Center for Biological Information's* Cn3D database. Here's how to access three-dimensional animated images of proteins from the database:

- Click http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dinstall.shtml to download the Cn3D-4.3.1_setup file (for Windows or Mac). The software will reside on your computer and will activate when you go to a macromolecule database search site.
- Click http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html to enter the protein structure database (Fig. 3.14):

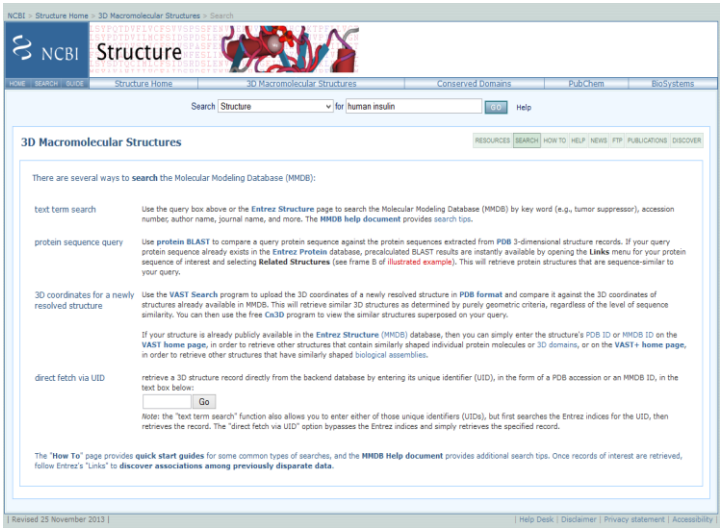


Fig. 3.14: Screen-shot of the NCBI protein structure database leading to 3D Macromolecular structures. http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html

- The search example shown above for human insulin takes you to this link: http://www.ncbi.nlm.nih.gov/structure/?term=human+insulin&SITE=NcbiHome&submit.x=12&submit.y=12 The website is shown below (Fig. 3.15):

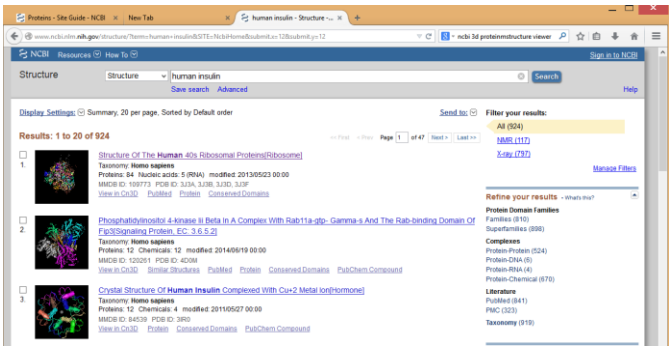


Fig. 3.15: Screen-shot of NCBI protein structure database search leading to available macromolecular structures.

- Click *View in Cn3D* for the desired protein. Fig. 3.16 shows a view of human insulin.

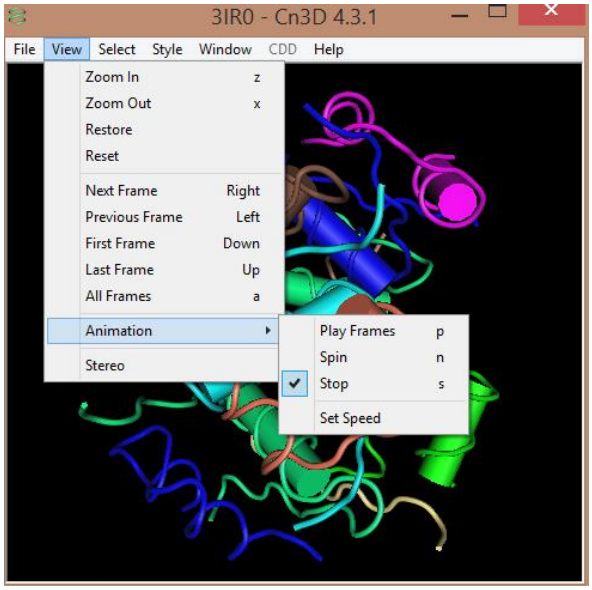


Fig. 3.16: Screen-shot of NCBI protein structure database search results for human insulin. http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html

- To rotate the molecule, click *View* then *Animation,* then *Spin*… and enjoy!

Some iText & VOP Key Words and Terms

A_β	functional groups	primary structure
α-carbon	gene family	prion
alpha helix	gene superfamily	protein folding
allosteric regulation	glycosylation	PrP
Alzheimer's disease	helix-turn-helix motif	quaternary structure
amino acid residues	hemoglobin	random coil
amino end	hydrophobic interactions	recombinatorial exchange
amyloid beta protein	levels of protein structure	salt bridges
amyloid plaques	Mad Cow disease	salt bridges
beta barrel	multimer	secondary structure
beta sheet	NCBI Cn3D database	sequence motifs
carboxyl end	neurofibrillary tangles	sickle cell anemia
catalysts	nucleoskeleton	side chains
chaperones	orders of protein structure	structural domain
configuration	Parkinson's disease	structural motif
Creutzfeldt-Jakob disease	peptide bonds	sulfhydryl groups
cytoskeleton	peptide linkages	tau protein
disulfide bonds	phosphorylation	tertiary structure
Edman degradation	pleated sheet	
enzymes	polypeptide backbone	

Chapter 4: Bioenergetics

Thermodynamics (Free Energy, Enthalpy and Entropy), Chemical Energy, Open vs. Closed Systems

4.1 Introduction

Three ***Laws of Thermodynamics*** describe the flow and transfer of energy in the universe. They are:

1. ***Energy can neither be created nor destroyed (also stated as 'universal energy is constant'***.
2. ***Universal entropy (disorder) is always increasing***.
3. ***Entropy declines with temperature*** -as temperatures approach absolute zero, so goes entropy.

In living systems, we do not have to worry about the third law because equations for energy exchange in living systems already reflect the temperature dependence of entropy change during reactions. Here we look at how we came to understand the basic thermodynamic principles and how they apply to living systems. First, we will look at different kinds of energy and at how ***redox*** reactions govern the flow of energy through living things. Next, we'll look at some simple arithmetic statements of the ***Laws of Thermodynamics*** for ***closed systems*** and at how they apply to chemical reactions conducted under ***standard conditions***. Finally, since there is really no such thing as a *closed system*, we look at the energetics of reactions occurring in ***open systems***. For an excellent discussion of how basic thermodynamic principles apply to living things, you can also check out Lehninger A. (1971) *Bioenergetics: The Molecular Basis of Biological Energy Transformation.* Benjamin Cummings, San Francisco.

Learning Objectives
When you have mastered the information in this chapter, you should be able to:

1. explain the difference between *energy transfer* and *energy transduction.*
2. compare and contrast *potential* vs. *kinetic* as well as other categories of energy (e.g., *mass, heat, light…,* etc.*).*
3. explain the reciprocal changes in universal *free energy* and *entropy*.
4. derive the algebraic relationship between *free energy*, *enthalpy* and *entropy.*
5. distinguish between *exothermic*, *endothermic*, *exergonic*, and *endergonic* reactions.
6. predict changes in free energy in a chemical reaction based on changes in the concentrations of reactants and products in *closed systems* as well as *open systems*.
7. predict how and when an endothermic biochemical reaction will release free energy (you should be able to do this after working some sample problems of closed system energetics).
8. explain how an exergonic reaction occurring in a cell might be energetically unfavorable.
9. explain and distinguish between the equilibrium and steady-state of reactions.

4.2 Kinds of Energy

We can easily recognize different kinds of energy around us like ***heat***, ***light***, ***electrical***, ***chemical***, ***nuclear***, ***sound***, etc. And you probably know that energy in its different forms is measurable (calories, joules, volts, decibels, quanta, photons…). Even mass is a form of energy, as you may recall from Albert Einstein's famous $e=mc^2$ equation (the *Law of Relativity*).

The problem in thinking about thermodynamics is that the universe is big and there are too many kinds of energy to contemplate at once! To simplify, imagine only two kinds of energy in the universe: ***potential energy*** and ***kinetic energy***. A helpful example is a dam. The water above the dam has *potential energy*. As the water flows over (or through) the dam, its potential energy is released as *kinetic energy*. In the old days the kinetic energy of flowing water could be used to power (i.e., turn) a millstone to grind wheat and other grains into flour. These days, water is more likely to flow through a hydroelectric dam where kinetic energy is converted (*transduced*) to electricity. In this simple view, heat (molecular motion), electricity (a current of electrons), sound (waves), and light (waves OR moving 'particles') are different forms of kinetic energy. The energy of mass or its position in the universe is potential energy. For example, the energy in a molecule of ATP is potential energy. Physicists talk a lot about potential energy and about kinetic energy flow and conversion. Here is an equally simple way to conceptualize energy: it is either ***useful*** *or* ***useless***. This concept led directly to the equations describing thermodynamic laws. In this utilitarian way of thinking about energy, useless energy is ***entropy*** while useful energy is any other energy form (potential or kinetic).

A key to understanding bioenergetics is to recognize the difference between closed and open systems in the universe. Systems such as biochemical reactions in a test tube are closed and will reach ***equilibrium***. ***Closed systems*** are artificial and only possible in a laboratory, where one can restrict and measure the amount of energy and mass getting into or escaping the system. Cells on the other hand (in fact every reaction or event in the rest of the universe) are ***open systems***. Open systems readily exchange energy and mass with their surroundings.

With this brief introduction, let's imagine ourselves to be early scientists trying to understand energy flow in the universe, asking how the ***Laws of Thermodynamics*** apply to living systems (***bioenergetics***). We'll see that the *Laws* can be demonstrated precisely because all kinds of energy can be measured and all unites of energy can be interconverted (e.g., volts into calories, light quanta into volts, joules into decibels, etc.).


138 Different Kinds of Energy; Chemical Equilibrium


4.3 Deriving Simple Energy Relationships

Thermodynamics Laws lead us to equations that help us understand how energy is moves (i.e., flows) between components of the universe. As we'll discuss, the universe is most easily thought of as a giant closed system within which energy can be transferred and even transduced (i.e., from one form to another) between those components. We can't measure energy flow in the universe as a whole. But we can isolate bits within the universe so that we can quantify energy as it is transferred or transduced within a smaller, more manageable closed system.

4.3.1 Energy in the Universe: the Universe is a *Closed System*

Consider an event, any event. I think we can agree that when stuff happens, the participants in the happening go from an unstable state to a relatively more stable state. For example, you are carrying a bag of marbles, the bag tears open and the marbles fall to the floor, roll and spread out, eventually coming to a stop. At that point, the marbles are in a more *stable state* than they were when you were holding the un-ripped bag.

We can all agree that gravity made the marbles fall from the bag. We might then say that the fallen marbles are in a more stable state than they were in the bag in your hands. That certainly seems true. If so, we could conclude that the drive to greater stability is what made the marbles fall! In fact, regardless of the force or impetus for the event, we say that the drive to achieve greater stability is what makes stuff

(i.e., events) happen! This is the essence of the ***Second Law of Thermodynamics***: all universal energy transfer events occur with an increase in stability…, that is, an increase in ***entropy***. We'll consider the Second Law and *entropy* in detail shortly.

The tendency of things to go from unstable to more stable is a natural, rational state of affairs…, like those marbles on the floor, or a messy bedroom with clothes strewn about. Intuitively, messy and disordered is more stable than ordered. Of course, marbles dropping or clothing going from folded and hung to wrinkled on the floor releases energy (potential energy) as they fall (kinetic energy). If you don't believe that this release of energy is real, just think of how much energy you will need to pick up the marbles or re-fold your clothes (after laundering them of course!).

We can model the flow of energy in the universe in a way that is consistent with thermodynamic laws. Since the ***First law of Thermodynamics*** says that *energy can be neither created nor destroyed*, a simple statement of the First Law could be:

$$E_{universal} = E_{light} + E_{heat} + E_{electrical} + E_{mass}....$$

The equation sums up the different kinds of energy in the universe. Consider the circle (also called a ***Venn diagram***) in Fig. 4.1 to be the universe.

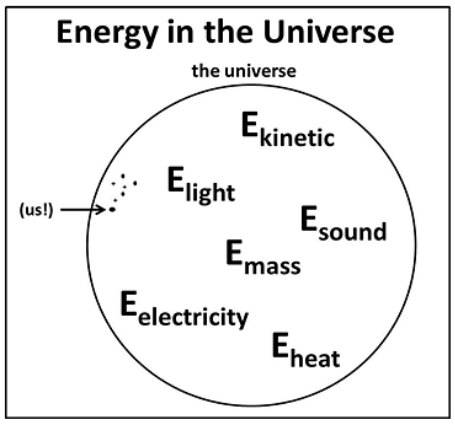


Fig. 4.1: The sum of all things contained in the universe (e.g., as mass and other, more familiar kinds of energy). In this simple "Venn" diagram, the universe is a closed system; nothing (mass, energy) can get into or exit the universe, consistent with the *First Law of Thermodynamics*.

From this we conclude that energy cannot get in or out of the universe, but energy can be transferred from one place to another or converted from one form to another. It follows then that $E_{universal}$ is the sum of all kinds of energy in the universe, and that this *sum* is a constant… in the words of the First law, *universal energy is constant*.

The equation below is consistent with this idea.

$$E_{light} + E_{heat} + E_{electrical} + E_{mass} + \ldots = \text{a constant}$$

This is a statement of the *First Law of Thermodynamics*.

139 First Law of Thermodynamics

If we go with the simpler binary notion of ***useful*** and ***useless*** energy, our equation shortens to the sum of just two kinds of energy in the universe:

$$E_{universal} = G_{universal} + TS_{universal}$$

In this equation, G is useful energy ("Gibbs" free energy), S is useless energy (entropy), and T is absolute temperature (included because of the third law). This is also a statement of the *First Law* as shown in the revised Venn diagram in Fig. 4.2.

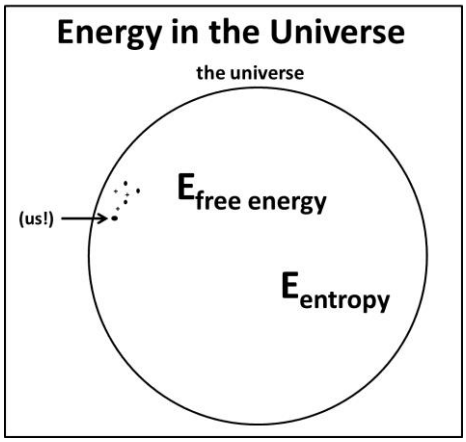


Fig. 4.2: Since even mass is a form of energy, the universe shown here consists of only two components, *entropy* and *free energy* (defined as available to do work), still consistent with the *First law of Thermodynamics*.

In the late 19th century, John Venn formalized this visual approach to segregating things and concepts into circles as a way of logically viewing relationships between them. The *Venn diagrams* used here to describe the universe are very simple. For

examples of more complex overlapping components of the universe that share some but not all attributes, “Google” Venn Diagrams. In this binary energy model, it follows that as universal entropy increases, free energy in the universe must decrease (as graphed in Fig. 4.3).

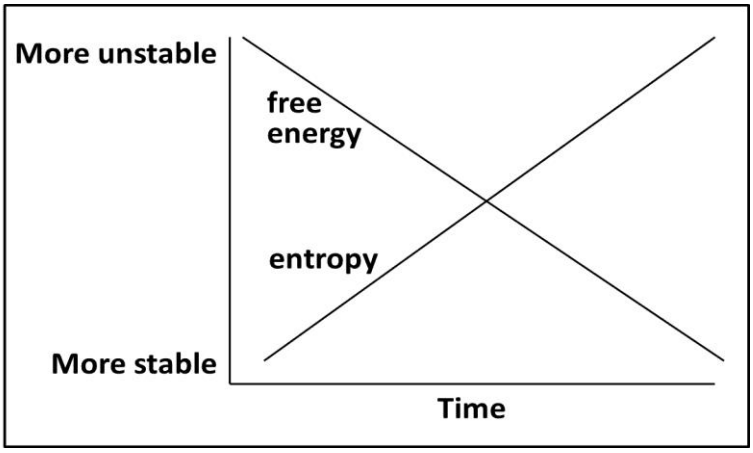


Fig. 4.3: The reciprocal relationship between entropy and free energy graphed over time. If all activities in the universe proceed by increasing entropy, and if the universe consists *only* of free energy and entropy, then as entropy increases, free energy must be decreasing in the universe.

Free or *potentially useful* energy is higher in more ordered, complex and therefore relatively unstable systems. Such unstable, ordered systems will release free energy *spontaneously*. In other words, stuff ***will*** happen!

140-2 Second Law of Thermodynamics

4.3.2 Energy is Exchanged Between Systems in the Universe

While the arithmetic statements about changes in energy are useful concepts, the parameters are of course, not measurable! But if we isolate a bit of the universe, we *can* measure energies and watch energy flow.
If we can measure the amount of energy put into or removed from ***a system within the universe***, we can write a more useful equation to follow the transfer of energy between a system and its surroundings:

$$\Delta H = \Delta G + T\Delta S$$

In this formula, **ΔH** is the change (Δ) in enthalpy, i.e., as energy entering/leaving the system in units of heat energy); **ΔG** is change in free energy and **ΔS** is change in entropy; **T** is the absolute temperature (°K).

Heat given off in a reaction (or other event) is often confused with entropy. It is true that much of the increase in entropy that occurs in living things is indeed in the form of random molecular motion, or heat. But remember that heat can have its uses; not all heat is entropic! Hence, it is more interesting (and accurate!) to think of energy in terms of changes in enthalpy, free energy and entropy during energy transfers. Thus, we must consider the arithmetic of energy transfers to involve not two, but three terms!

According to the equation **ΔH = ΔG + TΔS**, interacting systems in our universe would seem to be *closed systems*. Accordingly, energy put into or removed from the system (**ΔH**) will be exactly balanced by increases and/or decreases in the other two terms in the equation (**ΔG + TΔS)**. Recall that we refer to a system as closed *not* because it is really closed, but because we can isolate them well enough to account for energy flow into and out of the system. The value of this or any algebraic equation with three variables is that if you know two of the values, you can calculate the third! Here is a simple situation to illustrate the point: If I put a liter of water on a burner and light the flame, the water gets hot. If the temperature of the liter of water rises by 1°C, we know that it has absorbed 1000 calories (one Kcal, or one *food* Calorie) of the heat from the burner.

Since energy interactions depend on physical conditions such as temperature and air pressure, we need to standardize those conditions when conducting experiments that measure energy changes in experimentally isolated systems. For more on how standardizing these physical parameters enables measuring energy change in chemical reactions (in fact, any energy exchange), click the link below.

141 Deriving Closed System Thermodynamics

Turning to ***bioenergetics***, let's apply the equation **ΔH = ΔG + TΔS** to the conditions in which chemical reactions occur in cells. Because most life on earth lives at sea level where the air pressure is 1 atmosphere and the temperature is in the 20's (Celsius), typical determinations of **ΔH**, **ΔG**, and **ΔS** are made under defined and well-controlled ***standard conditions*** where T=298°K (25°C), an atmospheric pressure of pressures of 1 atm, and a constant pH of 7.0. The latter is so defined because the pH inside of a typical cell is close to neutral. In addition, measured values are adjusted to calculate unimolar quantities of reactants (see below). Our equation for reactions under *these* standard conditions becomes **ΔH = ΔGo + TΔS,** where **ΔGo** is the ***standard free***

energy change for a reaction conducted in a *closed system* under standard conditions. **ΔH** and **ΔS** are still the enthalpy and entropy changes, but determined under standard conditions.

What are *unimolar conditions* in practice? It means that if you measure the calories released by burning (oxidizing) *180 mg* of glucose, you must multiply the calories released by 1000 to get (**ΔH**). This will give you the calories that would have been released by burning *180 gm* (i.e., a whole mole) of the stuff.

Now we are ready to consider examples of how we determine the energetics of reactions.

4.3.3 How is Enthalpy Change (ΔH) Determined?

ΔH (heat released or absorbed) in a chemical reaction can easily be determined by conducting the reaction under standard conditions in a ***bomb calorimeter***, essentially an inner chamber surrounded by an outer chamber filled with water (Fig. 4.4).

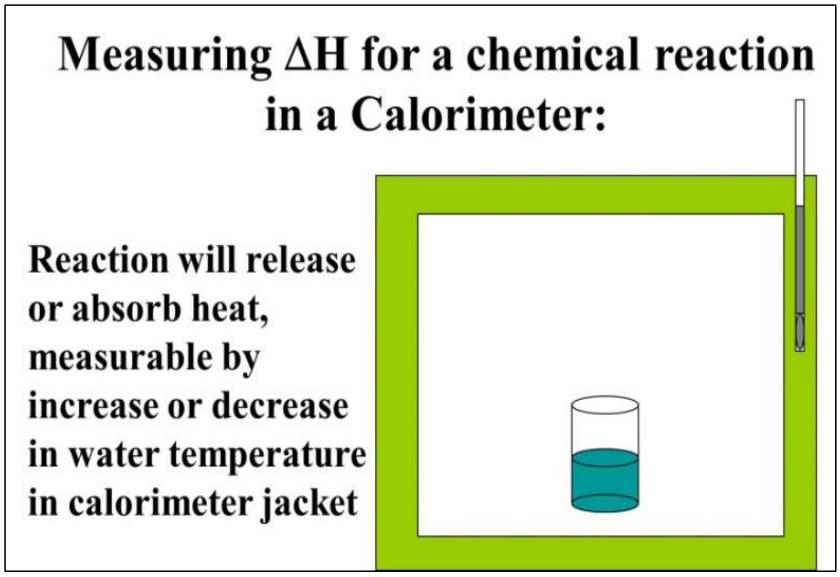


Fig. 4.4: Basic design of a *bomb calorimeter*, with an inner chamber for conducting reactions, and an outer chamber containing water with a thermometer to measure temperature change (heat absorption or release, or ΔH) during the reaction.

Food manufacturers determine the ***Calorie*** content of food by *bomb calorimetry*. As a reaction takes place in the beaker, it will either release or absorb heat, either heating or cooling the water in the calorimeter jacket, as measured by the thermometer. A closed system reaction that releases heat as it reaches *equilibrium* is defined as ***exothermic***; the **ΔH** for an *exothermic* reaction is negative. For example, a package says that a chocolate bar has 90 Calories. This means that burning the bar will generate 90 kilocalories (Kcal) of heat as measured in the calorimeter. Recall that one Calorie (with a capital C) = 1000 calories, or one Kcal. One calorie (lower case) is the energy needed to raise a gram of water by 1ºC).

You are probably most familiar with reactions that release heat, but some chemical reactions actually absorb heat. Take the common hospital cold pack for example. Squeeze it to get it going and toss it in the calorimeter and then watch the temperature in the calorimeter drop as the pack absorbs heat from the surroundings! Such reactions are defined as ***endothermic***, with a positive **ΔH**. OK, so we can determine the value of one of the energy parameters… we need to know at least one other, either **ΔGo** or **ΔS** before the equation **ΔH = ΔGo + TΔS** becomes useful.

4.3.4 How is Standard Free Energy Change (ΔG_0) Determined?

As it turns out, **ΔGo** or the standard free energy change is *directly proportional* to the concentrations of reactants and products of a reaction conducted to completion (i.e., ***equilibrium***) under standard conditions. Therefore, to determine **ΔGo**, we need to know or be able to measure the concentration of reactants and reaction products before and after a chemical reaction (i.e., when the reaction reaches *equilibrium*). Take the following generic chemical reaction:

$$2A + B \Longleftrightarrow 2C + D$$

The following equation relates ΔG_0 to the equilibrium concentrations of A, B, C and D:

$$\Delta Go = -RTlnKeq = RTln \frac{[C]^2[D]}{[A]^2[B]}$$

In this equation, **R** is the gas constant (1.806 cal/mole-deg), **T** is 298ºK and **Keq** is the ***equilibrium constant***. This is the ***Boltzman equation***. As you can see, the Keq for the reaction is the ratio of the product of the concentrations of the products (raised to their stoichiometric powers) to the product of the concentrations of the reactants (raised to *their* stoichiometric powers).

Once you know (or have determined) the equilibrium concentrations of reactants and products in a chemical reaction, you can use this equation to calculate **ΔGo** (*standard free energy change*) for a reaction. Remember, if the **ΔGo** is a negative number, the reaction is defined as ***exergonic***. We say that exergonic reactions release free energy. If the **ΔGo** is a positive number, the reaction absorbs free energy and is defined as ***endergonic***.

142 Determining ΔH & ΔG in Closed Systems

4.3.5 Working an Example Using These Equations for Closed Systems

Consider the following reaction:

$$X \rightleftharpoons Y$$

This reaction *endergonic*. If you do not know [X] and [Y] and therefore, ΔG_0, how can you tell?

If you are given [X] and [Y], you can also do the math. Assume we measure the concentrations of reactants and products for this reaction at equilibrium, with the following results:

[X] = 2.5 Kcal/Mole; [Y] = 500 cal/Mole

Try using the Boltzmann equation (above) to calculate the standard free energy for this reaction. What is the **Keq** for this reaction? What is the **ΔGo** for the reaction? If you did not come up with a Keq of 0.2 and an absolute value for the standard free energy for **|ΔGo|** of 866.2 Kcal/mole, re-calculate or collaborate with a classmate. Hint: make sure that you convert the units in your equation so that they are all the same!). Based on the calculated value of **ΔGo**, is this reaction in fact endergonic?

Now if you conduct the reaction in a bomb calorimeter, you find that it proceeds to equilibrium with a **ΔH** = -750 Kcal/Mole. What kind of reaction is this? Together with the *enthalpy change*, it is now possible to use the equation **ΔH = ΔGo + TΔS** to calculate an absolute value for the entropy change to be **|ΔS|** = 116.2 cal/mol-deg for the reaction. At equilibrium, the reaction proceeded with an increase in entropy under standard conditions. Again, if you did not get the correct answer, re-calculate or collaborate with a classmate.

143 Determining ΔS in Closed System

4.4 Summary: The Properties of Closed Systems

First, let's reiterate that there is no such thing as a closed system, unless of course the universe is one! What we call a closed system is simply one in which we can measure the energy going into or coming out of the system, and within which we can measure energy transfers and transductions (changes from one kind of energy to another. Features of closed systems can be defined by their properties:

- **Closed systems are experimentally *defined by an investigator*.**
- **Defined *Standard conditions* apply.**
- **Energy entering or leaving the system is *measurable*.**
- **Reactions reach *equilibrium* regardless of reaction rate.**
- **Product and reactant concentrations at equilibrium are *constant* (hence the Keq)**
- **Measured energy transfer/transduction values are *constant* (for a given set of standard conditions).**

Now let's turn our attention to open systems, since that's what cells are!

4.5 Open Systems and Actual Free Energy Change

Cells are ***open systems*** that are constantly exchanging mass and energy with their environment; They ***never reach equilibrium***. In addition, diverse organisms live under very different atmospheric conditions and maintain different body temperatures (e.g., your cat has a higher body temperature than you do!). Clearly, the conditions under which cells conduct their biochemical reactions are decidedly *non-standard*. However, while *open systems* do not reach equilibrium, they *do* achieve a ***steady state*** in which the rate of input of energy and matter is equal to the rate of output of energy and matter. Think of a biochemical pathway like glycolysis. If a cell's energy needs are constant, the pathway will reach a *steady state*. Of course, a cell's need for energy (as ATP) can change as energy needs change. If it does, then the steady state of ATP production will change to meet the needs of the cell.

Later we will be discussing just how energy flows through living things, from sunlight into chemical energy in nutrient molecules, into energy-rich fuels like ATP, and finally, into the performance of all manner of cellular work. We characterize open systems by their properties:

- **Open systems *exchange energy and mass with their surroundings*.**
- **Open systems *never reach equilibrium***
- **They *achieve steady state* where the energy input rate = output rate.**

- **The *steady state can change*.**
- **In open systems, *endergonic reactions can be energetically favorable* (spontaneous) and exergonic reactions can become energetically unfavorable.**

Fortunately, there is an equation to determine free energy changes in open systems. For our chemical reaction **2A + B <====> 2C + D**, this equation would be:

$$\Delta G' = \Delta Go + RT\ln \frac{[C]_{ss}^2 [D]_{ss}}{[A]_{ss}^2 [B]_{ss}}$$

Here, **ΔG'** is the ***actual free energy change*** for a reaction in an open system. **ΔGo** is the standard free energy change for the same reaction under standard conditions. In a closed system. **R** is again the gas constant (1.806 cal/mole-deg) and **T** is the absolute temperature in which the reaction is actually occurring. The subscript '**ss**' designates reactant and product concentrations measured under *steady state* conditions. You can see here that this equation states a relationship between **ΔGo** and **ΔG'**. So, to determine the actual free energy of a biochemical reaction in a cell (in fact in any living tissue), all you need to know are the ΔGo for the reaction, the steady state concentrations of reaction components in the cells/tissues, and the absolute T under which the reactions are occurring.

144 The Energetics of Open Systems

Elsewhere, we will use the reactions of the glycolytic pathway to exemplify the properties, as well as the energetics of open and closed systems. At that time, pay careful attention to the application of the terminology of energetics in describing energy flow in *closed* vs. *open systems*.

Some iText & VOP Key Words and Terms

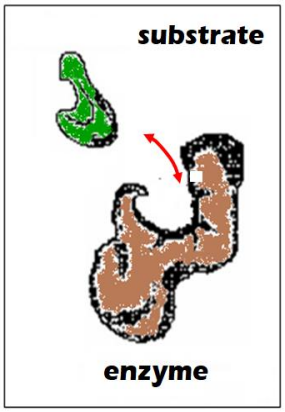
actual free energy	**endothermic**	**Law of Conservation**
ATP	**energy**	**Laws of Thermodynamics**
bioenergetics	**energy transduction**	**light**
Boltzman equation	**energy transfer**	**mass**
calories	**enthalpy**	**open system properties**
calorimeter	**entropy**	**open systems**
chemical energy	**equilibrium constant**	**order vs. entropy**

chemical equilibrium	exergonic	standard conditions
closed systems	exothermic	standard free energy
decibels	free energy	steady state
$e=mc^2$	gas constant	useful energy
electricity	Gibbs free energy	useless energy
endergonic	Keq	volts

Chapter 5: Enzyme Catalysis and Kinetics

Mechanism of Enzyme Catalysis, Induced Fit, Activation Energy, Determining and Understanding Enzyme Kinetics

Listen here:
https://www.youtube.com/watch?v=94M7B7bz-N0



Or here…
https://www.youtube.com/watch?v=bUfLHJg-CY0

"We belong to a mutual admiration society…"

5.1 Introduction

By definition, all catalysts accelerate chemical reactions, including enzymes. But enzymes and inorganic catalysts differ in important ways (Table 5.1 below).

Enzymes *vs* Inorganic Catalysts

Inorganic Catalysts	Enzymes
e.g., Ni, Pl, Ag, etc.	e.g., pepsin, trypsin, ATP synthase, ribonuclease, etc.
increase rxn rate	increase rxn rate
unchanged at end of rxn	unchanged at end of rxn
non-specific	highly specific
rigid, inflexible	flexible - can undergo *allosteric* change…
cannot be regulated	can be regulated

In this chapter, we look at the properties and mechanism of action of enzymes. These include allosteric change (***induced fit***, ***enzyme regulation***), energetic events (changes in ***activation energy***), and how enzymes work in open and closed (experimental) systems.

Most ***Enzymes*** are proteins, but as we'll see later, a few are RNAs. Enzymes are long polymers that can fold into intricate shapes. As a result, they can be more specific than inorganic catalysts in which substrates they recognize and bind to. Finally, enzymes are flexible and can be regulated in ways that rigid, inflexible, metallic inorganic catalysis cannot. The specificity of an enzyme lies in the structure and flexibility of its ***active site***. We will see that the active site of an enzyme undergoes conformational change during catalysis. The flexibility of enzymes also explains the ability of enzymes to respond metabolites in a cell that indicate its biochemical status. When such metabolites bind to an enzyme, they force a conformational change in the enzyme that can speed up or Slow down the catalytic rate of the reaction, a phenomenon called ***allosteric regulation***. As you might imagine, changing the rate of a biochemical reaction can change the rate of an entire biochemical pathway…, and ultimately the steady state concentrations of products and reactants in the pathway.

To understand the importance of allosteric regulation, we'll look at how we measure the speed of enzyme catalysis. As we consider the classic early 20th century enzyme kinetic studies of Leonor Michaelis and Maud Menten, we'll focus on the significance of the ***Km*** and ***Vmax*** values that they derived from their data. But before we begin our discussion here, remember that chemical reactions are by definition, reversible. The action of catalysts, either inorganic or organic, depends on this concept of reversibility.

Finally, let's give a nod to recent human ingenuity that enabled enzyme action to turn an *extracellular profit*! You can now find enzymes in household cleaning products like spot removers and detergents, where they digest and remove stains caused by fats and pigmented proteins. Enzymes added to meat tenderizers also digest (hydrolyze) animal proteins down to smaller peptides. Enzymes can even clean a clogged drain!

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. describe how the molecular flexibility of protein and RNA molecules make them ideal biological catalysts.
2. compare and contrast the properties of *inorganic* and *organic* catalysts.
3. explain why *catalysts do not change equilibrium concentrations* of a reaction conducted in a closed system.
4. compare the activation energies of catalyzed and un-catalyzed reactions and explain the roles of *allosteric effectors* in enzymatic reactions.
5. discuss how allosteric sites interact with an enzyme's active site and explain the concept of the rate limiting reaction in a biochemical pathway.

6. write simple rate equations for chemical reactions.
7. write the possible rate equations for catalyzed reactions.
8. distinguish between *Vmax* and *Km* in the Michaelis-Menten kinetics equation.
9. state what Vmax and Km say about the progress of an enzyme catalyzed reaction.
10. interpret enzyme kinetic data and the *progress of an enzyme-catalyzed reaction* from this data.
11. more accurately identify Leonor Michaelis and Maud Menten!

5.2 Enzymes and the Mechanisms of Enzyme Catalysis

Studies by George Beadle and Edward Tatum correlating mutations with enzyme deficiencies in *Neurospora crassa* (bread mold) and *Drosophila melanogaster* led them to propose the ***one-gene/one-enzyme hypothesis*** in 1941. By 1958, they shared Nobel Prize in Physiology and Medicine for this work and their hypothesis had already morphed twice… first into the ***one-gene/one-prot*ein**, and then the ***one-gene/one-polypeptide*** hypothesis. This rightfully revered history helped launch the age of molecular biology. The subsequent discovery of RNA catalysts came as quite a surprise! The revelation of RNA catalysts, dubbed ***ribozymes***, earned Sidney Altman and Thomas Cech a Nobel Prize in Chemistry in1989. *Ribozymes* are now known to catalyze RNA splicing (the removal of unwanted regions of a precursor RNA). They also catalyze a step in protein synthesis (translation) on ribosomes. In fact, almost no biochemical reaction exists that is not directly the result of enzyme catalysis, from the digestion of nutrients in your mouth, stomach and small intestines to pretty much every chemical reaction inside your cells [check out Kornberg A (1989) *Never a Dull Enzyme.* Ann. Rev. Biochem. 58:1-30]. In this chapter the focus on the long history of *protein enzyme catalysis*. But as you study, you may recognize that the mechanisms of enzyme catalysis described here involve common essential features seen in all biocatalysts.

Enzymes are generally soluble in or outside cells while a few are part of membranes or other cellular structures. In all cases, they bind to soluble ***substrates*** (the reactants in enzyme-catalyzed reactions). The large size and exquisite diversity of protein structures make enzymes highly specific catalysts. The specificity of an enzyme results from the shape of the ***active*** site of the enzyme, which is dependent on the three-dimensional arrangement of amino acids in and around the region. The *substrates* of a catalyzed biochemical reaction are bound to and held in place on the enzyme while rapid bond rearrangements take place. Their flexibility allows enzymes to change in shape at the active site during catalysis. In addition, this flexibility enables small metabolites in cells to interact with and change the shapes of many enzymes, changing their rates of catalysis. The latter phenomenon enables ***allosteric regulation***, allowing cells to control the rates and even the direction of biochemical reactions and pathways. As we will see, enzymes may also be bound to *prosthetic groups* or ions that contribute to the shape and activity of the enzyme.

Any, understanding the mechanism of catalysis must also include knowing something about the energetics of catalyzed reactions. We'll see that enzymes lower the ***activation energy*** of a chemical reaction, and that *activation energy* is an inherent *energy barrier* to the reaction. We will look at the energetics of enzyme action.

In sum, we describe the action of biological catalysis in terms of the structural features of the enzyme (active site shape, overall conformation, the *affinities* of the enzyme for its substrates), as well as free energy changes that occur during catalysis. Of course, structural and energy considerations of enzyme catalysis are related.

145 Enzymes vs. Other Catalysts

5.2.1 Structural Considerations of Catalysis

From a chemistry course, you may recall that the rate of an uncatalyzed reaction is dependent on the concentration of the reactants in solution. This is the *Law of Mass Action*, recognized in the19th century. Look at this simple reaction:

$$\mathbf{A + B \rightleftharpoons C + D}$$

The *Law of Mass Action* makes two key assumptions:
1) At any given time following the start of the reaction, the rate of product formation is proportional to the concentrations of the reactants and products ([A], [B], [C] and [D] in this case).
2) Chemical reactions in the laboratory (i.e., a '*closed system*') eventually reach equilibrium, at which point the net rate of formation of reaction products is zero. (in other words, the forward and reverse reactions occur at the same rate).

There are no products (i.e., C and D) at the start of the reaction written above. As there are no products yet, the reaction rate should be directly proportional only to the concentration of the reactants. The *Law of Mass Action* predicts that the chemical reaction above will occur faster when A and B are first mixed together…, that is, at higher concentrations of A & B. This is because there are more reactant molecules in solution and a greater likelihood that they will collide in an orientation that allows the bond rearrangements for the reaction to occur.

Of course, reactant concentrations decline as products accumulate over time and the rate of formation of C & D slows down. At some point, as products accumulate they should also influence the rate of their own production. Remember, all chemical reactions are inherently reversible! So rising levels of products will begin to push the reaction to form A and B, slowing down the net accumulation of C and D. You may

recall chemical rate equations from a chemistry course; these enable quantitation of reaction rates for our sample reaction. Here is the rate of formation of the products: **C & D formation rate = $k_1[A][B] - k_{-1}[C][D]$,** where **$k_1[A][B]$** is the rate of the forward reaction; **$k_{-1}[C][D]$** is the rate of the reverse reaction. In this way, the equation recognizes that the reaction is reversible. The equation states that the net reaction rate is equal to the *rate of the forward reaction* (**$k_1[A][B]$**) minus the *rate of the back reaction* (**$k_{-1}[C][D]$**). The equation is valid (applicable) at any time during the reaction. **k_1** and **k_{-1}** are ***rate constants*** for the forward and reverse reactions, respectively.

So how do catalysts work? Catalysts increase chemical reaction rates by bringing reactants together more rapidly than they would otherwise encounter each other based just on random molecular motion in solution. This is possible because catalysts have an ***affinity*** for their substrates. In the case of inorganic catalysts, relatively weak, generic forces account for the affinity of reactants and inorganic catalysts. Thus, a metallic catalyst (e.g., silver, platinum) attracts molecules with the appropriate (usually charge) configuration. If the attraction (*affinity*) is sufficient, the metal will hold reactants in place long enough to catalyze the bond rearrangements of a chemical reaction.

Unlike inorganic catalysts, enzymes have evolved highly specific shapes with physical-chemical properties. As a result, typical enzymes only attract the substrates necessary for a particular biochemical reaction. The active site of an enzyme has an exquisite, selective affinity for its substrate(s). This affinity is many times greater than those of inorganic catalysts for generic reactants. The result is that enzymes are more efficient and faster than inorganic catalysts. Early ideas of how substrate-enzyme interaction could be so specific suggested a ***Lock and Key*** mechanism (Fig. 5.1).

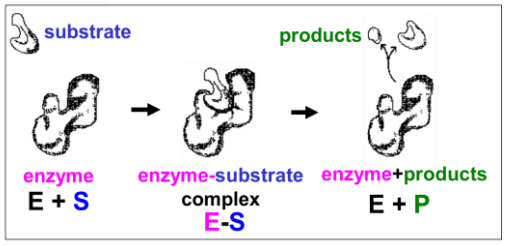


Fig. 5.1: Illustration of the early *Lock-&-Key* mechanism for enzyme-substrate interaction, in which the substrate *key* fits an enzyme *lock*.

According to this model, the affinity of enzyme for substrate engages the substrate '*key'* in the tumblers (i.e. active site) of enzyme '*lock'*. Thus engaged, the substrate(s) would undergo the bond rearrangements specific for the catalyzed reaction to

generate products and regenerate an unchanged enzyme. But X-ray crystallography of enzyme-substrate interactions reveal that the active site of the enzymes change shape during catalysis. This *allosteric change* suggested the ***Induced Fit*** mechanism of enzyme action (modeled in Fig. 5.2).

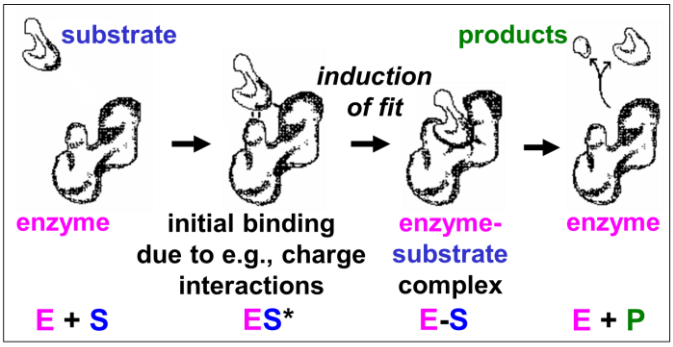


Fig. 5.2: The *Induced Fit* mechanism of enzyme-substrate interaction. Initial interaction of substrate with enzyme based on mutual affinity causes an allosteric change in the enzyme. This results in the induced 'better' fit of substrate to enzyme, to be followed by catalysis.

In this model, enzyme-substrate affinity causes the substrate to bind to the enzyme surface. Once bound, the enzyme undergoes an allosteric change, drawing the substrate(s) more tightly into the active site and catalyzing the reaction. Of course, after the reaction products come off, the enzyme returns to its original shape.

146-2 Induced Fit Mechanism of Enzyme Action

5.2.2 Energetic Considerations of Catalysis

Consider the random motion of substrates in solution that occasionally encounter one another. They even more rarely bump into one another in just the right orientation to cause a reaction. This explains why adding more reactants or increasing the temperature of a reaction can speed it up…, by increasing the number of random as well as productive molecular collisions. Unlike molecules and reactions in a test tube, living organisms do not have these options for increasing reaction rates.

But they have enzymes! *All* catalysts work by lowering the ***activation energy*** (***E*$_a$**) for a reaction, thereby increasing the rate of the reaction. Activation energy is essentially a barrier to getting substrates together to actually undergo a biochemical reaction.

Inorganic catalytic surfaces attract reactants where catalysis can occur. The attractions are weak compared to those of enzymes and their substrates. An enzyme's active site attracts otherwise randomly distributed substrates very strongly, making enzyme catalysis faster than inorganic catalysis. Again, cells cannot use inorganic catalysts, most of which are insoluble and would attract reactants indiscriminately... not a good way for cells to control metabolism! The advent of enzymes with their specificity and high rate of catalysis was a key event in *chemical evolution* required for the origins of life. As we saw, allosteric change during the 'induction of fit' enables specific catalysis. In fact, a catalyzed reaction will be faster than the same reaction catalyzed by a piece of metal, and of course much faster (millions of times faster!) than the uncatalyzed reaction. The energetics of catalysis helps to explain why. Take a look at the energetics of a simple reaction in which A & B are converted to C & D (Fig. 5.3).

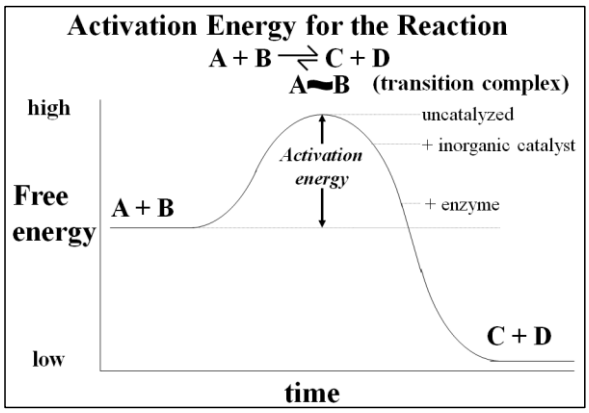


Fig. 5.3: Graph of change in free energy over time as chemicals A and B react. The high *Activation Energy* peak in the graph is the *free energy barrier* that A and B must overcome before much C and D can be made. This barrier is due mainly to thermal motion of the A and B molecules, with only rare encounters in relatively dilute solutions. Enzymes are even more efficient that inorganic catalysts in lowering the activation energy barrier to a reaction.

Conducted in a closed system, enzyme-catalyzed reactions reach their equilibrium more rapidly. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium concentrations of reactants and products of these reactions. The roughly 4000 biochemical reactions known to be catalyzed in cells are undoubtedly an underestimate! But remember too, that we estimate that the human genome has only 20,000 to 25,000 different genes!

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5.3 Enzyme Regulation

We noted that some enzymes are regulated, which just means that there are factors in the cell that can slow down or speed up their rate of catalysis. In this way the cell can respond quickly to metabolic needs reflected by the intracellular levels of these factors. Factors that slow down catalysis are ***inhibitors***. Those that speed up catalysis are called ***activators***. In addition to responding to intracellular molecular indicators of the metabolic status of the cell, enzymes may be inhibited by drugs, poisons or changes in the chemical milieu (e.g. pH). Since cellular reactions occur as part of biochemical pathways, regulating a single enzyme can affect an entire pathway (e.g., Fig. 5.4).

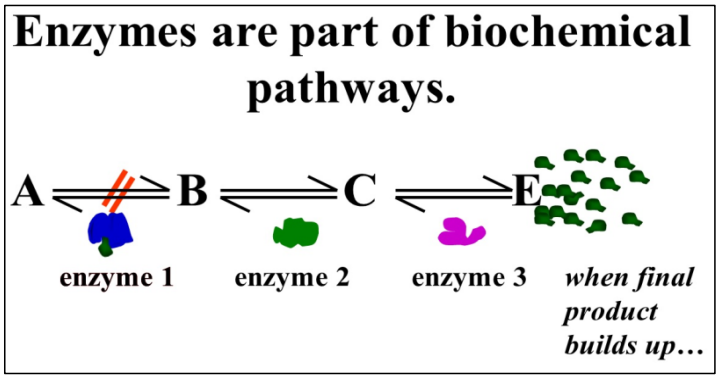

Fig. 5.4: A generic biochemical pathway; The end product of the pathway (E) accumulates. The pathway is regulated to prevent wasteful accumulation of E. When some of the accumulating E binds to enzyme 1, it blocks its catalysis, preventing the reaction from A to form B.

This pathway exists to produce substance **E**. Under normal conditions, another series of metabolic reactions would consume **E**. However, if the cell no longer needs so much **E**, it will accumulate in the cell. If there is an excess of **E** in the cell, some of the excess might bind to one of the enzymes. In the pathway shown, **E** binds to *enzyme 1.* This binding causes an allosteric change in the enzyme, inhibiting catalysis and slowing down the entire pathway. In this example of ***allosteric regulation***, the inhibitory regulation of **enzyme 1** evolved to control the rate of production of substance **E**. This common mode of *allosteric regulation* is called ***feedback inhibition***.

Enzymes can be regulated precisely because they can be *bent out of shape* (or into shape for that matter!). Some small metabolites become chemical information when they accumulate in cells, able to communicate cellular metabolic status. The result is a decrease or increase enzyme activities to achieve an appropriate cellular response.

Whether an activator or an inhibitor of enzyme catalysis, regulatory molecules typically bind to enzymes at ***allosteric regulatory*** sites, causing local conformational changes in the enzyme that are transmitted to the active site. *Enzyme inhibition* will occur if a change in shape reduces the affinity of enzyme for substrate… or if it reduces the rate of bond rearrangements after the substrate has entered the active site. *Enzyme activation* would occur if the allosteric effect were to increase this affinity and/or catalytic rate. The mechanism of allosteric regulation of enzyme activity is shown in Fig. 5.5.

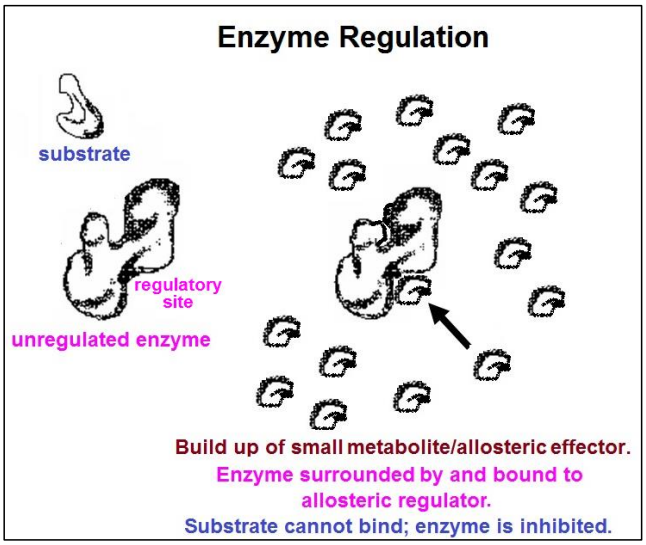

Fig 5.5: *Allosteric effectors* are small metabolites whose amounts in a cell reflect the cell's metabolic status. They bind to enzymes at a regulatory site, causing an *allosteric* (conformational) change in the enzyme that affects the shape of the *Active Site*, either inhibiting catalysis (as suggested here) or stimulating it.

148-2 Allosteric Regulation of Enzyme Activity

5.4 Enzyme Kinetics

Studying enzyme kinetics can tell us not only about the catalytic properties of a particular enzyme, but can also reveal important properties of biochemical pathways. Thus, one can determine a standard *rate-limiting reaction* under a given set of conditions by comparing kinetic data for each enzyme in a biochemical pathway. But what else can we do with kinetic data?

5.4.1 Why Study Enzyme Kinetics?

Apart from its value in understanding how an individual enzyme actually works, kinetic data has great clinical value. For example, if we know the kinetics of each enzyme in the biochemical pathway to the synthesis of a liver metabolite in a healthy person, we also know which enzyme is normally the *rate-limiting* (slowest) catalyst in the pathway. Consider a patient whose blood levels of this metabolite are higher than normal. Could this be because what was the normal rate-limiting reaction is no longer rate-limiting in the patient? Which enzyme in the patient then, is newly rate-limiting? Is there a possible therapy in having this information? One can ask similar questions of an alternate scenario in which a patient is producing too little of the metabolite. Reasons why a cellular biochemical would deviate from 'normal' levels include:

- ***Viral & bacterial infection or environmental poisons:*** these can interfere with a specific reaction in a metabolic pathway; remedies depend on this information!
- ***Chronic illness resulting from mutational enzyme deficiencies:*** treatments might include medications designed to enhance or inhibit (as appropriate) enzyme activity.
- ***Genetic illness tied to metabolic deficiency:*** if a specific enzyme is the culprit, investigation of a pre- and/or post-natal course of treatment might be possible (e.g., medication or perhaps even gene therapy).
- ***Life-style changes and choices:*** these might include eating habits, usually remediated by a change in diet.
- ***Life-Style changes brought on by circumstance rather than choice:*** these are changes due to aging. An all too common example is the onset of Type 2 Diabetes. This can be treated with medication and/or delayed by switching to a low carb diet favoring hormonal changes that improve proper sugar metabolism.

Knowing the rate-limiting reaction(s) in biochemical pathways can identify regulated enzymes. This in turn may lead to a remedy to correct a metabolic imbalance. As noted, ribozymes are RNA molecules that catalyze biochemical reactions; their kinetics can also be analyzed and classified. We will consider how enzymes are regulated later, when we discuss glycolysis, a biochemical pathway that most living things use to extract energy from nutrients. For now let's look at an overview of *experimental* enzyme kinetics and how we interpret kinetic data.

5.4.2 How We Determine Enzyme Kinetics and Interpret Kinetic Data

In enzyme kinetic studies, the enzyme is considered to be a reactant, albeit one that is regenerated by the end of the reaction. The reaction begins when substrate are added to the enzyme. In enzyme kinetic studies, the concentration of the enzyme is held constant while reaction rates are measured after adding different amounts of

substrate. As a consequence, all catalyzed reactions will reflect *saturation* of the enzyme at high concentration of substrate. This is the basis of saturation kinetics illustrated below in Fig.5.6.

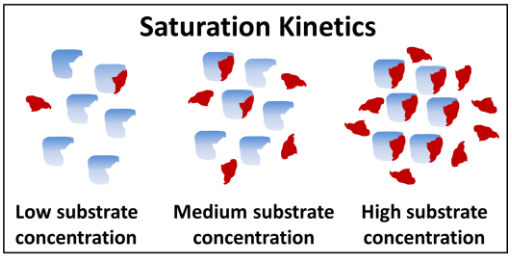

Fig. 5.6: *Saturation kinetics* of an enzyme-catalyzed reaction. At high substrate concentrations, all the active sites of all enzymes are occupied. Under these conditions, the reaction occurs at its fastest rate.

From the illustration, the active sites on all the enzyme molecules are bound to substrate molecules at high substrate concentration. Under these conditions, a catalyzed reaction is proceeding at its fastest. Let's generate some kinetic data to see saturation in action. The experiment illustrated below (Fig. 5.7, below) will determine the kinetics of the conversion of S to P by enzyme E.

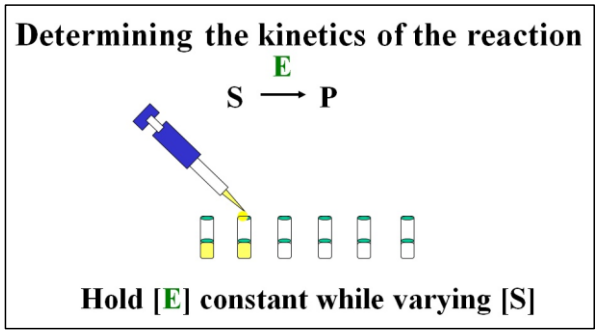

Fig. 5.7: Experimental protocol for determining the kinetics of an enzyme-catalyzed reaction.

A series of reaction tubes are set up, each containing the same concentration of enzyme ([E]) but different concentrations of substrate ([S]). The concentration of P ([P]) produced at different times beginning just after the start of the reaction in each tube is plotted to determine the *initial rate* of P formation for each concentration of substrate tested. Fig. 5.8 below is such a plot.

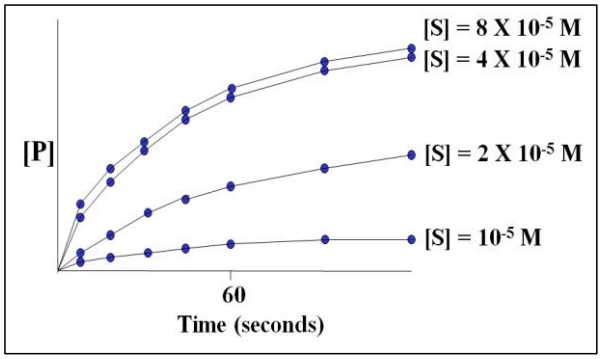

Fig. 5.8: This graph plots the rate of product (P) formation at 4 different substrate concentrations, while holding the enzyme concentration constant.

In this hypothetical example, the rates of the reactions (amount of P made over time) do not increase at substrate concentrations higher than 4 X10^{-5} M. The upper curves thus represent the maximal rate of the reaction at the experimental concentration of enzyme. We say that the maximal reaction rate occurs at ***saturation***.

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Next, we can estimate the initial reaction rate (v_o) at each substrate concentration by plotting the slope of the first few time points through the *origin* of each curve in the graph. Consider the graph of the initial reaction rates estimated in this way in Fig. 5.9 (below).

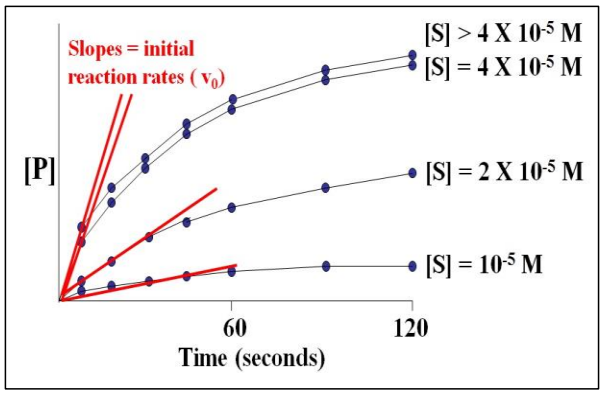

Fig. 5.9: This graph shows the slopes of the initial reaction rates (red) taken from the curves in the previous graph (Fig. 5.8).

The slope of each straight line is the v_o for the reaction at a different [S], near the very beginning of the reaction when [S] is high and [P] is vanishingly low. Next, we plot these rates (slopes, or v_o values) against the different concentrations of S in the experiment to get the curve of the reaction kinetics in Fig. 5.10.

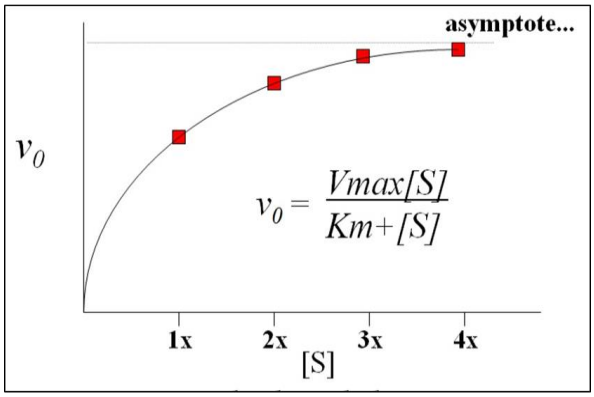

Fig. 5.10: This graph plots the initial reaction rates (slopes, or v_0) for the reactions plotted in Fig. 5.9. The formula shown for this curve is that of a *rectangular hyperbola*.

This is an example of Michaelis-Menten kinetics common to many enzymes, named after the two biochemists who realized that the curve described ***rectangular hyperbola***. Put another way, the equation mathematically describes the mechanism of catalysis of the enzyme. The equation below mathematically describes a rectangular hyperbola:

$$y = \frac{xa}{x+b}$$

You might be asked to understand the derivation of the Michaelis-Menten equation in a Biochemistry course. You might even be asked to do the derivation yourself! We won't make you do that, but suffice it to say that Michaelis and Menten started with some ***simple assumptions*** about how an enzyme-catalyzed reaction would proceed and then wrote reasonable chemical and rate equations for those reactions. The goal here is to understand those assumptions, to see how the kinetic data support those assumptions, and to realize what this tells us about how enzymes really work. Here is one way to write the chemical equation for a simple reaction in which an enzyme (E) catalyzes the conversion of substrate (S) to product (P):

$$S \underset{E}{\rightleftharpoons} P$$

Michaelis and Menten rationalized that this reaction might actually proceed in three steps. In each step enzyme **E** is treated as a reactant in the conversion of S to P. The resulting chemical equations are shown below.

$E + S \rightleftharpoons E\text{-}S$	binding of enzyme and substrate
$E\text{-}S \rightleftharpoons E\text{-}P$	conversion of substrate to product
$E\text{-}P \rightleftharpoons E + P$	dissociation of product and enzyme

Reasoning that the middle reaction (the conversion of E-S to E-P) would be the fastest one and therefore would not be the *rate-limiting reaction of catalysis*, they only considered the first and third reactions to be relevant in determining the overall kinetics of product formation. Then they wrote the following rate equations for just these two chemical reactions (as one would in an introductory chemistry course):

$$v_{\text{E-S formation}} = k_1[E][S] - k_{-1}[E\text{-}S]$$

$$v_{\text{P formation}} = k_2[E\text{-}S] - k_{-2}[E][P]$$

Both of these equations describe a straight line, which does not describe the observed hyperbolic reaction kinetics. Solving either equation for [E-S] and substituting the solution for [E-S] in the other equation left a single equation. But this equation also described a straight line. Again, not the expected rectangular hyperbola. To arrive at a chemical rate equation consistent with a rectangular hyperbola, Michaelis and Menten made several assumptions, including those made by G. E. Briggs and J. B. S. Haldane about how E, S and P would behave in a catalyzed reaction.

It was those assumptions allowed them to re-write each equation, combine and rewrite them into a single mathematical equation that did indeed describe a rectangular hyperbola. Here are Briggs and Haldane's assumptions:

1. **[S] >> [E] at the start and during the 'steady state'.**
2. **[P] << [S] at the start of a reaction.**
3. **All E is bound to S at the start of the reaction.**
4. **$[E]_{total} = [E]_{free} + [E\text{-}S]$ at all times.**

We have already seen the equation that Michaelis and Menten derived and now known as the Michaelis-Menten equation:

$$v_0 = \frac{Vmax[S]}{Km + [S]}$$

The take-home message here is that the assumptions about an enzyme-catalyzed reaction are a good approximation of how the reaction proceeds over time.

Michaelis and Menten defined Vmax and Km as key kinetic factors in enzyme-catalyzed reactions. In the generic example of substrate conversion to product, we saw that increasing [S] results in a higher rate of product formation because a higher rate of encounters of enzyme and substrate molecules. At some point however, increasing [S] does not increase the initial reaction rate any further. Instead, v_0 ***asymptotically*** approaches a theoretical maximum for the reaction, defined as ***Vmax***, the *maximum initial rate*. As we have already seen, *Vmax* occurs when all available enzyme active sites are saturated (occupied by substrate). At this point, the intrinsic catalytic rate determines the *turnover rate* of the enzyme. The substrate concentration at which the reaction rate has reached ½*Vmax* is defined as K_M (the *Michaelis-Menten constant*). The Km is a ratio of rate constants remaining after rewriting the rate equations for the catalyzed reaction.

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To recapitulate, the two most important kinetic properties of an enzyme are:

1. how quickly the enzyme becomes saturated with a particular substrate, which is related to the *Km* for the reaction, and
2. the maximum rate of the catalyzed reaction, described by the *Vmax* for the reaction.

Knowing these properties suggests how an enzyme might behave under cellular conditions, and can show how the enzyme should respond to allosteric regulation by natural inhibitory or activating factors…, and to poisons or other noxious chemicals.

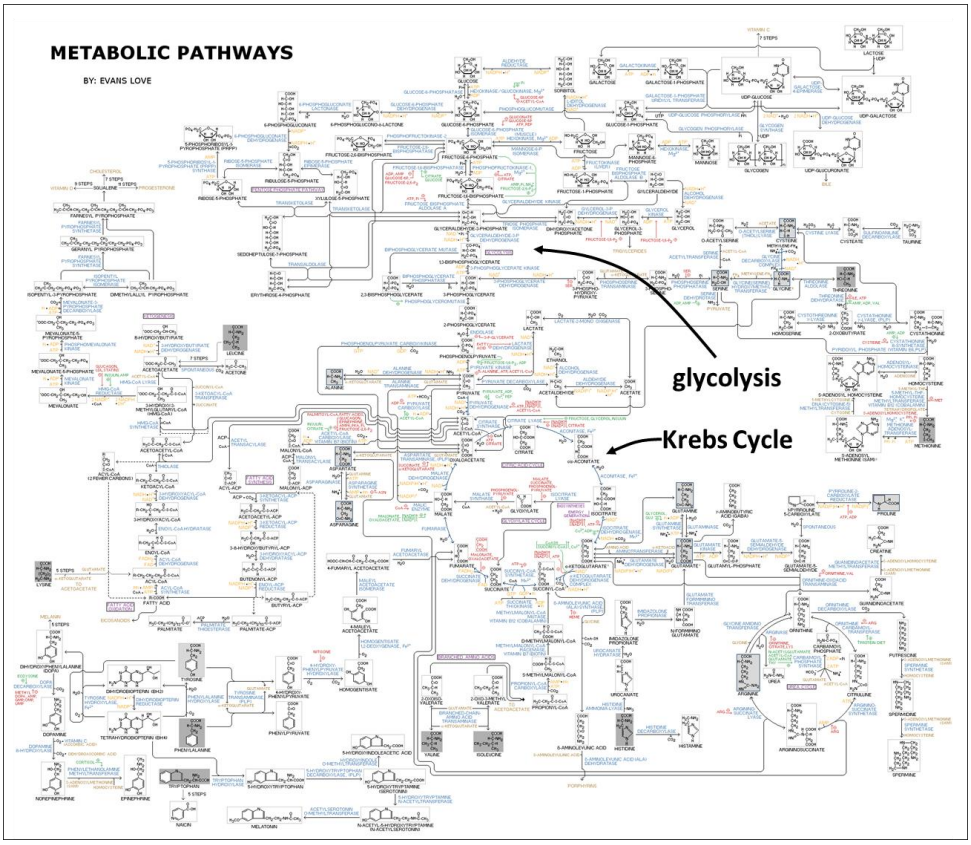
For clear, detailed explanations of enzyme catalytic mechanisms, check out Jencks WP 1987, *Catalysis in Chemistry and Enzymology*. Mineola, NY, Courier Dover Publications]. You can also find more details of how kinetic equations are derived (an important step in understanding how the enzyme works) in any good biochemistry textbook, or check out the *Michaelis-Menten Kinetics* entry in in the Enzymes Wikipedia link.

Some iText & VOP Key Words and Terms

activation energy	enzyme	Michaelis-Menten constant
active site	enzyme activation	Michaelis-Menten kinetics
allosteric change	enzyme inhibition	rate-limiting reaction
allosteric regulation	enzyme kinetics	ribozyme
allosteric site	enzyme regulation	saturation kinetics
biochemical pathway	induced fit	substrate specificity
catalytic RNAs	inorganic catalyst	substrates
conformation	Km	Vmax

Chapter 6: Glycolysis, the Krebs Cycle and the Atkins Diet

Glycolysis, Gluconeogenesis & the Krebs Cycle - Getting Energy from Food;
Enzyme Regulation & the Bioenergetics of Cellular Free Energy Capture;
Liver Cells in Glucose metabolism; Fooling Your Body - Atkins (& Similar) Diets



This (in fact any!) metabolic pathways chart highlights the centrality of the Krebs (citic acid) cycle

6.1 Introduction

We used to get metabolic pathways charts like the one you see here free from vendors of biochemical reagents. This one is a high-resolution image; if you zoom in, you can actually read the content…, but don't feel you must! The big picture is correct in macro-detail, but the chart is likely out of date in small new details. In this chapter, we 'zoom in' on the small region in the middle of the chart, encompassing glycolysis and the Krebs cycle.

We have looked at the principles governing ***thermodynamics*** (the flow of energy in the universe) and *bioenergetics* (energy flow in living systems). We saw evidence that energy can be exchanged between components in the universe, but that it can be *neither created nor destroyed*. That makes the universe a *closed system*, a conclusion codified as the **F*irst Law of Thermodynamics***. Personally, I find it troubling that there is no escape from the universe…, that is, until I remind myself that the universe is a pretty big place, and I am only a small part of a small system. You can define systems for yourself: the solar system, planet earth, the country you pledge allegiance to, your city or village, your school, a farm or homestead…! Then you may derive comfort from the realization that you can move from one system to another and even exchange goods and services between them. This is a metaphor for energy flow between systems in the universe. We also saw that the *First Law* applies to ***closed systems*** *within the universe*…, and that there are no closed systems in the universe! Any system in the universe is open, always exchanging energy and mass with neighboring systems. What we mean by the term closed system is that we can define and *isolate* some small part of the universe, and then *measure* any energy that this isolated system gives up to its environment, or takes in from it. The simplest demonstration of the *First Law* in action was the *bomb calorimeter* that measures heat released or absorbed during a chemical reaction.

The second concept said that energy flows from one place to another only *when it can*. In the vernacular, we say that ***energy flows downhill***. Anything that happens in the universe (a galaxy moving through space, a planet rotating, getting out of bed in the morning, coffee perking, your cells sugar burning, DNA replicating…) does so because energy flows downhill. We saw that by definition, any happening or event in the universe, however large or small, is ***spontaneous***. That is, it occurs with a release of ***free energy***. Remember, *spontaneous* means "by itself" and not necessarily instantaneous or fast! Finally, we noted that that when enzymes catalyze biochemical reactions in a closed system, the reactions still reach equilibrium, despite the higher rate of the catalyzed reaction. What does this tell you about the energetics of catalyzed reactions in *closed systems*?

With this brief reminder about energy flow and what enzymes do, we'll look at how our cells capture ***nutrient free energy***. This will include examples of the energetics of closed systems that reach equilibrium, and open systems that don't! First, we tackle ***glycolysis***, an ***anaerobic fermentation*** pathway for generating chemical energy from glucose, as well as the first of several ***aerobic*** pathways of ***respiration***. We'll see that most of the energy from glycolysis and respiration is captured in molecules of ATP, the universal energy currency of life, used by cells to… live! Then we look at ***Gluconeogenesis***, a regulated reversal of glycolysis. We ask when, where and why we would want to make, rather than burn glucose. Finally, we begin a discussion of respiration with a look at the ***Krebs Cycle***. The complete respiratory pathway can be summarized by the following equation:

$$C_6H_{12}O_6 + 6O_2 \rightleftharpoons 6CO_2 + 6H_2O$$

The **standard free energy change** for this reaction *(ΔGo*) is about -687Kcal/mole. This is the maximum amount of nutrient free energy that is (at least in theory) available from the complete respiration of a mole of glucose. Given a cost of about 7.3 Kcal to make each mole of ATP (*adenosine triphosphate*), how many moles of ATP might a cell produce after burning a mole of glucose? We'll figure this out here.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain the difference between fermentation and respiratory glycolysis and the role of redox reactions in both processes.
2. Calculate, compare and contrast *ΔGo and ΔG'* for the same reaction, and explain any differences in free energy in open and closed systems.
3. describe and explain the major events of the first stage of glycolysis and trace the free energy changes through the formation of G-3-P.
4. describe and explain the major events of the second stage of glycolysis and trace the free energy changes through the formation of pyruvate and lactic acid.
5. state the role of *redox reactions* in glycolysis and fermentation.
6. compare and contrast glucose (i.e., carbohydrates in general), ATP, NADH and FADH2 as *high-energy* molecules. [Just for fun, click Power in the Primordial Soup to read some *far out* speculations on prebiotic high-energy molecules that might have been around when ATP was being hired for the job!].
7. explain why only a few cell types in the human body conduct *gluconeogenesis*.
8. explain how gluconeogenesis, an energetically unfavorable pathway, can occur at all.
9. explain why the *Atkins Diet* (and similar diets) work and speculate on any downsides.
10. explain the concept of a *super-catalyst* and explain why a *super-catalyst* like the *Krebs Cycle* would have evolved.
11. explain the role of high energy linkages and electron carriers in the Krebs cycle.

12. compare *phosphate ester linkages* in ATP and GTP, and *thioester linkage* in *acetyl-S-CoA* and *succinyl-S-CoA* in terms of energetics and their biochemical reactions.
13. speculate on why the *Krebs Cycle* in *E. coli* generates GTP molecules and why it generates ATP molecules eukaryotes.

6.2 Glycolysis, a Key Pathway in Energy Flow through Life

One of the properties of life is that living things require energy. The pathways of energy flow through life are shown in Fig. 6.1 (below). To begin with, recall that the most common intracellular *energy currency* with which livings things "pay" for cellular work is **ATP**. The energy to make ATP on planet earth ultimately comes from the sun via *photosynthesis*. Recall that light energy fuels the formation of glucose and O_2 from CO_2 and water in green plants, algae, cyanobacteria and a few other bacteria. Photosynthesis even produces some ATP directly, but not enough to fuel all cellular and organismic growth and metabolism. In fact, all cells, even plant cells, use ***fermentation*** and/or ***respiration*** (***anaerobic*** or ***aerobic*** processes respectively) to capture nutrient free energy (mostly) as ATP.

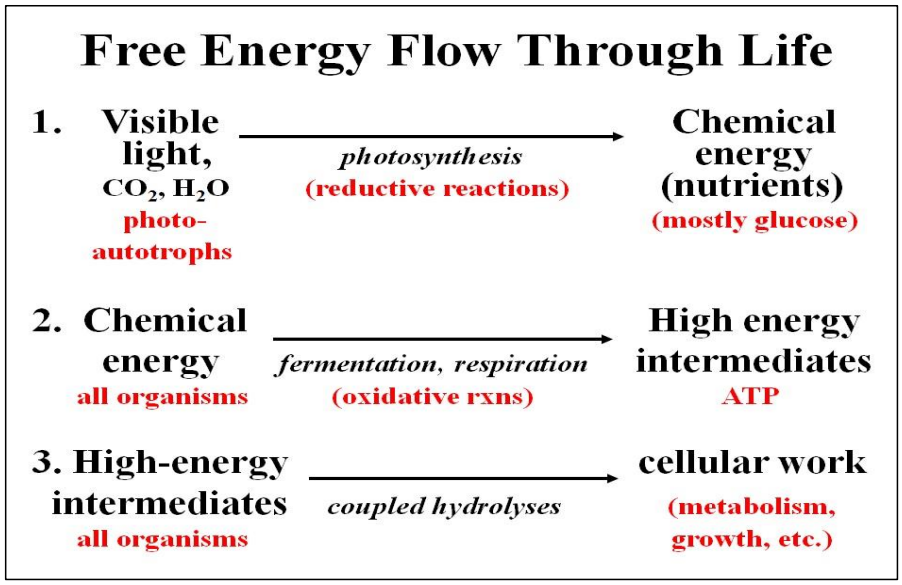


Fig. 6.1: The *flow of free energy* through life, from visible light to chemical energy (e.g., photosynthesis of glucose) to the high energy intermediates like ATP (fermentation or respiration) and finally to cellular work.

ATP is called a ***high-energy intermediate*** because its hydrolysis releases a large amount to free energy. In the condensation reactions that make ATP, it takes about 7.3 Kcal of free energy to link a phosphate to ADP in a *phosphate ester* linkage.

Having captured nutrient free energy in a form that cells can use, ATP hydrolysis then releases that free energy to fuel cellular work. Cellular work includes bending cilia, whipping flagella, contracting muscles, transmitting neural information, building polymers from monomers, and more. The free energy needed to make ATP in animal cells comes exclusively from nutrients (sugars, fats, proteins). As noted, plants get free energy directly from sunlight, but they mobilize nutrient free energy they make in much the same way as the rest of us get it from what we eat! The energetics of ATP hydrolysis and synthesis are summarized in Fig. 6.2 below.

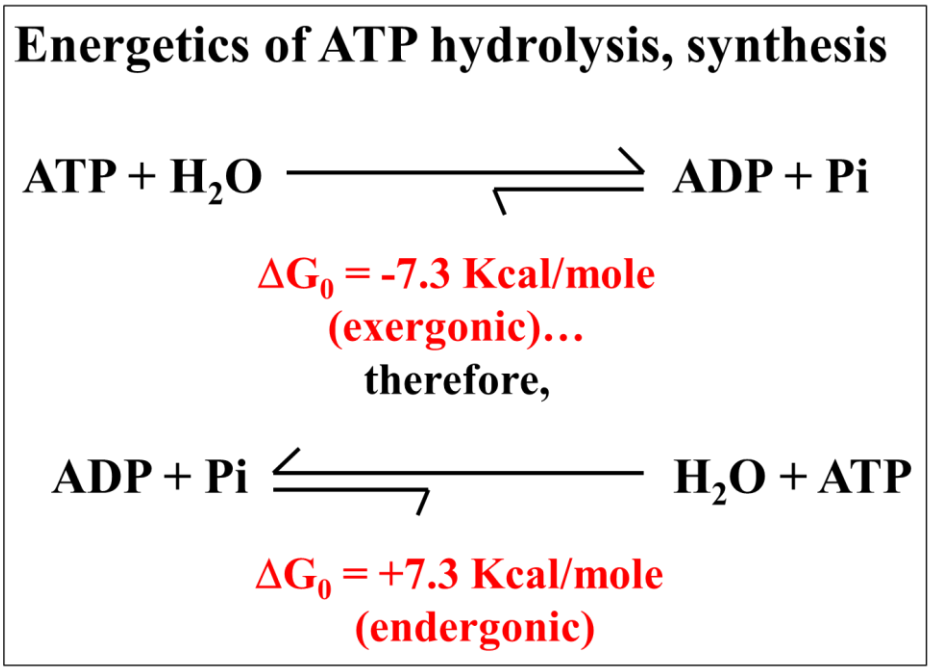


Fig. 6.2: The energetics of ATP synthesis and hydrolysis.

In all living things, glucose oxidation releases a considerable amount of free energy, enough to synthesize many molecules of ATP (Fig. 6.3, below).

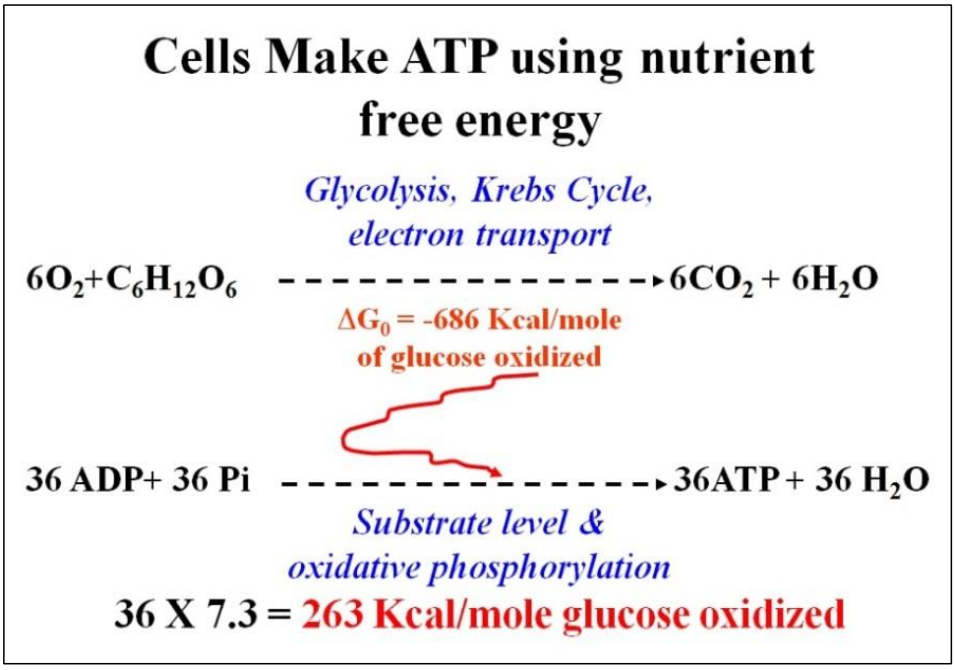


Fig. 6.3: Stoichiometry of glucose metabolism and ATP productions by glycolysis, the Krebs Cycle, electron transport and oxidative phosphorylation.

Cellular respiration, the oxidation of glucose, starts with ***glycolysis*** (from the Greek *glyco* (sugar) *lysis* (separation), or sugar breakdown. Otto Myerhoff and Archibald V. Hill shared a Nobel Prize in Physiology or Medicine with in 1923 for isolating enzymes of glucose metabolism from muscle cells. Thanks to the efforts of others (e.g., Gustav Embden, Otto Meyerhof, Otto Warburg, Gerty Cori, Carl Cori), all the enzymes and reactions of the *glycolytic pathway* were known by 1940, when the pathway became known as the *Embden-Myerhoff Pathway*. As we will see, glycolysis is an evolutionarily conserved biochemical pathway used by all organisms to capture a small amount of nutrient free energy. For more detail, check out Fothergill-Gilmore LA [(1986) *The evolution of the glycolytic pathway*. Trends Biochem. Sci. 11:47-51]. The glycolytic pathway occurs in the cytosol of cells where it breaks down each molecule of glucose ($C_6H_{12}O_6$) into two molecules of ***pyruvic acid*** (***pyruvate***; $CH_3COCOOH$). This occurs in two stages, capturing nutrient free energy in two ATP molecules per glucose molecule that enters the pathway.

Fig 6.4 below summarizes glycolysis, highlighting its *two stages*.

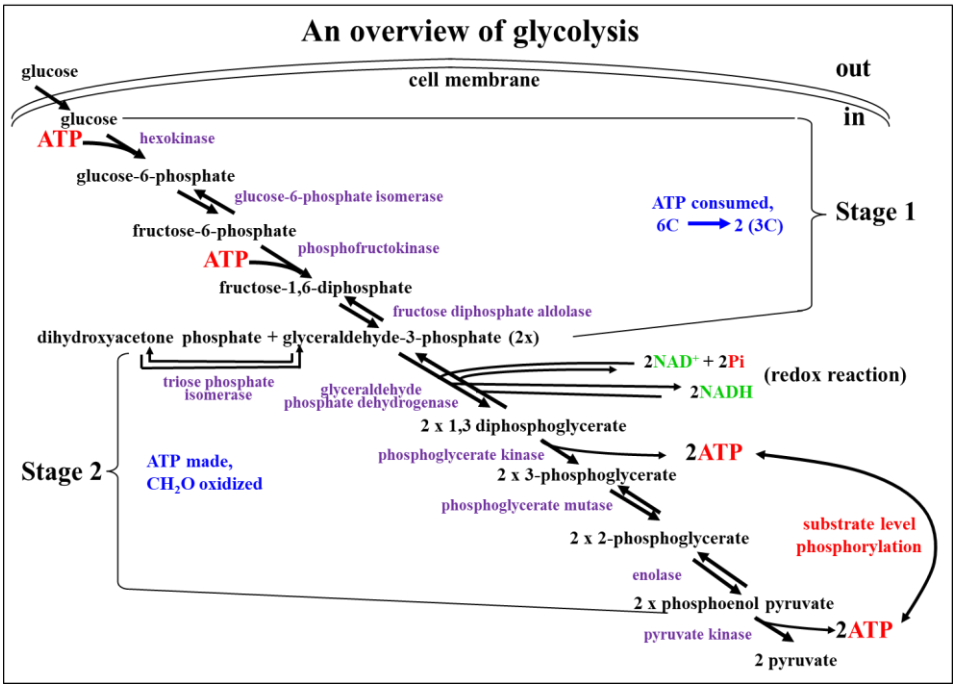


Fig. 6.4: *Glycolysis*: Glucose (6-C) becomes 2 pyruvates (3-C); 2 ATPs are consumed in Stage 1 but 4 are made in Stage 2; The oxidation of glyceraldehyde -3-P marks the start of Stage 2.

Stage 1 of glycolysis actually consumes ATP. Phosphates are transferred from ATP first to glucose and then to fructose-6-phosphate, reactions catalyzed by ***hexokinase*** and ***phosphofructokinase*** respectively. So, these *Stage 1* phosphorylations *consume* free energy. Later, in ***Stage 2*** of glycolysis, nutrient free energy is captured in ATP and ***NADH*** (reduced *nicotinamide adenine dinucleotide*). NADH forms in ***redox reactions*** in which **NAD^+** is reduced as some metabolite is oxidized. In *Stage 2*, it is *glyceraldehyde-3-phosphate* that is oxidized…, but more later!

To summarize, by the end of glycolysis, a single starting glucose molecule has been split into two molecules of ***pyruvate*** while four molecules of ATP and two molecules of NADH have been produced. Pyruvate will be metabolized either anaerobically or aerobically. The alternate fates of pyruvate are summarized in Fig. 6.5, below.

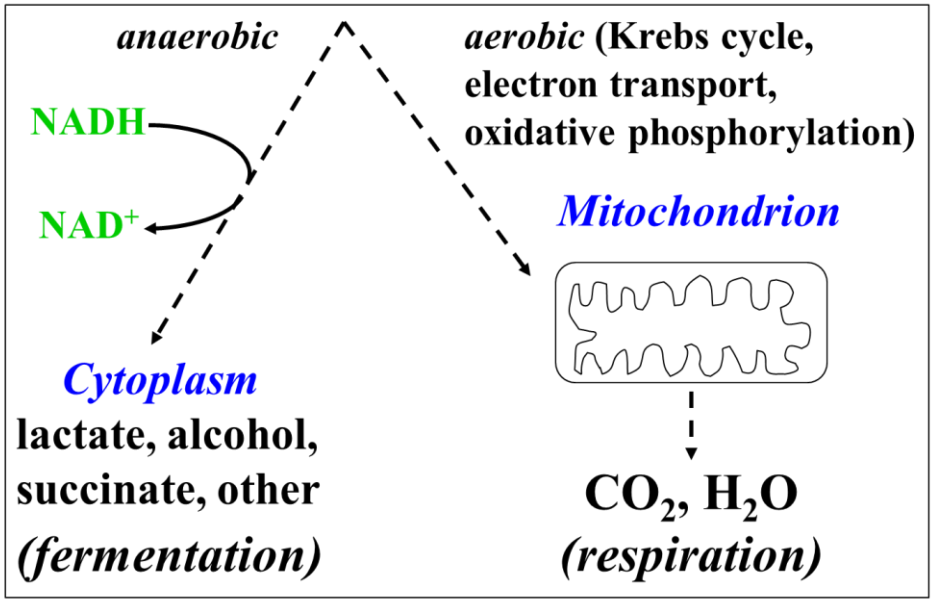


Fig. 6.5: Alternate Fates of Pyruvate: *Fermentation* (the anaerobic reduction to e.g., alcohol, lactate, etc.) or *Respiration* (aerobic oxidation of pyruvate using oxygen as a final electron acceptor, resulting in H_2O and CO_2 production).

151 Overview of Glycolysis

Anaerobic* (*complete*) *glycolysis is a *fermentation*. Two NADH molecules are made per glucose and two will reduce pyruvate at the end of the fermentation pathway. Thus by the end complete glycolysis there is no consumption of O_2 and no net oxidation of nutrient (i.e., glucose). A familiar anaerobic glycolytic pathway is the production of alcohol by yeast in the absence of oxygen. Another is the production of lactic acid by skeletal muscle during strenuous exercise, which leads to the *muscle fatigue* you might have experienced after especially vigorous workout. Muscle fatigue is due to a buildup of lactic acid in the muscle cells that, under these conditions, can't oxidize pyruvate, instead reducing it to lactate. Other cell types produce different fermentative end-products while still capturing free energy in two ATPs per starting glucose. Next, we'll also consider ***Gluconeogenesis***, a pathway that essentially reverses the glycolysis and results in glucose synthesis. Gluconeogenesis occurs both under normal conditions, during in high-

protein/low carb diets, and during fasting or starvation. Finally, we learn that ***respiration***, the aerobic oxidation of pyruvate after ***incomplete glycolysis*** occurs in mitochondria in eukaryotic cells. We look at the role of the Krebs Cycle (also called the TCA, or tricarboxylic acid cycle in the complete oxidation of pyruvate, and why it takes a cycle, and think about how Hans Krebs revealed the cyclic pathway to do it! The Krebs cycle begins a ***respiratory*** pathway that oxidizes glucose to CO_2 and H_2O, leaving no carbohydrates behind. As we look at the reactions of glycolysis and the Krebs cycle, watch for redox reactions in both pathways.

6.3 Some Details of Glycolysis

Let's take a closer look at glycolysis by focusing on the enzyme-catalyzed reactions and free energy transfers between pathway components. We will consider the energetics and enzymatic features of each reaction.

6.3.1 Glycolysis, Stage 1

Reaction 1: In the first reaction of glycolysis, the enzyme ***hexokinase*** rapidly phosphorylates glucose entering the cell, forming *glucose-6-phosphate* (**G-6-P**). As shown in Fig. 6.6, the overall reaction is ***exergonic***; the *free energy change* for the reaction is -4 Kcal per mole of G-6-P synthesized.

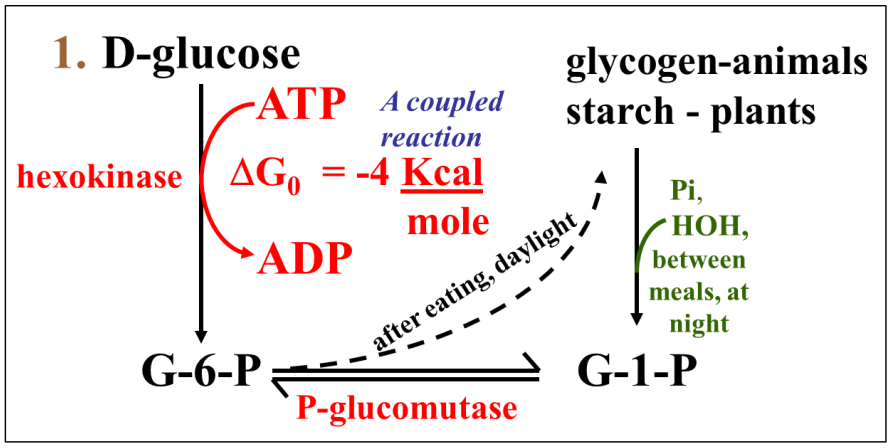


Fig. 6.6: In Reaction 1, phosphorylation of glucose to make glucose-6-P (G-6-P) consumes a molecule of ATP. If cellular energy needs are being met, G-6-Pwill be polymerized to make storage polysaccharides. The G-6-P can be retrieved by polysaccharide breakdown (hydrolysis).when the cells require nutrient energy; at that time, the G-6-P will resume glycolysis.

The hexokinase reaction is a ***coupled reaction***, in which *phosphorylation* of glucose is coupled to ATP hydrolysis. The free energy of ATP hydrolysis (an energetically favorable reaction) fuels glucose phosphorylation (an energetically *un*favorable reaction). The reaction is also ***biologically irreversible***, as shown by the single vertical arrow.

Excess dietary glucose can be stored in most cells (especially liver and kidney cells) as a highly branched polymer of glucose monomers called ***glycogen***. In green algae and plants, glucose made by photosynthesis is stored as polymers of starch. When glucose is necessary for energy, glycogen and starch hydrolysis form glucose-1-phosphate (**G-1-P**) which is then converted to **G-6-P**. Let's look at the energetics (free energy flow) of the hexokinase-catalyzed reaction. This reaction can be seen below as the sum of two reactions in Fig 6.7.

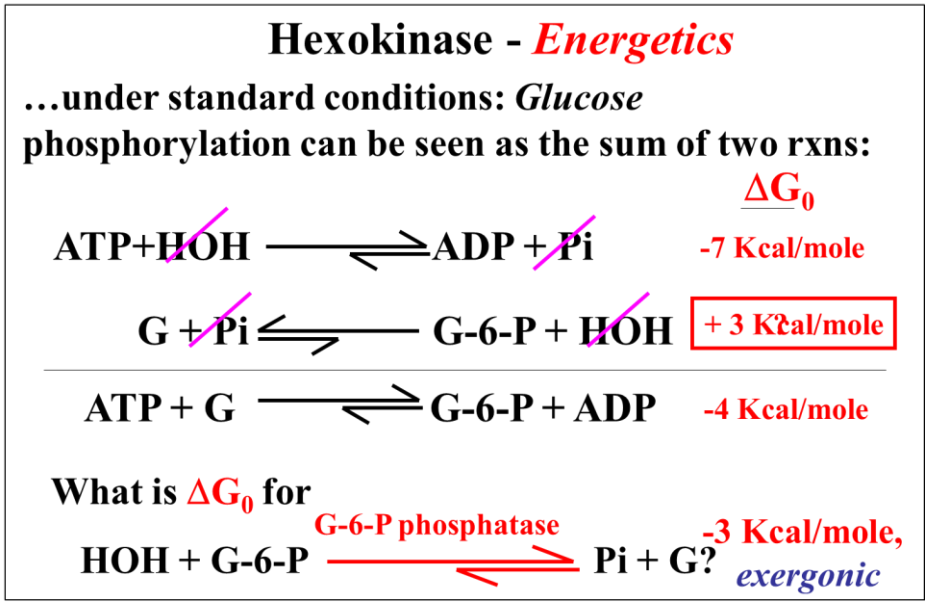


Fig. 6.7: Free energy flow (exchange) for Reaction1 of glycolysis.

Recall that ATP hydrolysis is an *exergonic reaction*, releasing ~7 Kcal/mole (rounding down!) in a closed system under standard conditions. The condensation reaction of glucose phosphorylation occurs with a ΔGo of +3 Kcal/mole. This is an *endergonic*

reaction under standard conditions. Summing up the free energy changes of the two reactions, we can calculate the overall ΔGo of -4 Kcal/mole for the coupled reaction under standard conditions in a closed system.

The reactions above are written as if they are reversible. However, we said that the overall coupled reaction is *biologically irreversible*. Where's the contradiction? To explain, we say that an enzyme-catalyzed reaction is biologically irreversible when the products have a relatively low affinity for the enzyme active site, making catalysis (acceleration) of the reverse reaction very inefficient. While enzymes catalyzing biologically irreversible reactions don't allow going back to reactants, they are often allosterically regulated. This is the case for hexokinase. Imagine a cell that slows its consumption of G-6-P because its energy needs are being met. As a result, G-6-P levels rise in cells. You might expect the hexokinase reaction to slow down so that the cell doesn't unnecessarily consume a precious nutrient energy resource. The *allosteric regulation* of hexokinase is illustrated below (Fig. 6.8).

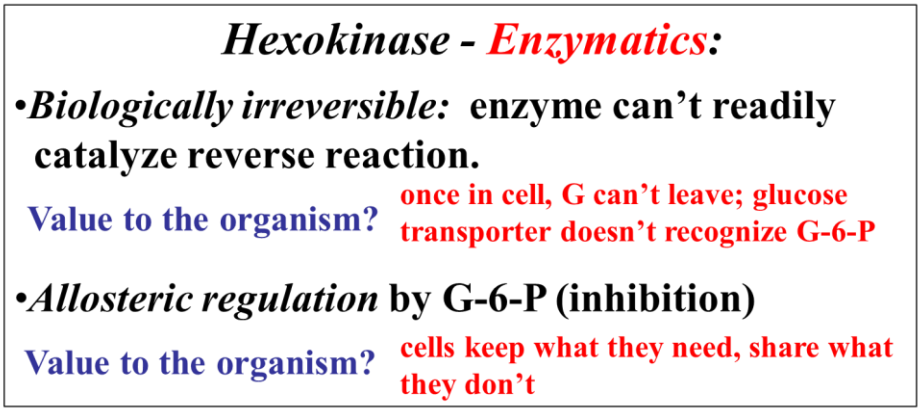


Fig. 6.8: Enzymatics of the Hexokinase reaction. This enzyme catalyzes a *biologically irreversible* reaction and is allosterically regulated.

As G-6-P concentrations rise in the cell, *excess* G-6-P binds to an allosteric site on hexokinase. The conformational change in the enzyme is then transferred to the active site, inhibiting the reaction.

152-2 Glycolysis Stage 1, Reaction 1

Reaction 2: In this slightly endergonic and reversible reaction, *isomerase* catalyzes the isomerization of **G-6-P** to *fructose-6-P* (**F-6-P**) (Fig. 6.9, below).

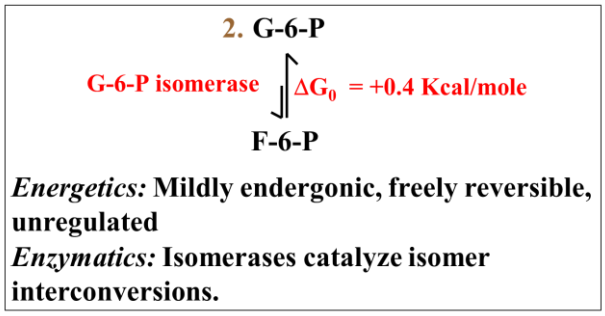


Fig. 6.9: Reaction 2 of glycolysis. G-6-P isomerase catalyzes the isomerization of G-6-P to F-6-P (i.e., the isomerization of glucose to fructose). The reaction is endergonic and reversible.

Reaction 3: In this biologically irreversible reaction, *6-phosphofructokinase* (6-P-fructokinase) catalyzes the phosphorylation of F-6-P to make *fructose 1,6 di-phosphate* (**F1,6 diP**). In this is a *coupled reaction* ATP again provides the second phosphate. The overall reaction is written as the sum of two reactions in Fig.6.10.

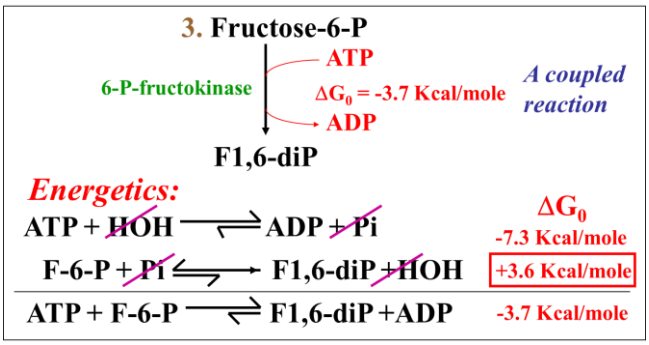


Fig. 6.10: Reaction 3 of glycolysis. A kinase catalyzes phosphorylation of F-6-P to F1,6-di-P in a biologically irreversible reaction, consuming a molecule of ATP.

Like the hexokinase reaction, the *6-P-fructokinase* reaction is a coupled, exergonic and allosterically regulated reaction. Multiple ***allosteric effectors***, including ATP, ADP and AMP and long-chain fatty acids regulate this enzyme.

Reactions 4 and 5: These are the last reactions of the first stage of glycolysis. In *reaction 4*, F1,6 diP (a 6-C sugar) is reversibly split into *dihydroxyacetone phosphate* (**DHAP**) and *glyceraldehyde-3-phosphate* (**G-3-P**). In *reaction 5* (also reversible), DHAP is converted into another G-3-P. These reactions are shown in Fig. 6.11.

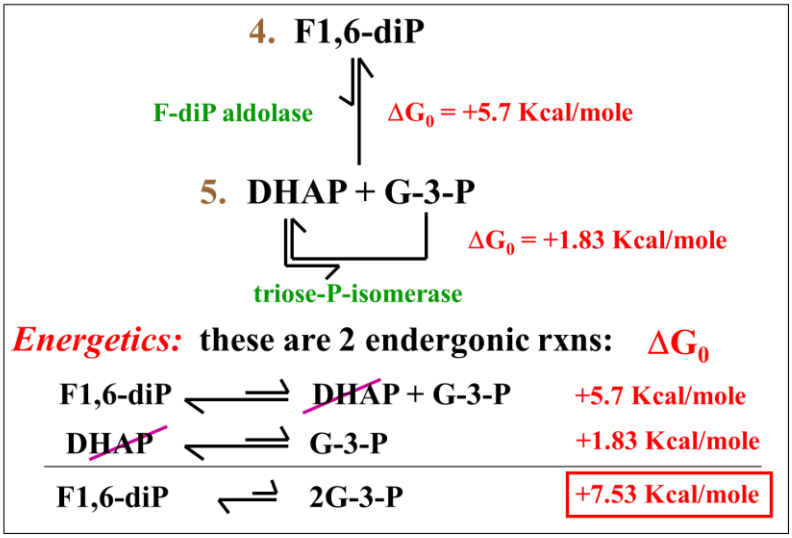


Fig. 6.11: Reactions 4 and 5 of glycolysis. In reaction 4, F1,6-di-P is split into dihydroxyacetone phosphate (DHAP) and glyceraldeghyde-3-P (G-3-P) by an aldolase enzyme. In reaction 5, DHAP is isomerized to G-3-P. Both reactions are endergonic, consuming free energy. These reactions mark the end of Stage 1 of glycolysis.

The net result is the formation of two molecules of G-3-P in the last reactions of *Stage 1* of glycolysis. The enzymes *F-diP aldolase* and *triose-P-isomerase* both catalyze freely reversible reactions. Also, both reactions proceed with a positive free energy change and are therefore *endergonic*. The sum of the free energy changes for the splitting of F1,6 diP into two G-3-Ps is a whopping +7.5 Kcal per mole, a very energetically unfavorable process.

Summing up, by the end of *Stage 1* of glycolysis, two ATP molecules have been consumed and one 6C carbohydrate has been split into two 3-C carbohydrates. We have also seen two biologically irreversible and allosterically regulated enzymes.

153 Glycolysis Stage 1; Reactions 2-5

6.3.2 Glycolysis, Stage 2

We will follow just one of the two molecules of G-3-P generated by the end of *Stage 1* of glycolysis, but remember that both are proceeding through *Stage 2* (the rest of) glycolysis**.**

Reaction 6: This is a redox reaction. G-3-P is oxidized to *1,3, diphosphoglyceric acid* (**1,3, diPG**) and NAD^+ is reduced to NADH. The reaction catalyzed by the enzyme g*lyceraldehyde-3-phopsphate dehydrogenase* is shown in Fig. 6.12.

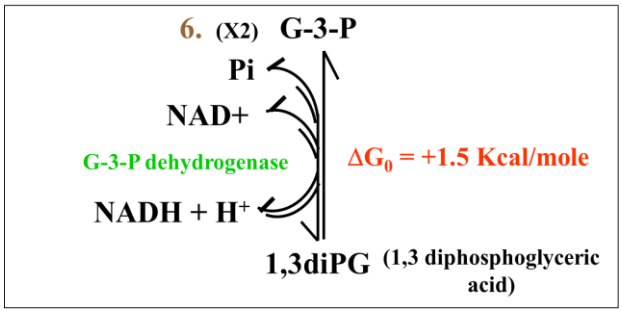


Fig. 6.12: Reaction 6, a redox reaction catalyzed by G-3-P dehydrogenase, is freely reversible. G-3-P is oxidized to1,3 diphosphoglyceric acid (1,3 diPG), transferring electrons as a hydride (H^-) ion to NAD^+ to make NADH.

In this *freely reversible endergonic* reaction, a hydrogen molecule (**H_2**) is removed from G-3-P, leaving behind phosphoglyceric acid. This short-lived oxidation intermediate is phosphorylated to make *1,3 diphosphoglyceric acid* (**1,3diPG**). At the same time, the hydrogen molecule is split into a hydride ion (**H^-**) and a proton (**H^+**). The H^- ions reduce **NAD^+** to **NADH**, leaving the protons behind in solution. Remember that all of this is happening in the active site of the same enzyme!

Even though it catalyzes a reversible reaction, *G-3-P dehydrogenase* is allosterically regulated. However, in contrast to the regulation of hexokinase, that of G-3-P dehydrogenase is more complicated! The regulator is NAD^+. The mechanism of regulation of *G-3-P dehydrogenase* by NAD^+ is called ***negative cooperativity***. It turns out that the higher the [NAD^+] in the cell, the lower the affinity of the enzyme for more NAD^+ and the faster the reaction in the cell! The mechanism is discussed at the link below.

154 Glycolysis Stage 2; Reaction 6

Reaction 7: This reaction, catalyzed by *phosphoglycerate kinase,* is freely reversible and exergonic (Fig. 6.13), yielding ATP and *3-phosphoglyceric acid* (**3PG**).

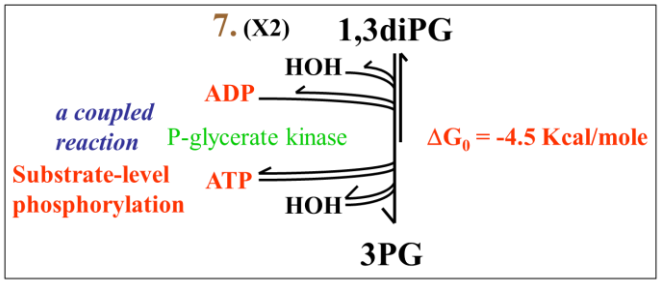


Fig. 6.13: Reaction 7, a reversible, *exergonic*, *coupled* reaction generates ATP using free energy released by the hydrolysis of one of the phosphates from 1,3diPG. The mechanism of ATP synthesis here is called *substrate-level phosphorylation*.

Catalysis of phosphate group transfer between molecules by kinases is called ***substrate-level phosphorylation***, one way of phosphorylating ADP to make ATP. In this *coupled reaction* the free energy released by hydrolyzing a phosphate from 1,3diPG is used to make ATP. Remember that this reaction occurs twice per starting glucose. Two ATPs have been synthesized to this point in glycolysis. We call 1,3diPG a ***very high-energy phosphate compound***.

Reaction 8: This freely reversible endergonic reaction moves the phosphate from the number 3 carbon of 3PG to the number 2 carbon (Fig. 6.14).

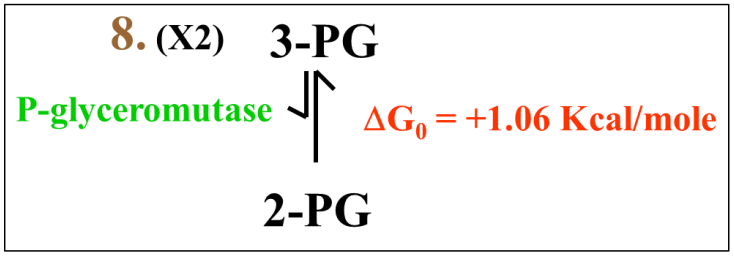


Fig. 6.14: Reaction 8 is a reversible, *endergonic* reaction catalyzed by a mutase. Mutases catalyze transfer of a chemical group from one part of a molecule to another; here, 3-PG is converted to 2PG.

Mutases like *phosphoglycerate mutase* catalyze transfer of functional groups within a molecule.

Reaction 9: In this reaction (Fig. 6.15), *enolase* catalyzes the conversion of 2PG to *phosphoenol pyruvate* (**PEP**).

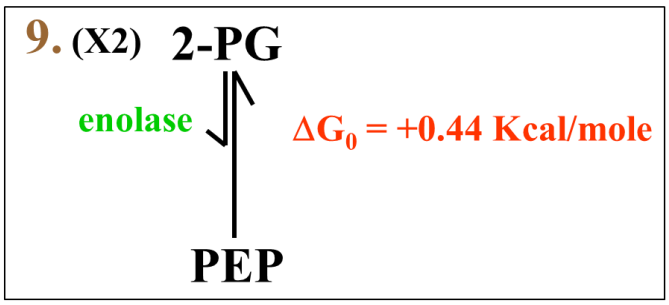


Fig. 6.15: Reaction 9 is a reversible *endergonic* reaction. An *enolase* catalyzes the conversion of 2-PG to phosphoenol pyruvate (PEP).

Reaction 10: This reaction results in the formation of *pyruvic acid* (***pyruvate***), illustrated in Fig. 6.16 below).

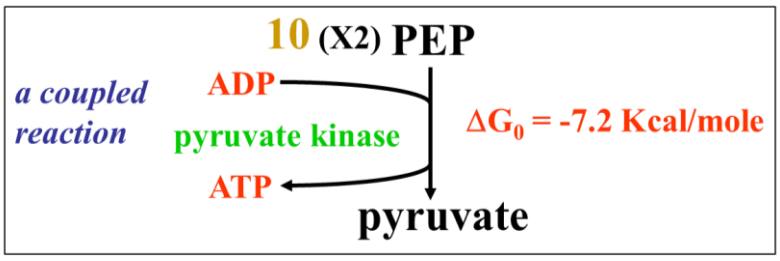


Fig. 6.16: Reaction 10 is biologically irreversible. In a coupled reaction, pyruvate kinase catalyzes a highly exergonic transfer of a phosphate on PEP to ADP to make ATP

Remember again, two pyruvates are produced per starting glucose molecule. The enzyme *pyruvate kinase* couples the *biologically irreversible,* exergonic hydrolysis of a phosphate from PEP and transfer of that phosphate to ADP in a *coupled reaction*. The reaction product PEP is another *very high-energy* phosphate compound. Pyruvate kinase is allosterically regulated by ATP, citric acid, long-chain fatty acids, F1,6 diP and one of its own substrates, PEP.

155-2 Glycolysis Stage 2; Reactions 7-10

As we have seen, there are alternate fates of pyruvate. a product of incomplete glycolysis. One is the aerobic mitochondrial oxidation of pyruvate following *incomplete glycolysis*. The other is an anaerobic ***fermentation***, or *complete glycolysis* in which pyruvate is reduced to one or another end product. Recall that muscle fatigue results when skeletal muscle uses anaerobic fermentation to get energy during vigorous exercise, reducing pyruvate to ***lactic acid***. It is the accumulation of lactic acid in skeletal muscle cells that causes muscle fatigue. The enzyme LDH (***L****actate* ***De****Hydrogenase*) that catalyzes the reduction of pyruvate to lactate is regulated… but not allosterically. Instead, different types of muscle tissues regulate LDH by making different versions of the enzyme. Click the Link below for a more detailed explanation.

156 Fermentation: Regulation of Pyruvate Reduction is NOT Allosteric!

6.4 A Chemical and Energy Balance Sheet for Glycolysis

Compare the balance sheets for *complete* glycolysis (fermentation) to lactic acid and *incomplete* (aerobic) glycolysis, showing chemical products and energy transfers (Fig. 6.17).

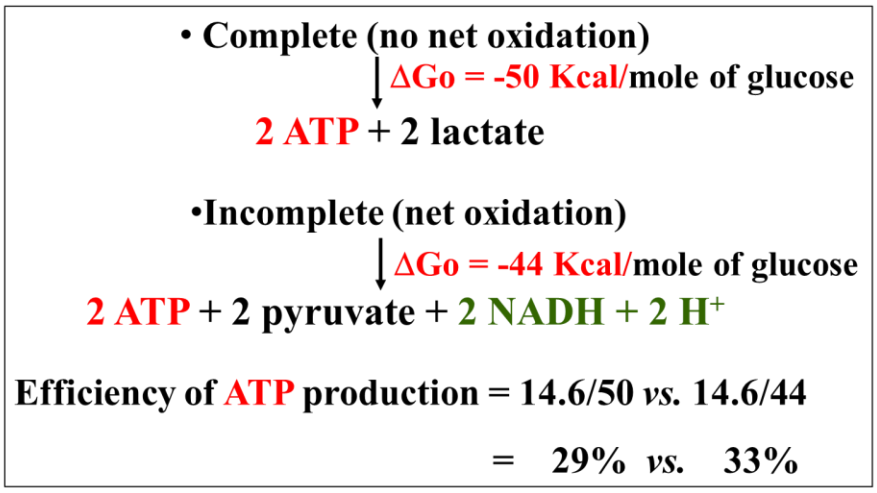


Fig. 6.17: Free energy and ATP yields of complete glycolysis (a fermentation) and incomplete glycolysis (i.e., respiration). Percentages represent the efficiency of ATP production. They are based on the ratios of the free energy captured as ATP to the free energy released by the different pathways for metabolizing glucose. From the data, incomplete glycolysis is a more efficient way to extract nutrient free energy.

There are two reactions in *Stage 2* of glycolysis that each yields a molecule of ATP, each occurring twice per starting glucose molecule. Stage 2 of glycolysis thus produces four ATP molecules per glucose. Since *Stage 1* consumed two ATPs, the net yield of chemical energy as ATP by the end of glycolysis is two ATPs, whether complete to lactate or incomplete to pyruvate! Because they can't make use of oxygen, anaerobes have to settle for the paltry 15 Kcal worth of ATP that they get from a fermentation. Since there are 687 Kcal potentially available from the complete combustion of a mole of glucose, there is a lot of free energy left to be captured during the rest of respiration (i.e., pyruvate oxidation.

157-2 Balance Sheet of Glycolysis

Remember also that the only redox reaction in aerobic glycolysis is in Stage 2. This is the oxidation of G-3-P, a 3C glycolytic intermediate. Now check out the redox reaction a fermentation pathway. Since pyruvate, also a 3C intermediate, was reduced, there has been *no net oxidation of glucose* (i.e., glycolytic intermediates) in complete glycolysis.

By this time, you will have realized that glycolysis is a *net* energetically favorable (*downhill*, *spontaneous*) pathway in a closed system, with an overall negative ΔGo. Glycolysis is also normally spontaneous in most of our cells, driven by a constant need for energy to do cellular work. Thus the actual free energy of glycolysis, or ΔG', is also negative. In fact, glycolysis in actively respiring cells proceeds with release of more free energy than it would in a closed system. In other words, the ΔG' for glycolysis in active cells is more negative than the ΔGo of glycolysis! Feel free to investigate the truth of this statement on your own.

Before we proceed to discuss the aerobic fate of pyruvate, let's look at gluconeogenesis, the Atkins Diet and some not-so-normal circumstances when glycolysis essentially goes in reverse, at least in a few cell types. Under these conditions, glycolysis is energetically unfavorable, and even the otherwise exergonic reactions of glycolysis will be proceeding with a negative ΔG'.

6.5 Gluconeogenesis

In a well-fed animal, most cells can store a small amount of glucose as glycogen. All cells break glycogen down when needed to retrieve nutrient energy as G-6-P. Glycogen hydrolysis, or ***glycogenolysis***, produces G-1-P that is converted to G-6-P, as we saw at the top of *Stage* 1 of glycolysis. But glycogen in most cells is quickly used up between meals. Therefore, most cells depend on a different, external source of glucose other than diet. Those sources are liver (and to a lesser extent kidney) that can store large amounts

of glycogen after meals. In continual feeders (cows and other ruminants, glycogenolysis is ongoing. Liver cell glycogenolysis supplies glucose to the blood for 6-8 hours between meals in *Intermittent feeders* (like us), to be distributed as needed to all cells of the body. Thus, you can expect to use up liver and kidney glycogen reserves after a good night's sleep, a period of intense exercise, or any prolonged period of low carbohydrate intake (fasting or starvation). Under these circumstances, animals use ***gluconeogenesis*** (literally, *new glucose synthesis*) in liver and kidney cells to provide systemic glucose to nourish other cells. In healthy individuals, the hormones glucagon and insulin regulate blood *glucose homeostasis* by raising or lowering blood glucose levels based on cellular glucose (energy) needs, thus protecting the organism from *hypoglycemia* (low blood sugar) and *hyperglycemia* (high blood sugar) respectively. The gluconeogenic pathway produces glucose from carbohydrate and non-carbohydrate precursors that include pyruvate, lactate, glycerol and *gluconeogenic amino acids*. The latter are amino acids that can be converted to alanine. The reactions of glycolysis and gluconeogenesis are shown side-by-side in Fig. 6.18.

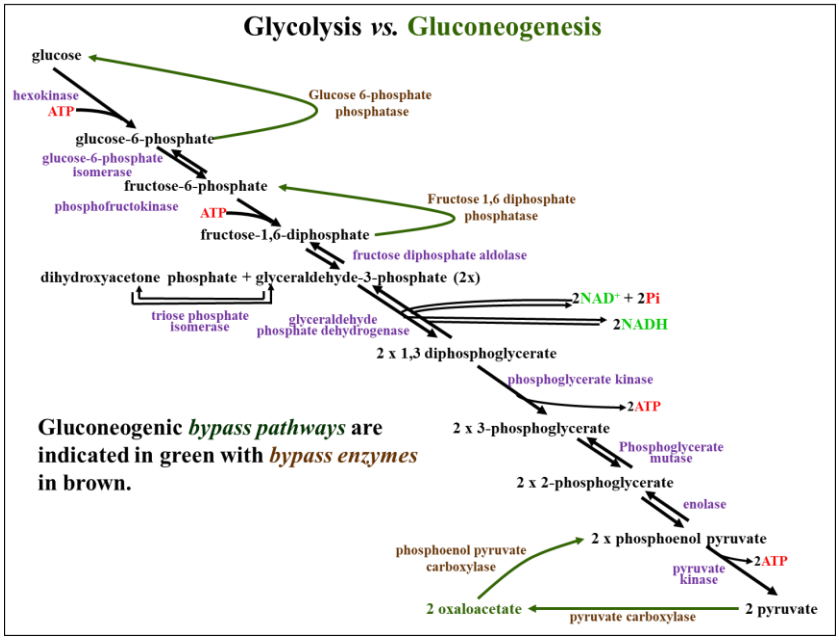


Fig. 6.18: Comparison of incomplete glycolysis to gluconeogenesis. The two pathways are essentially the reverse of one another, except for the *bypass enzymes* in gluconeogenesis (shown in green), required to get around biologically irreversible enzymes of glycolysis.

Look for the *bypass* reactions catalyzed by *carboxylases* and *phosphatases* and the glycolytic reactions that function in reverse during gluconeogenesis. If glycolysis is an exergonic pathway, then gluconeogenesis must be an endergonic one. In fact, while glycolysis through two pyruvates generates a net of two ATPs, gluconeogenesis from two pyruvate to glucose costs 4 ATPs and two GTPs!

Likewise, gluconeogenesis is only possible if the bypass enzymes are present. These are necessary to get around the three biologically irreversible reactions of glycolysis. Except for the *bypass reactions*, gluconeogenesis is essentially a reversal of glycolysis.

As drawn in the pathways above, glycolysis and gluconeogenesis would seem to be cyclic. In fact this apparent cycle was recognized by Carl and Gerti Cori, who shared the 1947 Nobel Prize for Medicine or Physiology with Bernardo Houssay for discovering how glycogen is broken down to pyruvate in muscle (in fact most) cells) to be re-converted to glucose in liver cells. Named after the Coris, The ***Cori Cycle*** (Fig. 6.19) recognizes the interdependence of liver and muscle in glucose breakdown and re-synthesis.

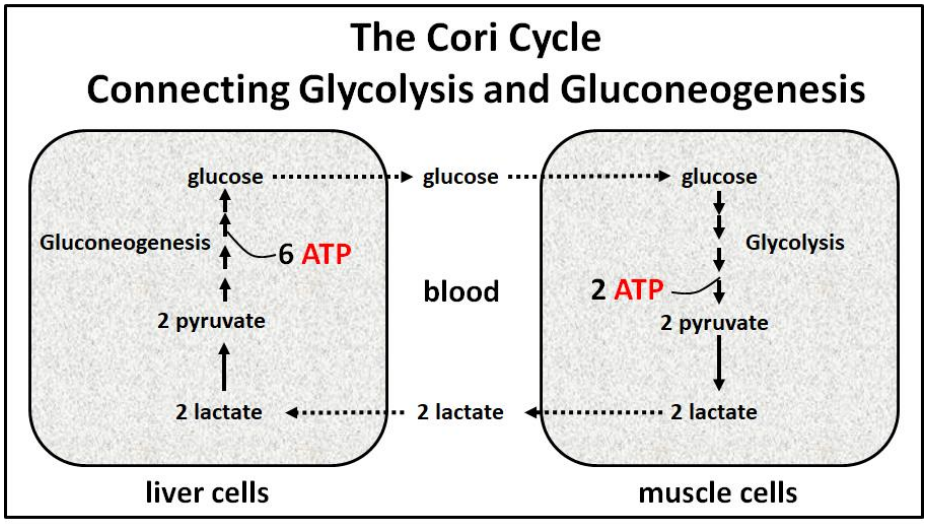


Fig. 6.19: The *Cori cycle* reveals the relationship between glycolysis and gluconeogenesis. Lactic acid produced by complete glycolysis in active skeletal muscle goes to the liver where it could be converted to pyruvate, and then to glucose .

In spite of this free energy requirement, gluconeogenesis is energetically favorable in liver and kidney cells! This is because the cells are open systems. The accumulation of pyruvate in liver cells and a rapid release of new glucose into the blood drive the

energetically favorable reactions of gluconeogenesis forward. Therefore, under gluconeogenic conditions, glucose synthesis occurs with a negative ΔG', a decline in actual free energy. Of course, glycolysis and gluconeogenesis are not simultaneous! Which pathways operate in which cells is tightly controlled. Glycolysis is the norm in all cell types, even in liver and kidney. However, the cessation of glycolysis in favor of gluconeogenesis in the latter cells is under hormonal control. Hormonal control of gluconeogenesis is illustrated in Fig. 6.20, below.

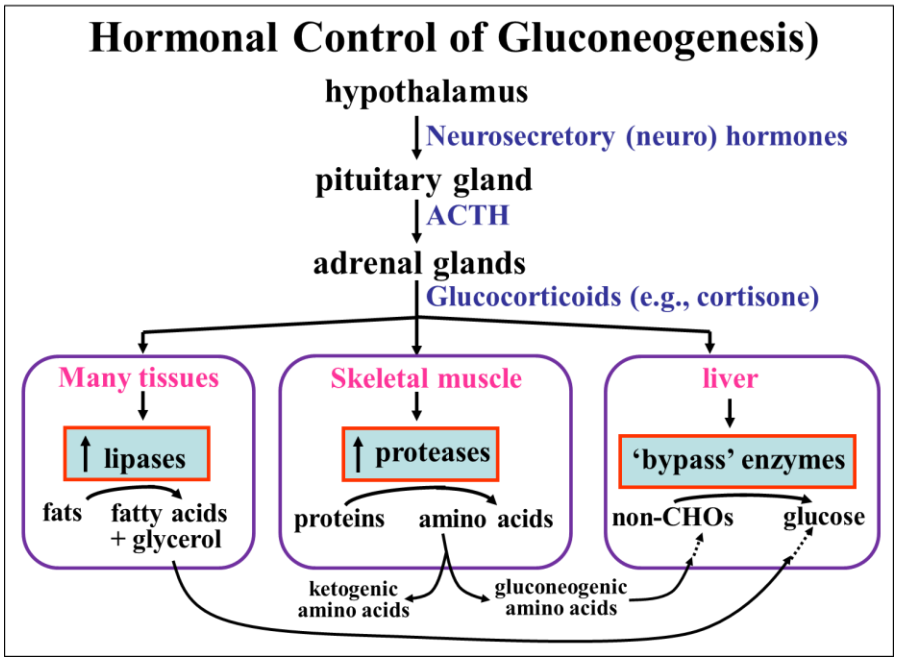


Fig. 6.20: Control of gluconeogenesis by hormones of the *hypothalamic-pituitaryaxis*. A need for glucose for energy stimulates hypothalamic hormones that in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH then stimulates release of glucocorticoids rom the adrenal glands. Glucocorticoid hormones stimulate many tissues to control gluconeogenic glucose production as well as the use alternate nutrient fuels by many cells in the body.

Key in turning on liver gluconeogenesis is the role of glucocorticoid hormones. What causes the secretion of glucocorticoids? A long night's sleep, fasting and in the extreme, starvation are forms of *stress*. Stress responses start in the *hypothalamic-pituitary axis*. Different stressors cause the *hypothalamus* to secrete a *neurohormone* that in turn, stimulates the release of *ACTH* (*adrenocorticotropic hormone*) from the *pituitary gland*.

ACTH then stimulates the release of cortisone and other glucocorticoids from the cortex (outer layer) of the adrenal glands. As the name glucocorticoid suggests, these hormones participate in the regulation of glucose metabolism. Here is what happens at times of low blood sugar (e.g., when carbohydrate intake is low):

1. Glucocorticoids stimulate the synthesis of gluconeogenic bypass enzymes in liver cells.
2. Glucocorticoids stimulate *protease* synthesis in skeletal muscle, causing hydrolysis of the peptide bonds between amino acids. Gluconeogenic amino acids circulate to the liver where they are converted to pyruvate, a major precursor of gluconeogenesis. Some amino acids are ketogenic; they are converted to Acetyl-S-CoA, a precursor to *ketone bodies*.
3. Glucocorticoids stimulate increased levels of enzymes including *lipases* that catalyze hydrolysis of the ester linkages in triglycerides (fat) in adipose and other cells. This generates *fatty acids* and *glycerol*.
4. Glycerol circulates to liver cells that take it up convert it to G-3-P, augmenting gluconeogenesis. Fatty acids circulate to liver cells where they are oxidized to Acetyl-S-CoA that is then converted to ketone bodies. and released to the circulation.
5. Most cells use fatty acids as an alternate energy nutrient when glucose is limiting. Heart and brain cells depend on glucose for energy, though under extreme conditions (prolonged fasting, starvation) brain cells can use ketone bodies as energy source of last resort.

Thus, essential roles of glucocorticoids include

1. enabling most cells to oxidize fats (fatty acids) for energy
2. allowing brain cells to use gluconeogenic glucose for energy, and in the extreme, ketone bodies as an alternate energy source
3. allowing cardiac muscle to use gluconeogenic glucose as its energy source.

It's a pity that we humans can't use fatty acids as gluconeogenic substrates! Plants and some lower animals have a *glyoxalate cycle* pathway that can convert fatty acid oxidation products directly into gluconeogenic carbohydrates substrates. Lacking this pathway, we (and higher animals in general) cannot convert fats to carbohydrates, in spite of the fact that we can all too easily convert the latter to the former!

The dark side of bad eating habits is prolonged starvation that will eventually overwhelm the gluconeogenic response. You see this in reports from third world regions suffering starvation due to drought or other natural disaster, or war. The spindly arms and legs of starving children result from muscle wasting as the body tries to provide the glucose necessary for survival. When the gluconeogenic response is inadequate to the task, the

body can resort to ketogenic fat metabolism. Think of this as a *last* resort, leading to the production of ketone bodies and the "acetone breath" in starving people or people with severe eating disorders (e.g. *anorexia nervosa*).

6.6 The Atkins Diet and Gluconeogenesis

You may know that the *Atkins Diet* is an ultra-low carb diet. It is one of several low-carb *ketogenic diets*. The glucocorticoid hormones released on a low carb diet trick the body into a constant gluconeogenic state. While the liver can produce enough glucose for brain and heart cells, the rest of the cells in our bodies switch to burning fats, hence the weight loss. Carried to an extreme, a severe restriction of carbohydrate intake will result high blood levels of ketones and "acetone breath". Though discredited some years ago, the Atkins Diet (and similar ones e.g., Paleo diet, South Beach diet) are now back in favor.

When not exaggerated, low carb diets are important in the control of diabetes. In older folks, type 2 (adult-onset) diabetics can control their disease with a low carb diet and a drug called *metformin*, which blocks gluconeogenesis and therefore prevents glucose synthesis from gluconeogenic substrates, at the same time stimulating cellular receptors to take up available glucose. For more details on the mechanism of *metformin* action, check out Hundal RS et al. [(2000) *Mechanism by Which Metformin Reduces Glucose Production in Type 2 Diabetes.* Diabetes 49 (12): 2063–9]. Given the prevalence of obesity and type 2 diabetes in the U.S., it's likely that someone you know is taking *metformin* or other similar medication!

158-2 Gluconeogenesis & the Atkins Diet

6.7 The Krebs/TCA/Citric acid cycle

Glycolysis through fermentative reactions produces ATP anaerobically. The evolution of respiration (the aerobic use of oxygen to efficiently burn nutrient fuels) had to wait until photosynthesis created the oxygenic atmosphere we live in now. Read more about the source of our oxygenic atmosphere in Dismukes GC et al. [(2001) *The origin of atmospheric oxygen on earth: the innovation of oxygenic photosynthesis.* Proc. Nat. Acad. Sci. USA 98:2170-2175].

The ***Krebs cycle*** is the first pathway of mitochondrial oxygenic respiration in eukaryotic cells. Evolution of the Krebs cycle no doubt occurred a few reactions at a time, perhaps at first as a means of protecting anaerobic cells from the 'poisonous' effects of oxygen. Later, natural selection fleshed out the aerobic Krebs cycle, electron transport and

oxidative phosphorylation metabolic pathways we see today. Whatever its initial utility, these reactions were an adaptive response to the increase in oxygen in the earth's atmosphere. As we've seen, respiration is much more efficient pathway than glycolysis for getting energy out of nutrients. Animals rely on it, but even plants and photosynthetic algae use the respiratory pathway when sunlight is not available! Here we focus on oxidative reactions in mitochondria, beginning with pyruvate oxidation and continuing to the redox reactions of the Krebs cycle.

After entering the mitochondria, *pyruvate dehydrogenase* catalyzes pyruvate oxidation to ***Acetyl-S-Coenzyme A*** (***Ac-S-CoA***). Then the Krebs cycle completely oxidizes the *Ac-S-CoA*. These mitochondrial redox reactions generate CO_2 and lot of reduced electron carriers (NADH, $FADH_2$). The free energy released in these redox reactions is coupled to the synthesis of only one ATP per pyruvate oxidized (i.e., two per the glucose we started with!). It is the NADH and $FADH_2$ molecules that have captured most of the free energy in the original glucose molecules. The entry of pyruvate into the mitochondrion and its oxidation are summarized in Fig. 6.21.

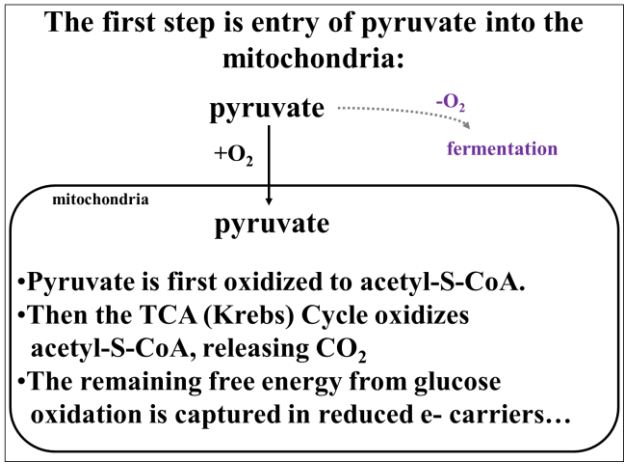


Fig. 6.21: Entry of pyruvate into mitochondrion, followed by its oxidation to acetyl-S-Coenzyme A (acetyl-S-CoA).

Pyruvate oxidation converts a 3C carbohydrate into acetate, a 2C acetate molecule, releasing a molecule of CO_2. In this highly exergonic reaction, CoA-SH forms a *high-energy thioester linkage* with the acetate in Ac-S-CoA. The oxidation of pyruvic acid results in the reduction of NAD+, the production of ***Ac-S-CoA*** and a molecule of CO_2 (Fig. 6.22 below).

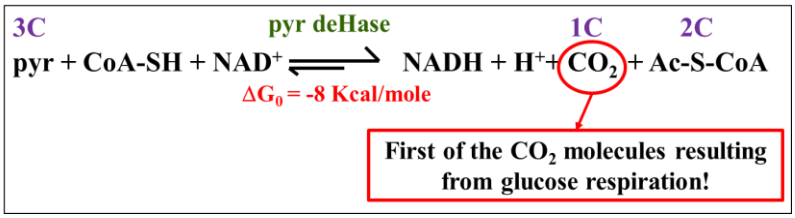


Fig. 6.22: Pyruvate dehydrogenase catalyzes pyruvate oxidation to Ac-S-CoA, releasing a molecule of CO_2 and reducing NAD^+ to NADH.

The Krebs Cycle as it occurs in animals is summarized below in Fig. 6.23. Ac-S-CoA from the oxidation of pyruvate enters the Krebs cycle in the first reaction, condensing with oxaloacetate (OAA) to form citric acid (from which the cycle gets its first name).

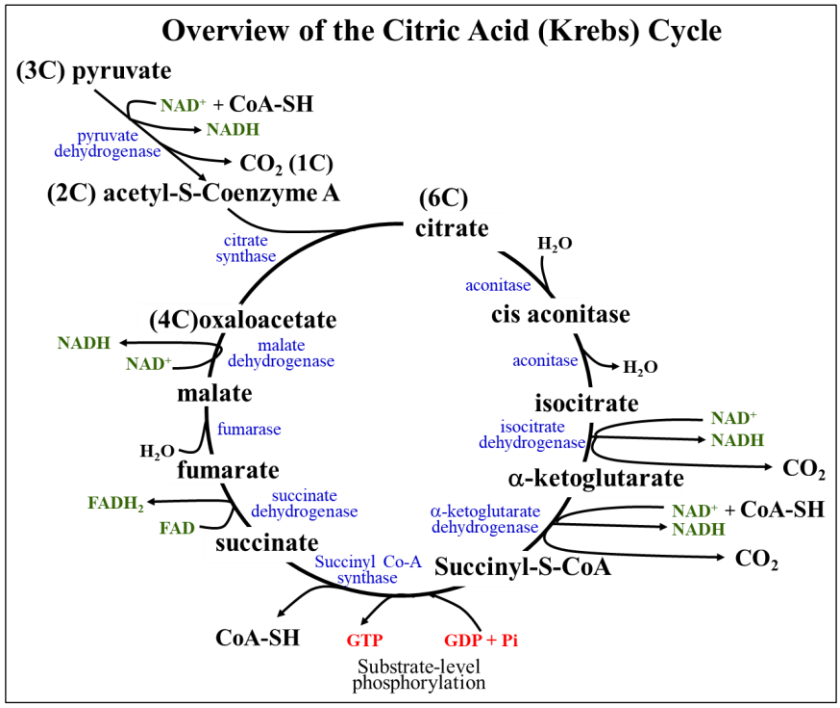


Fig. 6.23: Highlights of the Krebs Cycle. The first reaction is condensation of Acv-S-CoA and oxaloacetate (OAA) to form citric acid (citrate). Four of the reactions in the cycle are redox reactions that create reduced electron carriers (NADH, FADH2), while one reaction is coupled to GTP synthesis by substrate level phosphorylation.

The **Krebs cycle** functions during respiration to oxidize Ac-S-CoA and to reduce NAD^+ and FAD to NADH and $FADH_2$ (respectively). Intermediates of the Krebs cycle also function in amino acid metabolism and interconversions. All aerobic organisms alive today share the Krebs cycle we see in humans. This is consistent with its spread early in the evolution of our oxygenic environment. Because of the central role of Krebs cycle intermediates in other biochemical pathways, parts of the pathway may even have pre-dated the complete respiratory pathway. This centrality of the Krebs cycle to cellular metabolism is emphasized in the biochemical pathways chart shown at the top of this chapter.

After the oxidation of pyruvate, the Ac-S-CoA enters the Krebs cycle, condensing with *oxaloacetate* in the cycle to form *citrate*. There are four redox reactions in the Krebs cycle. As we discuss the Krebs cycle, look for the accumulation of reduced electron carriers ($FADH_2$, NADH) and a small amount of ATP synthesis by substrate-level phosphorylation. Also, follow the carbons in pyruvate into CO_2. To help you understand the events of the cycle,

1. find the two molecules of CO_2 produced in the Krebs cycle itself.
2. find GTP (which quickly transfers its phosphate to ADP to make ATP). Note that in bacteria, ATP is made directly at this step.
3. count all of the reduced electron carriers (NADH, $FADH_2$). Both of these electron carriers carry a pair of electrons. If you include the electrons on each of the NADH molecules made in glycolysis, how many electrons have been removed from glucose during its complete oxidation?

Remember that glycolysis produces two pyruvates per glucose, and thus two molecules of Ac-S-CoA. Thus, the Krebs cycle turns twice for each glucose entering the glycolytic pathway. The *high-energy thioester bonds* formed in the Krebs cycle fuel ATP synthesis as well as the condensation of oxaloacetate and acetate to form citrate in the first reaction. Each NADH carries about 50 Kcal of the 687 Kcal of free energy originally available in a mole of glucose; each $FADH_2$ carries about 45 Kcal of this free energy. This energy will fuel ATP production during electron transport and oxidative phosphorylation.

159-2 Highlights of the Krebs Cycle

Finally, the story of the discovery of the Krebs cycle is as interesting as the cycle itself! Albert Szent-Györgyi won a Nobel Prize in 1937 for discovering some organic acid oxidation reactions initially thought to be part of a linear pathway. Hans Krebs did the

elegant experiments showing that the reactions were part of a cyclic pathway. He proposed (correctly!) that the cycle would be a ***supercatalyst*** that would catalyze the oxidation of yet another organic acid. Some of the experiments are described by Krebs and his coworkers in their classic paper: Krebs HA, et al. [(1938) *The formation of citric and α-ketoglutaric acids in the mammalian body.* Biochem. J. 32: 113–117].

Hans Krebs and Fritz Lipmann shared the 1953 Nobel Prize in Physiology or Medicine. Krebs was recognized for his discovery of TCA cycle, which more commonly carries his name. Lipmann was recognized for proposing ATP as the mediator between food (nutrient) energy and intracellular work energy, and for discovering the reactions that oxidize pyruvate and synthesize Ac-S-CoA, bridging the Krebs Cycle and oxidative phosphorylation (to be considered in the next chapter).

160 Discovery of the Krebs Cycle

You can read Krebs' review of his own research in Krebs HA [(1970) *The history of the tricarboxylic acid cycle*. Perspect. Biol. Med. 14:154-170]. For a classic read on how Krebs described his supercatalyst suggestion, click Hans Krebs Autobiographical Comments. For more about the life of Lipmann, check out the brief Nobel note on the Fritz Lipmann Biography.

Some iText & VOP Key Words and Terms

Acetyl-S-coenzyme A (Ac-S-CoA)	**free energy capture**	**phosphatase enzymes**
ADP, ATP, GDP, GTP	**fructose**	**phosphate-ester linkage**
aerobic	**G, G6P, F6P, F1,6-diP**	**redox reactions**
anaerobic	**gluconeogenesis**	**reducing agent**
Atkins Diet	**gluconeogenic amino acids**	**respiration**
biochemical pathways	**glycolysis**	**SDH (succinate dehydrogenase)**
bioenergetics	**glyoxalate cycle**	**spontaneous reaction**
bypass reactions, enzymes	**high energy bond (linkage)**	**stage 1**
$C_6H_{12}O_6$ (glucose)	**high energy molecules**	**stage 2**
cells as open systems	**isomerase enzymes**	**standard conditions**
Cori Cycle	**kinase enzymes**	**steady state**

dehydrogenase enzymes	Krebs (TCA, citric acid) cycle	stoichiometry of glycolysis
DHAP, G3P,1,3-diPG, 3PG, 2PG, PEP, Pyr	metabolic effects of low carb diet	substrate level phosphorylation
diabetes	metformin	Succinyl-S-CoA
energetics of glycolysis	mitochondria	super-catalyst
energy flow in cells	mutase enzymes	synthase enzymes
equilibrium	NAD+ (oxidized nicotinamide adenine di-Phosphate)	thioester linkage
FAD (oxidized nicotinamide adenine di-Phosphate)	NADH (reduced nicotinamide adenine di-Phosphate)	ΔG' (actual free energy change)
$FADH_2$ (reduced flavin adenine di-Phosphate)	nutrients	ΔGo (standard free energy change)
fermentation	oxidation, reduction	
free energy	oxidizing agent	

Chapter 7: Electron Transport, Oxidative Phosphorylation, Photosynthesis

Mitochondrial Electron Transport and Oxidative Phosphorylation; oxidizing NADH and $FADH_2$; A Chemiosmotic Mechanism and Protein Motors Make ATP; Photosynthesis as a precursor to respiration; Reducing CO_2 in chloroplasts with electrons from H_2O; Light-dependent and Light-independent reactions

Click here: https://www.youtube.com/watch?v=3rYoRaxgOE0

7.1 Introduction

We have seen that glycolysis generates two pyruvate molecules per glucose molecule, and that the subsequent oxidation of each pyruvate generates two Ac-S-CoA molecules. After the further oxidation of each Ac-S-CoA by the *Krebs cycle*, aerobic cells have captured about 30 Kcal out of the 687 Kcal potentially available from a mole of glucose in two molecules of ATP. Not much for all that biochemical effort! However, a total of 24 H+ ions (protons) pulled from glucose in *redox reactions* have also been captured, in the *reduced electron carriers* NADH and $FADH_2$. We start here with a look at ***electron transport*** and ***oxidative phosphorylation***, the linked ("coupled") mechanism that transfers much of nutrient free energy into ATP. We will see that the free energy released by the transport of electrons from the reduced electron carriers is captured in a ***proton (H^+) gradient***. Then we'll see how dissipation of this gradient releases free energy to fuel ATP synthesis by *oxidative phosphorylation*. Next, we will contrast mitochondrial oxidative phosphorylation with the *substrate-level phosphorylation* we saw in glycolysis and the Krebs cycle. After presenting an energy balance sheet for respiration, we look at how cells capture of free energy from alternate nutrients, and by ***photosynthesis*** (overall the opposite of respiration). We conclude by comparing photosynthesis and respiration.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain the *centrality* of the Krebs Cycle to aerobic metabolism.
2. identify *sources of electrons in redox reactions* leading to and within the Krebs cycle.
3. illustrate the *path of electrons* from the Krebs cycle to and through the electron transport chain.
4. trace the *evolution of the electron transport chain* from its location on an aerobic bacterial membrane to its location in eukaryotic cells.
5. list the expected properties of a *proton gate* and a *proton pump.*
6. *interpret experiments* involving redox reactions, ATP synthesis and ATP hydrolysis conducted with intact mitochondria and separated mitochondrial membranes.
7. distinguish between the *pH, H+ and electrical gradients* established by electron transport.
8. explain the *chemiosmotic mechanism of ATP synthesis* and contrast it with *substrate-level phosphorylation.*
9. compare and contrast the role of electron transport in respiration and photosynthesis and discuss the evolution of each.
10. trace and explain the different paths that electrons can take in photosynthesis.
11. explain the presence of similar (or even identical) biochemical intermediates in respiration and photosynthesis.

7.2 Electron Transport Chains

All cells use an ***electron transport chain*** (**ETC**) to oxidize substrates in *exergonic* reactions. The electron flow from reduced substrates through an ETC is like the movement of electrons between the poles of a battery. In the case of the battery, the electron flow releases free energy to power a motor, light, cell phone, etc. During mitochondrial respiration, reduced electron carriers (NADH, $FADH_2$) are oxidized and electrons flow down an ETC to molecular oxygen (O_2) to make water. In plants and other photosynthetic organisms, electron flow down ETC oxidizes NADPH (a phosphorylated version of NADH), eventually reducing CO_2 to sugars.

In both respiration and photosynthesis, the oxidation of energy-rich reduced electron carriers releases ***free energy***. This free energy of electron flow is coupled to the active transport of protons (H+ ions) across a membrane by ***proton pumps***. The proton pumps create a chemical (proton) gradient and consequently a pH and electrical gradient. In a kind of shorthand, we say that the free energy once in reduced substrates is now in an ***electrochemical gradient***. The proton gradient (in fact any gradient) is a source of free energy. The proton gradients of respiration and photosynthesis will be used to make ATP by ***oxidative phosphorylation*** and ***photophosphorylation***, respectively.

The ***Chemiosmotic Mechanism*** explains how the electrochemical gradient forms and how its free energy ends up in ATP. For his insight, Peter Mitchell won the Nobel Prize in Chemistry in 1978. Read Mitchell's original proposal of the *chemiosmosis model* of mitochondrial ATP synthesis in Mitchell P (1961) *Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism.* Nature 191:144-148.

7.3 Electron Transport in Respiration

Here we focus on the details of respiration as it occurs in the mitochondria of eukaryotic cells. The end products of electron transport are NAD^+, FAD, water and protons. The protons end up outside the mitochondrial matrix because they are pumped across the cristal membrane using the free energy of electron transport. ***Electron transport*** and ***oxidative phosphorylation*** are summarized in Fig. 7.1 below.

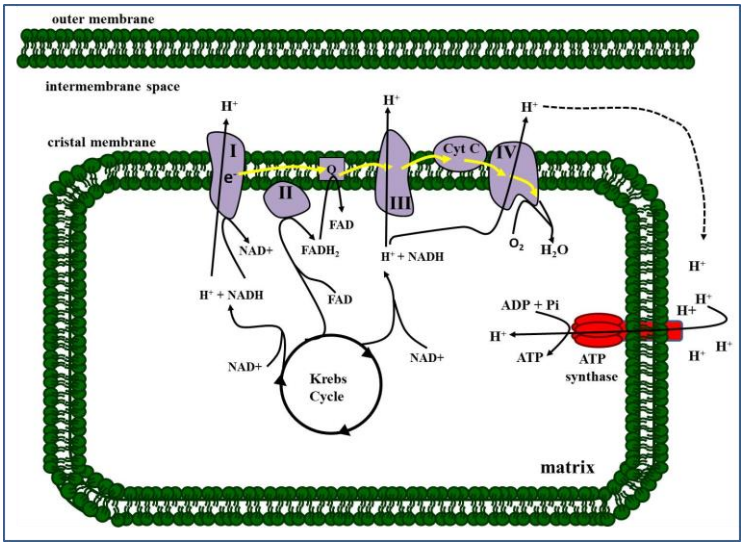


Fig. 7.1: Overview of electron transport and oxidative phosphorylation. Oxidation of NADH and $FADH_2$ feeds electrons into electron transport, releasing free energy that powers proton (H^+ ions) pumps to force H^+ ions out of the mitochondrion. The resulting H^+ gradient fuels ATP synthesis as the protons flow into the mitochondrial matrix through a regulated *ATP synthase* in the cristal membrane (at the right in the drawing).

Roman numbered protein complexes in the drawing above, along with Coenzyme Q ("Q") and cytochrome C (Cyt C) constitute the ETC. The role of the respiratory ETC is to oxidize NADH or $FADH_2$ to NAD+ and FAD respectively. The electrons from these reduced electron carriers are transferred from one ETC complex to the next. At the end

of chain, electrons, protons and oxygen unite in complex IV to make water. Under standard conditions in a closed system, electron transport is downhill, with an overall release of free energy (a negative ΔGo) at equilibrium.

161 Electron Transport Oxidizes Reduced Electron Carriers

162-2 Finding the Free Energy of Electron Transport

163-2 Separating Electron Transport from Oxidative Phosphorylation

In the illustration above, we can see three sites in the respiratory ETC that function as H^+ pumps. At these sites, the negative change in free energy of electron transfer is large and coupled to the action of a proton pump that forces protons out of the mitochondrial matrix across the cristal membrane. Because the outer mitochondrial membrane is freely permeable to protons, the gradient created is in effect, between the cytoplasm and the mitochondrial matrix. The flow of protons back into the mitochondrial matrix through lollipop-shaped ATP synthase complexes on the cristal membrane releases the gradient free energy that will be harnessed to make ATP by oxidative phosphorylation.

164-2 Proton Pumps Store Free Energy of the ETC in Proton Gradients

7.4 Oxidative Phosphorylation in Respiration

Oxidative phosphorylation is the mechanism by which ATP captures the free energy in the mitochondrial proton gradient. Most of the ATP made in aerobic organisms is made by oxidative phosphorylation, rather than by substrate phosphorylation (the mechanism of ATP synthesis in glycolysis or the Krebs cycle). Some aerobic biochemistry may have evolved in response to the toxic effects of rising environmental oxygen levels. Later elaboration of respiratory metabolism was undoubtedly selected because it turns out to be more efficient at making ATP than anaerobic fermentations such as 'complete' glycolysis. In other words, oxidative phosphorylation is more efficient than substrate-level phosphorylation.

To summarize here, the movement of electrons down the electron transport chain fuels three proton pumps that establish a proton gradient across the *cristal* membrane that stores free energy. Oxidative phosphorylation then couples controlled diffusion of protons back into the mitochondrial matrix through cristal membrane ATP synthases, fueling ATP production. We say that the proton gradient has a ***proton motive force***, recognizing the three-part gradient – proton and pH gradient and a difference in electric potential.

The use of this proton motive force to make ATP is regulated. Conditions in the cell control when the energy stored in this gradient will be released to make ATP. The switch that allows protons to flow across the cristal membrane to relieve the proton gradient is an *ATP synthase*, a tiny, complex enzymatic protein motor. For a clear discussion of this complex enzyme, see P. D. Boyer (1997) *The ATP synthase – a splendid molecular machine.* Ann. Rev. Biochem. 66:717-749. The capture of free energy of protons flowing through this lollipop-shaped complex is shown in Fig. 7.2.

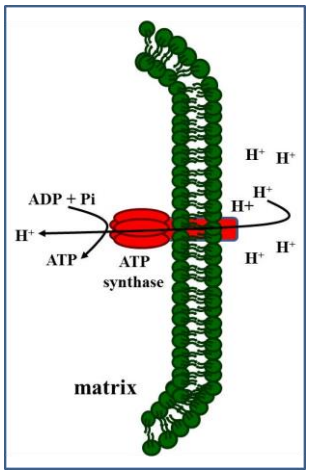


Fig. 7.2: The flow of protons through the cristal membrane ATP synthase relieves the proton gradient, releasing free energy that fuels ATP synthesis in the mitochondrial matrix.

In mitochondria, the protons **pumped** out of the mitochondrial matrix (using the free energy released by electron transport) can then flow back into the matrix through the *ATP synthase.* If the three ETC sites in the cristal membrane that actively transport protons are **proton pumps**, then the cristal membrane ATP synthase complexes function as regulated **proton gates** that catalyzes ATP synthesis when protons are allowed to flow through them. For their discovery of the details of ATP synthase function, P. D. Boyer and J. E. Walker shared the Nobel Prize in Chemistry in 1997.

165-2 Proton Gates Capture Proton Gradient Free Energy as ATP

The ratio of ATP to ADP concentrations regulates proton flow through the ATP synthase gates. A high *ATP/ADP* ratio in the mitochondrial matrix indicates that the cell does not need more ATP, closing the proton gate so that the proton gradient cannot be relieved. On the other hand, a low *ATP/ADP* ratio in the matrix means that the cell is hydrolyzing a

lot of ATP, and that the cell needs more. Then the proton gate opens, and protons flow through cristal membrane ATP synthases back into the matrix down a concentration gradient. As they flow, they release free energy to power a protein motor in the enzyme that in turn, activates ATP synthesis.

Just as we did for glycolysis, we can count the ATPs and see how much free energy we get from aerobic respiration, i.e., the complete oxidation of glucose. You can see this in the link below.

166 A Balance Sheet for Respiration

Recall that according to the *endosymbiotic theory*, aerobic bacteria are the evolutionary ancestor to mitochondria; in fact, the cell membrane of aerobic bacteria house an ETC and *chemiosmotic mechanism* of ATP generation much like that in mitochondria. And, proton gradients do not only power ATP synthesis, but can also power cellular work quite directly. Consider the bacterial flagellum driven directly by proton flow through cell membrane proton gate/molecular motor complex (Fig. 7.3, below). Electron transport in the cell membrane creates the gradient; relief of the gradient powers the flagellum.

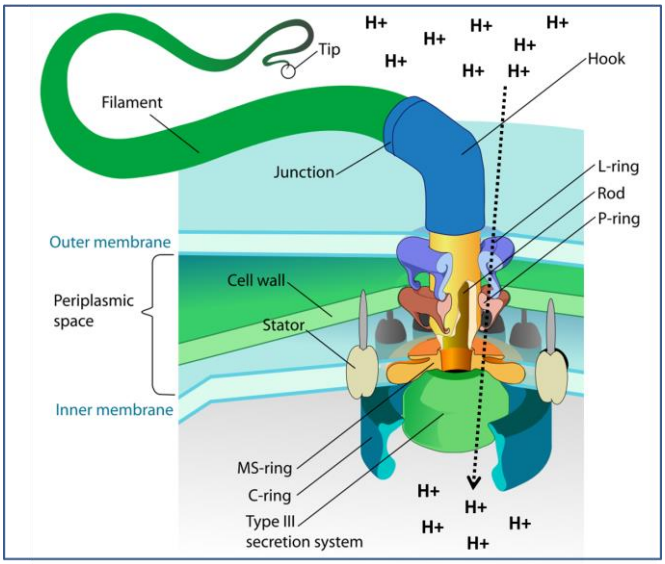


Fig. 7.3: The plasma membrane ATPase of motile bacteria is a molecular motor that can e.g., spin a flagellum powered by protons flowing into the matrix to relieve a proton gradient.

7.5 Photosynthesis

Chemically, photosynthesis is the reverse reaction of respiration. Compare the two reactions:

1. $C_6H_{12}O_6 + 6O_2 \rightleftharpoons 6CO_2 + 6H_2O$ ($\Delta Go = -687$Kcal/mole)
2. $6CO_2 + 6H_2O \rightleftharpoons C_6H_{12}O_6 + 6O_2$ ($\Delta Go = +687$Kcal/mole)

If respiration (reaction 1) is the complete oxidation of glucose to H_2O and CO_2, then photosynthesis (reaction 2) is the reduction of CO_2 using electrons from H_2O to make glucose. Photosynthesis is thus an endergonic reaction. During photosynthesis, sunlight, specifically visible light, fuels the reduction of CO_2 (Fig. 7.4, below).

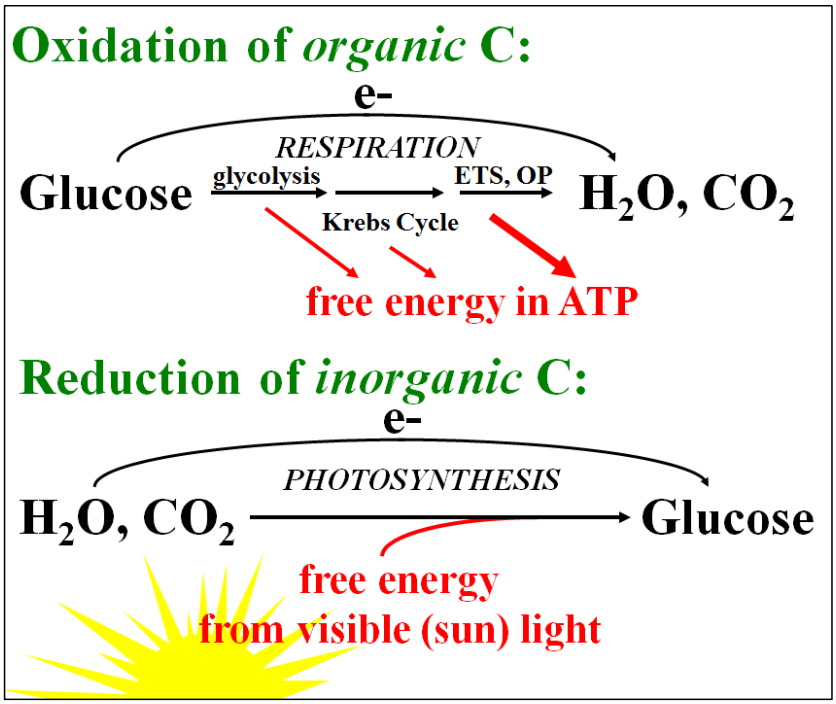


Fig. 7.4: Summary of carbon flow through life. Organic carbon is oxidized to release free energy and make ATP. Sunlight provides free energy for photosynthesis to turn inorganic carbon (CO_2) into organic carbon (e.g., glucose).

Photosynthesis began in the absence of oxygen; it came before oxygenic respiration on earth. Increasing oxygen in the atmosphere led to selection of oxygenic respiratory pathways (the Krebs cycle, electron transport and oxidative phosphorylation). When we look at photosynthesis in some detail, we will see that photosynthesis and respiration have electron transport-ATP synthesizing systems with similar features. This suggests that they share a common evolutionary ancestry. Elsewhere, we will consider what a common ancestral system might have looked like. Two biochemical pathways make up photosynthesis:

- ***Light-dependent reactions*** that use visible light energy to remove electrons from water, reduce electron carriers, pump protons and make ATP;
- ***Light-independent reactions*** that use ATP to transfer electrons from the reduced electron carriers to CO_2 to synthesize glucose.

These two pathways are summarized in Fig. 7.5.

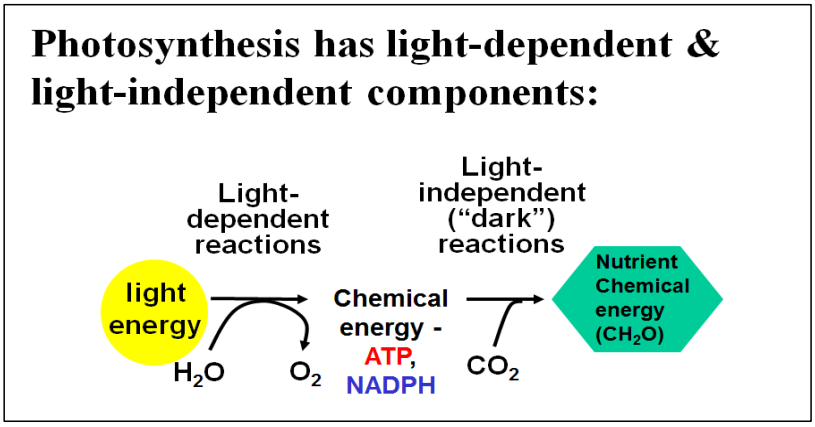


Fig. 7.5: The *light dependent* and *light independent* ('dark') reactions of photosynthesis. The light-dependent reactions 'split' water, releasing oxygen and protons. The light-independent reaction 'fix' CO_2 in organic molecules.

7.5.1 The Light-Dependent Reactions

Colored substances contain **pigments** that reflect the colors that we see and at the same time, absorb all the other colors of visible light. Early studies asked which plant pigments absorbed light that allowed (we say supported) photosynthesis. Chlorophyll, the abundant pigment we see in plant tissues, is actually two separate green pigments, ***chlorophyll a*** and ***chlorophyll b***. One might therefore predict that light absorbed by either or both chlorophylls will support photosynthesis. Fig. 7.6 (below) illustrates the experiment that tested this hypothesis.

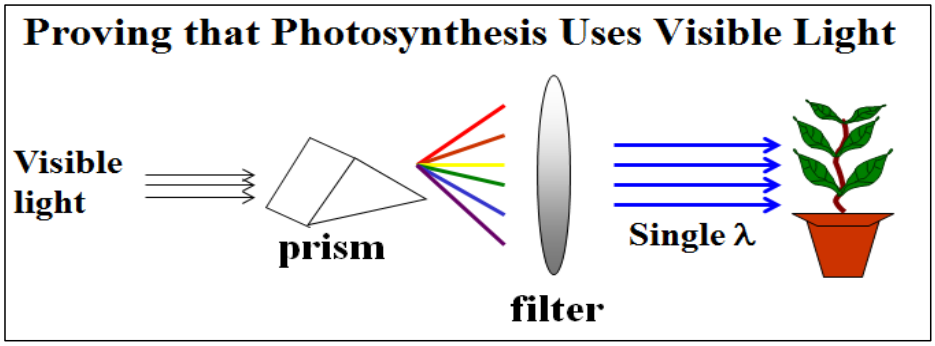


Fig. 7.6: Experimental design to test the hypothesis that photosynthesis is supported by absorption of light by chlorophyll pigments.

The ***action spectrum*** of photosynthesis (Fig. 7.7) plots the results of this experiment.

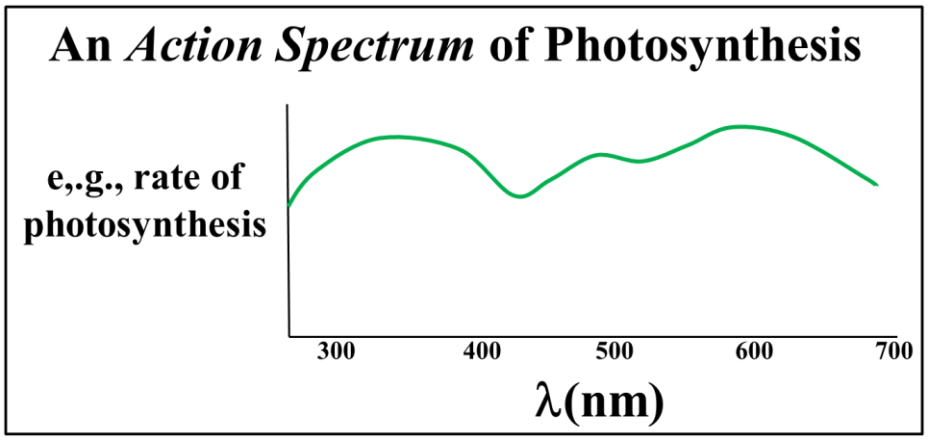


Fig. 7.7: Graph showing the *action spectrum* of photosynthesis, showing that photosynthesis is supported across a wide range of visible light wavelengths.

The spectrum shows that all wavelengths of visible light energy can support photosynthesis. In addition, other experiments revealed that radiation other than visible light (e.g., ultraviolet and infrared light) do not support photosynthesis. One can conclude that chlorophylls alone are likely not the only pigments to support photosynthesis.

Chlorophylls are easily purified from leaves. The graph below (Fig. 7.8) shows an average ***absorbance spectrum*** of chlorophylls. The absorbance of *chlorophyll a* and c*hlorophyll b* are slightly different, but center at wavelengths at 450nm and 675nm.

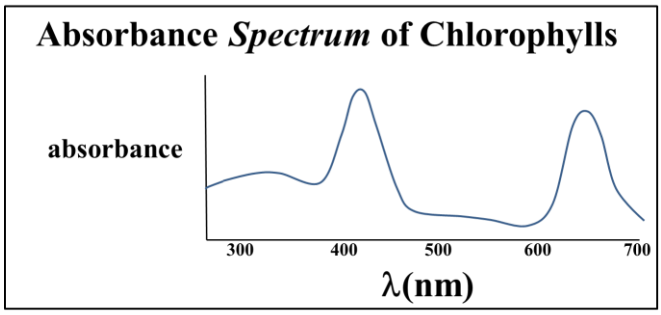


Fig. 7.8: Graph plotting the absorbance spectrum of purified chlorophylls, showing two main peaks of absorbance.

From such *absorbance spectra* we can conclude that the chlorophylls support photosynthesis, but that chlorophylls alone do not account for *the action spectrum* of photosynthesis! In fact, other pigments absorbing elsewhere in the visible spectrum also support photosynthesis. Of course, we knew that these other pigments are present in leaves and other photosynthetic plant tissues, many of which we see as fall colors. All of these pigments (including chlorophylls) are found in **chloroplasts**, the organelles that conduct photosynthesis in plants. Take another look at the structure of chloroplasts in the electron micrographs in Fig. 7.9.

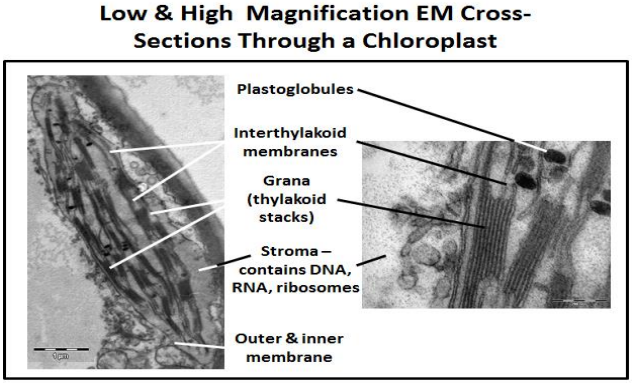


Fig. 7.9: Low (LEFT) and high (RIGHT) power transmission micrographs of typical chloroplasts, thylakoid membranes and grana (thylakoid stacks).

Fig. 7.10 shows that the visible light *absorbance spectra* of the *chlorophylls*, ***carotenoids*** and possibly other plant pigments coincide with the ***action spectrum*** of photosynthesis. This implies that absorption of light by those pigments is responsible for photosynthesis.

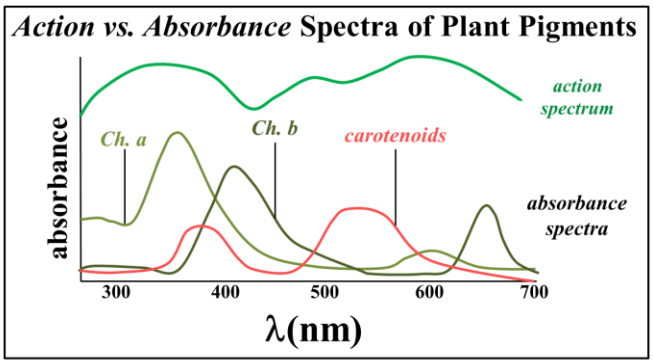


Fig. 7.10: Graph superimposing the action spectrum of photosynthesis over the absorbance spectra of different plant pigments. The multiple absorbance peaks of different chloroplast pigments are consistent with the action spectrum.

In fact, *chlorophyll a*, *chlorophyll b*, carotenoids and yet other ***accessory pigments*** participate in capturing light energy for photosynthesis. Two clusters of pigments, called ***reaction centers***, capture light energy. The reaction centers are part of ***photosystems 1*** **(PSI)** and ***photosystem 2*** **(PSII)** on **thylakoid membranes** of chloroplasts. Johann Deisenhofer, Robert Huber and Hartmut Michel first determined the 3D structure of a bacterial reaction center. Then they unraveled the relationship between the structure of the proteins in the center and the membrane in which they were embedded. For this, they shared the 1988 Nobel Prize in Chemistry.

The activities of PSI are animated at Photosystem 1 Action. You should see light (a photon) excite electron (e^-) pairs from PSI pigments that then transfer their energy from pigment to pigment, ultimately to ***chlorophyll a P700***. The impact of the electron pair excites a pair of electrons from *chlorophyll a P700* that is captured by a *PSI* e^- acceptor, an event referred to as ***charge separation***. Next, the reduced PSI acceptor is oxidized as electrons move down a short ETC, eventually reducing $NADP^+$ to NADPH. Electrons on NADPH will eventually be used to reduce CO_2 to a carbohydrate. So far, so good! But that leaves an electron deficit in PSI. The Z-Scheme illustrated in Fig. 7.11 (below) follows electrons *taken from water* (absorbed through roots) into PSII, which will replace those missing from PSI.

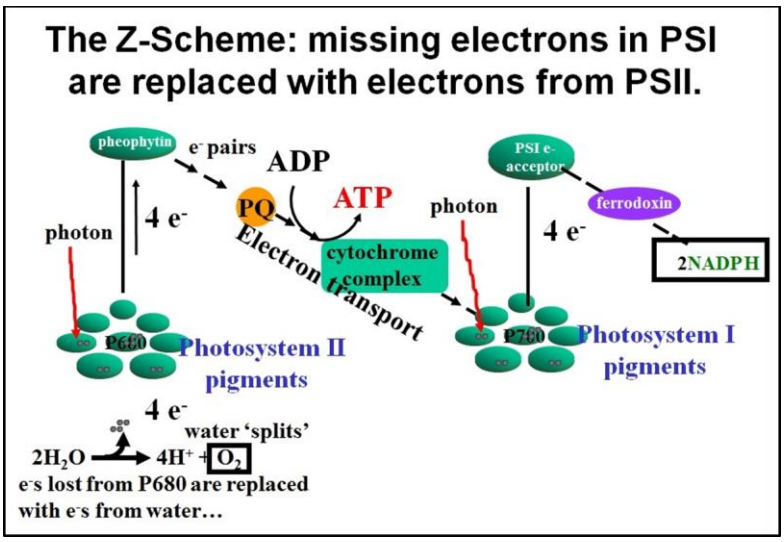


Fig. 7.11: The 'Z-scheme' of photosynthesis. Photosystems I and II (PSI, PSII) pigments absorb light energy that excites electrons captured by electron acceptors. Electrons excited from PSI reduce NADPH, the starting point of the light-independent reactions. Electrons excited out of PSII come from water ('splitting it to release oxygen). They will flow down a photosynthetic electron transport chain to replace electrons lost from PSI. Along the way, free energy from those excited electrons fuels ATP synthesis.

Let's summarize the flow of electrons from water through the Z-scheme. Light excites an e^- pair from the ***P680*** form of *chlorophyll a* in PSII. A PSII electron acceptor in the thylakoid membrane, identified as ***pheophytin***, captures these electrons; this is another photosynthetic *charge separation*. An electron transport chain oxidizes the pheophytin, transferring e^- pairs down to PSI. Some of the free energy released then pumps protons from the *stroma* into the luminal space of the thylakoid membranes. The gradient free energy fuels ATP synthesis as protons flow back into the stroma through a chloroplast ***ATP synthase***. The link at Action in the Z-Scheme animates the entire Z-Scheme, showing first how PSI electrons reduce NADP+ and then how PSII electrons replace missing PSI electrons, making ATP along the way. The oxygen released by splitting water ends up in the atmosphere.

Since the goal of photosynthesis is to make and store glucose, photosynthesizing cells get ATP for their energy needs by ***photophosphorylation*** during electron transport through Z-scheme. However, ***Cyclic photophosphorylation*** can occur when the cell's need for ATP exceeds the capacity of the Z-scheme to supply it.

Cyclic photophosphorylation is illustrated in Fig. 7.12 below.

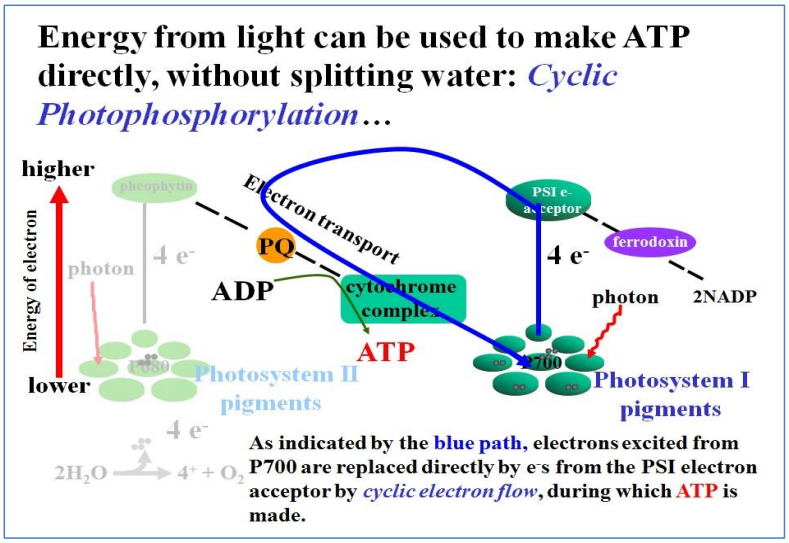


Fig. 7.12: In *Cyclic Photophosphorylation* electrons excited from PSI byp[ass PSII and are captured by the photosynthetic electron transport chain and returned to return to PSI. ATP made by the Cycle are used for cellular work when there is no need or capacity to make more sugar.

Cyclic Photophosphorylation is a variation on the light dependent reactions, a kind of time-out to make ATP without reducing NADP+ (and ultimately, without reducing CO_2 to make sugar). The *cycle* simply takes electrons excited to the PSI electron acceptor, and instead of sending them to NADP+, deposits them on PC (*plastocyanin*) in the electron transport chain between PSII and PSI. These electrons then flow down this 'long line' of the Z, right back to PSI, releasing their free energy to make ATP. In light, the electrons just go up and around, hence the name *Cyclic Photophosphorylation*. The path of electrons shown in Fig. 7.12 is also animated at Action in Cyclic Photophosphorylation.

7.5.2 The Light-Independent ("Dark") Reactions

As we have seen, the light-dependent reactions of photosynthesis require light energy and water to generate O_2, ATP and NADPH. In the ***light-independent*** (or ***'dark'***) reactions, the ATP and NADPH will provide free energy and electrons (respectively) for carbon fixation (the reduction of CO_2 to make carbohydrates). There are three main pathways for the so-called "Dark Reactions".

7.5.2.a C3 Photosynthesis - Light-Independent Reactions

CO_2 enters photosynthetic tissues through ***stomata***. Stomata are pores in leaves that can be open or closed, depending on light, temperature and water availability. In addition to allowing CO_2 into photosynthetic tissues, stomata also function in ***transpiration***, which allows excess water in cells to leave the plants by transpiration (sometimes called *evapotranspiration*). C3 photosynthesis is so named because its first carbohydrate product is a 3-C molecule, 3-phosphoglyceric acid (3-PG). You should recognize 3-PG; it is also a glycolytic intermediate. The Calvin Cycle is the most common light-independent reaction pathway (Fig. 7.13). For its discovery, M. Calvin received the Nobel Prize in Chemistry in 1961.

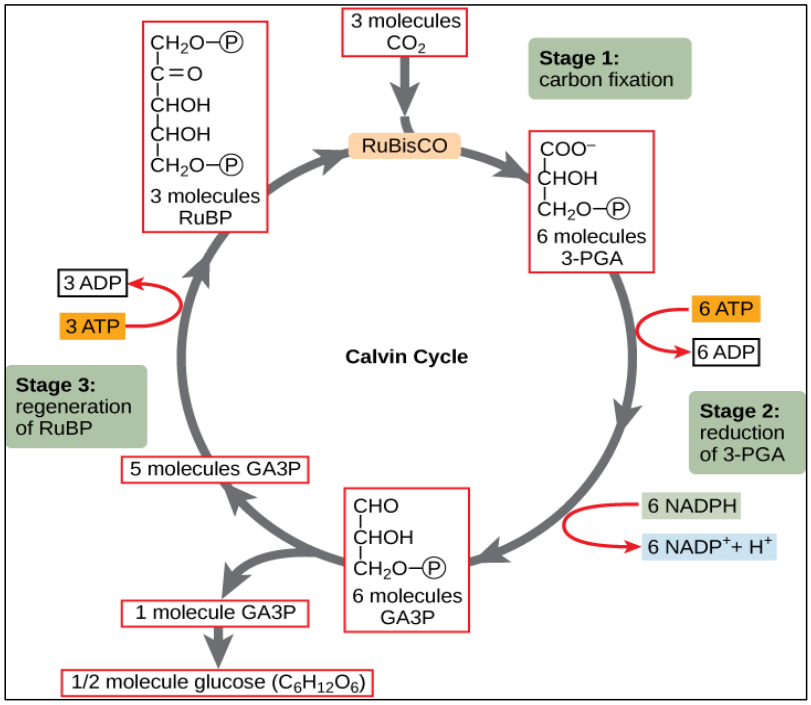


Fig. 7.13: Overview of the *Calvin Cycle* for carbon fixation in *C3* (most) plants. Three RuBP molecules *fix* 3 CO_2 molecules, catalyzed by ribulose bisphosphate carboxylase –oxygenase (RUBISCO). The three 6C molecules produced split into six 3C carbohydrates, becoming six glyceraldehyde-3-P (GA3P in the illustration). Five of these go on to regenerate 3 RuBP molecules.; the 6th GA3P waits for the Calvin Cycle to repeat, producing another GA3P. Two GA3Ps from 2 turns of the cycle are substrates for glucose synthesis.

Check the animation at Action in the Calvin Cycle. Each carbon dioxide entering the Calvin cycle is "fixed" to a 5-carbon *ribulose bisphosphate* molecule (RuBP), catalyzed by the enzyme ***RuBP carboxylase-oxygenase***, or ***RUBISCO*** for short. The expected 6-C molecule must quickly split into two 3-C molecules since it has not been found as an intermediate to date! The first detectable products are two molecules of 3-phosphoglyceric acid (3PGA). Each 3PGA is in turn reduced to glyceraldehyde-3-phosphate (GA3P in the illustration, or G-3-P). Some of the Calvin Cycle intermediates should look familiar! The cycle regenerates the RuBP ***and*** produces glucose. Fig. 7.14 below may help with the arithmetic of the Calvin Cycle.

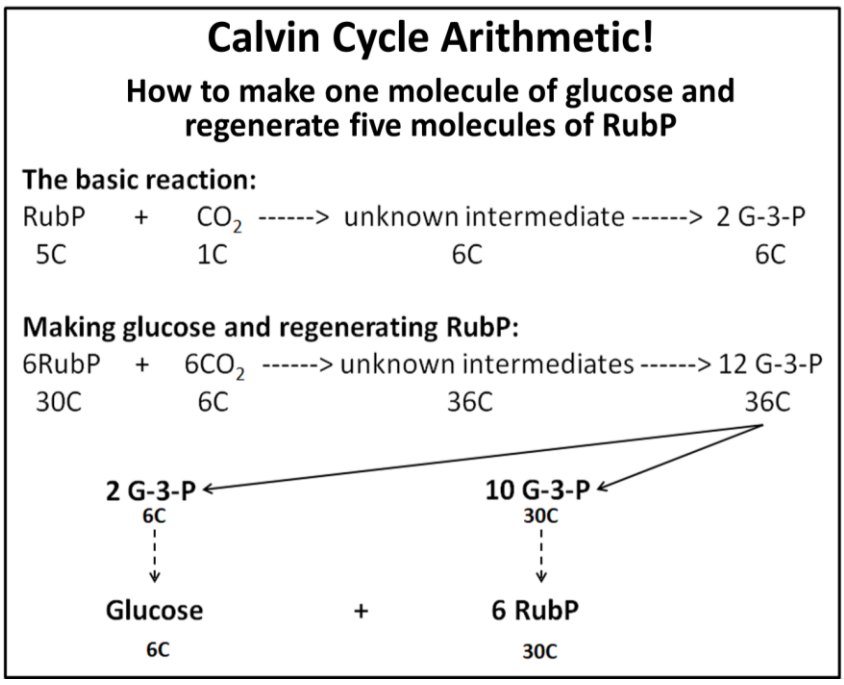


Fig. 7.14: *Calvin Cycle* Arithmetic: Making one glucose and regenerating 5 RubP molecules.

Perhaps the easiest way to see this is to imagine the cycle going around 6 times, fixing 6 molecules of CO_2, as shown in the illustration. In fact, a photosynthetic cell must fix six CO_2 carbons onto 6 Rubp molecules to generate one 6C glucose. This leaves 30 carbons on the table (as G-3-P molecules) with which to make six 5-carbon RubP molecules.

There are times that even plants in temperate environments suffer prolonged hot, dry spells. Perhaps you have seen a lawn grow more slowly and turn brown after a dry heat wave in summer, only to grow and re-green after the rains resume. C3 plants resort to ***photorespiration*** during drought and dry weather, closing their stomata to conserve water. Under these conditions, CO_2 can't get into the leaves… and O_2 can't get out! As CO_2 levels drop and O_2 levels rise in photosynthetic cells, the Calvin Cycle slows down. Instead of fixing CO_2, the enzyme ***RUBISCO*** now catalyzes "*O_2 fixation*" using its *oxygenase* activity. The combination of RuBP with O_2 splits RuBP into a 3-carbon and a 2-carbon molecule: **3-phosphoglyceric acid** (3-PG) and **phosphoglycolate** respectively (Fig. 7.15).

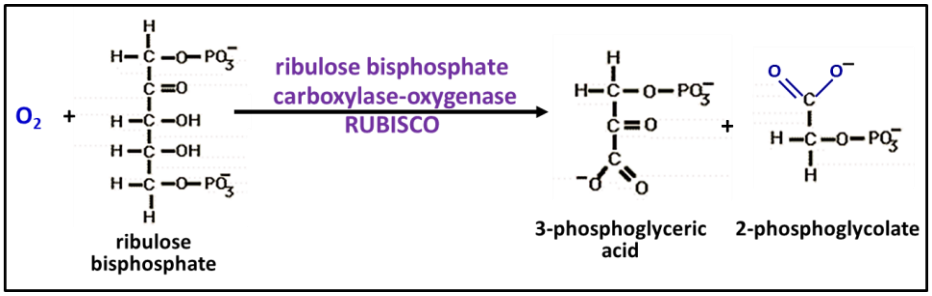


Fig. 7.15: Photorespiration is a C3 plant strategy to survive a dry spell or a heat wave. Water-starved C3 plants resort to *fixing* oxygen using RuBP. This reaction is catalyzed by RUBISCO, the same enzyme that fixes CO_2 to RuBP, but using its oxygenase activity.

Under these conditions, not only does photorespiration result in only one 3-carbon carbohydrate (compared to two in the Calvin Cycle), but the phosphoglycolate produced is *cytotoxic* (not healthy for cells!). Removing the phosphate and metabolizing the remaining *glycolic acid* costs energy. Therefore, photorespiration can only be sustained for a short time. On the other hand, plants that have adapted to live in hot arid environments all the time have evolved one of two alternate pathways. One is the ***CAM*** (***Crassulacean Acid Metabolism***); the other is the ***C4*** pathway. Each is an alternative to C3 carbon fixation.

7.6.2b CAM Photosynthetic Pathway - Light-Independent Reactions

Crassulacean acid metabolism (**CAM**) was discovered in the *Crassulaceae*. These are succulents like sedum (a common ground cover), cactuses and jade plants, and some orchids. This pathway was selected in evolution to allow plants to conserve water, especially during the high daytime temperatures.

The CAM pathway is shown in Fig. 7.16 (below).

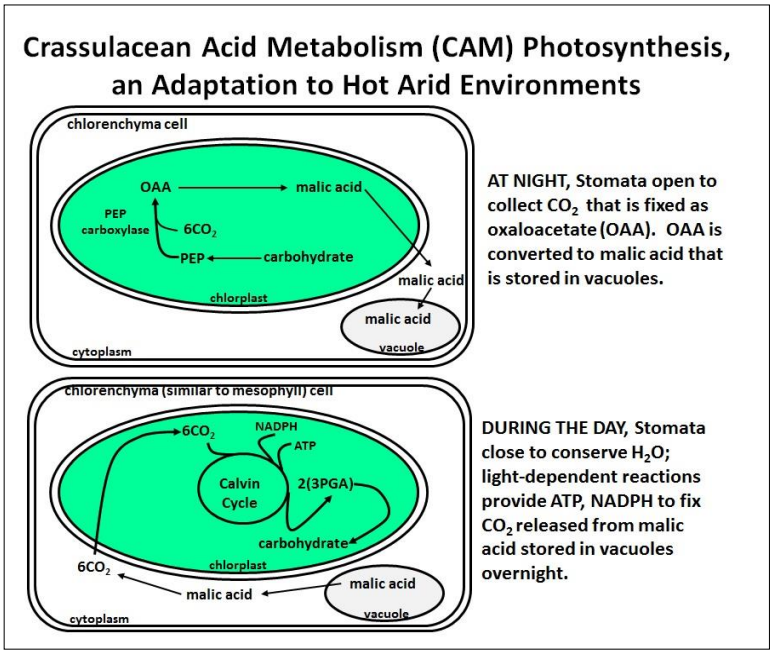


Fig. 7.16: Crassulacean Acid Metabolism in CAM plants. To survive in arid climates and minimize water loss, CAM plants o[pen their stomata and fix CO_2 in oxaloacetate (OAA) that is converted to malic acid to be stored until daylight. Stomata close at daybreak to conserving water, while malic acid releases CO_2 to be *re-fixed* to make glucose.

Stomata in chlorenchymal (***mesophyll***) leaf cells close during the day to minimize water loss by ***transpiration***. The stomata open at night, allowing plant tissues to take up CO_2. CAM plants fix CO_2 by combining it with ***PEP*** (*phosphoenol pyruvate*) to make ***oxaloacetate*** (***OAA***). This eventually produces ***malic acid*** that is stored in plant cell vacuoles. By day, stored malic acid retrieved from the vacuoles splits into pyruvate and CO_2. The CO_2 then enters chloroplasts and joins the Calvin Cycle to make glucose and the starches. In sum, CAM plant *mesophyll* cells

- open stomata to collect, fix and store CO_2 as an organic acid at night.
- close stomata to conserve water in the daytime.
- re-fix the stored CO_2 as carbohydrate using the NADPH and ATP from the light reactions the next the day.

7.6.2.c C4 Photosynthetic Pathway - Light-Independent Reactions

Malic acid is the *4-carbon* end product of the **C4** CO_2 fixation pathway. The ***C4 pathway*** is similar to the CAM pathway! In both, PEP carboxylase catalyzes carbon fixation, converting phosphoenol pyruvate (PEP) to oxaloacetate (OAA). The difference between the CAM and C4 pathway is in what happens to malic acid. Fig. 7.17 below shows the role of malic acid in the C4 the carbon fixation pathway.

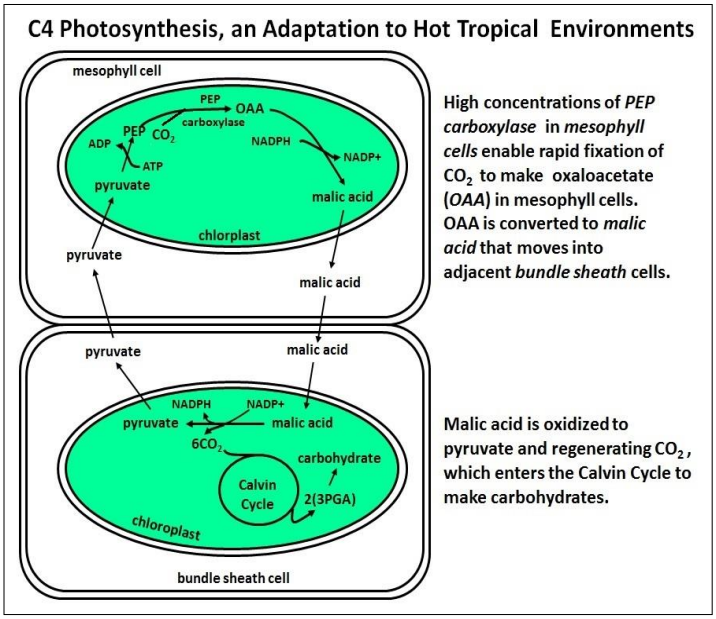


Fig. 7.17: *C4 Photosynthesis* pathway reactions in tropical plants. C4 plants separate their 'dark' reactions into 2 cell types. *Mesophyll* cells can keep stomata open part of the day, allowing CO_2 into the cells where it is fixed to PEP to make OAA and then malate. Unlike CAM plants, the malate is not stored, but transferred to *bundle sheath* cells where it can release CO_2 to be *re-fixed* by the Calvin Cycle.

C4 metabolism diverges from CAM pathway after malic acid formation. PEP carboxylase catalysis is rapid in C4 plants, in part because malic acid does not accumulate in the *mesophyll* cells. Instead, it is rapidly transferred from mesophyll to adjacent ***bundle sheath*** cells, where it enters chloroplasts. The result is that C4 plants can keep stomata open for CO_2 capture (unlike CAM plants), but closed at least part of the day to conserve water. The 4-carbon malic acid is oxidized to

pyruvate (three carbons) in the bundle sheath cell chloroplasts. The CO_2 released enters the Calvin cycle to be rapidly fixed by RUBISCO. Of course, this system allows more efficient water use and faster carbon fixation under dry high heat conditions than does C3 photosynthesis. Corn is perhaps the best-known C4 plant! By the way, can you recognize several more intermediates common to respiration and the light-independent photosynthetic reactions?

7.6 Thoughts on the Origins and Evolution of Respiration and Photosynthesis

We can assume that the abundance of chemical energy on our cooling planet favored the formation of cells that could capture free energy from energy-rich nutrients in the absence of any oxygen. For a time, we thought that the first cells would have extracted nutrient free energy by non-oxidative, fermentation pathways. But they would have been voracious feeders, quickly depleting their environmental nutrient resources. In this scenario, the evolution of autotrophic life saved heterotrophic life from an early extinction! That is because autotrophs could create organic molecules by extracting free energy from inorganic molecules or from light. An alternative scenario that is gaining traction, suggests that the first cells may have started with oxidative reactions that used something other than oxygen as a final electron acceptor. In this scenario (to be considered in more detail elsewhere), non-oxygenic 'oxidative' chemistries came first, followed by the evolution of anoxic fermentative chemistries, then followed by photosynthesis, and finally respiratory pathways. In either scenario, we can safely assume that photosynthesis existed before oxygenic respiration.

We also assume that oxygenic photoautotrophs that capture free energy from light would become the most abundant autotrophs, if for no other reason than sunlight is always available (at least during the day), and oxygen is abundant in the air! The early photoautotrophs were likely the ancestors of today's cyanobacteria. In fact, a phylogenetic study of many genes including "plastid-encoded proteins, nucleus-encoded proteins of plastid origin…, as well as wide-ranging genome data from cyanobacteria" suggests a common ancestry of freshwater cyanobacteria and eukaryotic chloroplasts (Ponce-Toledo, R.I. et al., 2017, *An Early-Branching Freshwater Cyanobacterium at the Origin of Plastids.* Current Biology 27:386-391).

Finally, let's think about the origins of respiratory metabolism and the endosymbiotic origins of mitochondria? Let's start by asking how respiration co-opted photosynthetic electron transport reactions that captured the electrons from H_2O needed to reduce CO_2, turning those reactions to the task of burning sugars back to H_2O and CO_2. As photosynthetic organisms emerged and atmospheric oxygen increased, elevated oxygen levels would have been toxic to most living things. Still, some autotrophic cells must have had a pre-existing genetic potential to conduct detoxifying respiratory chemistry. These

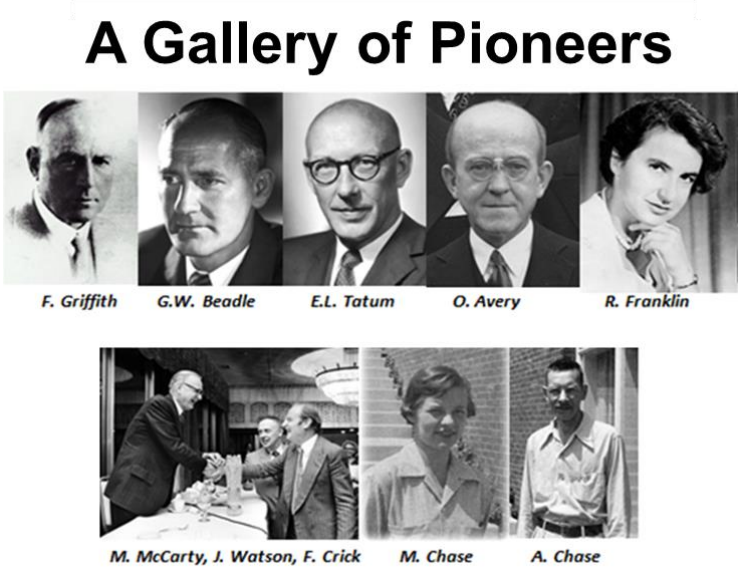
would have been facultative aerobes with the ability to switch from photosynthesis to respiration when oxygen levels rose. Today's *purple non-sulfur bacteria* (e.g., *Rhodobacter sphaeroides*) are just such facultative aerobes! Perhaps we aerobes descend from the ancestors of *Rhodobacter*-like cells that survived and spread from localized environments where small amounts of oxygen threatened their otherwise strictly anaerobic neighbors. Is it possible that the endosymbiotic critter that became the first mitochondrion in a eukaryotic cell was not just any aerobic bacterium, but a purple photosynthetic bacterium?

Some iText & VOP Key Words and Terms

active transport of protons	energy efficiency of glucose metabolism	PEP carboxylase
ATP synthase	energy flow in glycolysis	pH gradient
bacterial flagellum	energy flow in the Krebs Cycle	pheophytin
C4 photosynthesis	F1 ATPase	photosynthesis
Calvin Cycle	FAD	Photosystems
CAM photosynthesis	$FADH_2$	proton gate
carotene	Light-dependent reactions	proton gradient
chemiosmotic mechanism	Light-independent reactions	proton pump
Chlorophyll a	Malic acid	PSI electron acceptor
Chlorophyll b	mitochondria	PSII electron acceptor
complex I, II, III, IV	molecular motor	redox reactions
Crassulaceae	NAD+	reduced electron carriers
cristal membrane	NADH	RUBISCO
Cyclic photophosphorylation	NADP+	RuBP
cytochromes	NADPH	Splitting water
Dark Reactions	outer membrane	stoichiometry of glycolysis
electrochemical gradient	oxidative phosphorylation	stoichiometry of the Krebs Cycle
electron transport system (chain)	oxidative phosphorylation	substrate-level phosphorylation
endosymbiotic theory	P_{680}	Z-scheme
energy efficiency of glucose metabolism	P_{700}	

Chapter 8: DNA, Chromosomes and Chromatin

The "Stuff of Genes"; The Double Helix; Chromosomes and Chromatin; Naked, Circular Bacterial DNA



8.1 Introduction

Here we look at classic experiments that led to our understanding that genes are composed of ***DNA***. We already knew that genes were on chromosomes (*chromo* – colored; *soma*-body), that the shapes of chromosomes of a species were unique, and that we could characterize a species by the appearance (***karyotype***) of its chromosomes. Early 20th century ***gene mapping*** even showed the relative location (***locus***) of genes on linear eukaryotic chromosomes. We'll see that eukaryotic chromosomes are highly *condensed* structures composed of DNA and protein, visible only during ***mitosis*** or ***meiosis***. During the much longer ***interphase*** portion of the eukaryotic ***cell cycle***, chromosomes de-condense to ***chromatin***, a less organized form of protein-associated DNA in the nucleus. Compared to eukaryotic cells, bacteria contain a tiny amount of much more simply organized DNA. Nevertheless, we'll learn here that bacterial *gene mapping* first revealed that the *bacterial '*chromosome' to be just a small closed, naked circular DNA double helix. Since cells must alter patterns of *gene expression* over time and in response to environment, understanding the structure and organization of DNA in

cells is essential to an understanding of how and when cells turn genes on and off. While we consider how cells regulate gene expression in an upcoming chapter, keep in mind that that every cell of a eukaryotic organism contains the same species-specific DNA, as does each cell of a given prokaryotic species. Here we'll consider that eukaryotic chromatin is the gatekeeper of *gene activity*, a situation quite different from bacterial cells.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. summarize the evidence that led to acceptance that genes are made of DNA.
2. discuss how Chargaff''s DNA *base ratios* support DNA as the "stuff of genes".
3. interpret the results of Griffith, Avery et al. and Hershey & Chase, in historical context.
4. outline and explain how Watson and Crick built their model of a DNA double helix.
5. distinguish between conservative, semiconservative and dispersive replication.
6. describe and/or draw the progress of a *viral infection.*
7. trace the fate of $^{35}SO_4$ *(sulfate)* into proteins synthesized in cultured bacteria.
8. distinguish between the organization of DNA in chromatin and chromosomes and speculate on how this organization impacts replication.
9. list some different uses of karyotypes.
10. compare and contrast euchromatin and heterochromatin structure and function.
11. outline an experiment to purify *histone H1* from chromatin.
12. formulate an *hypothesis* to explain why chromatin is found only in eukaryotes.
13. describe the roles of different histones in nucleosome structure.
14. explain the role of Hfr strains in mapping genes in *E. coli*.
15. explain the chemical rationale of using different salt concentrations to extract 10 nm nucleosome fibers *vs*. 30nm solenoid structures from chromatin.

8.2 The Stuff of Genes

That all eukaryotic cells contain a nucleus was understood by the late 19th century. By then, histological studies had shown that nuclei contained largely proteins and DNA. At around the same time, the notion that the nucleus contains genetic information was gaining traction. Mendel's Laws of Inheritance, presented in 1865, were not widely understood, probably because they relied on a strong dose of arithmetic and statistics, at a time when the utility of quantitative biology was not much appreciated. But, following the re-discovery three decades later, the number of traits in any given organism and their Mendelian patterns of inheritance increased rapidly. But evidence that DNA was the *stuff of genes* was slow to follow.

In 1910, Albrecht Kossel received the 1910 Nobel Prize in Physiology or Medicine for his discovery of the adenine, thymine, cytosine and guanine (the four DNA bases), as well as of uracil in RNA. At that time, DNA was known as a small, simple molecule, made up of

only the four nucleotides (see *DNA Structure* below for additional historical perspective). So, the question was how could such a small, simple account for the inheritance of so many different physical traits? The recognition that enzyme activities were inherited in the same way as morphological characteristics led to the ***one-gene-one enzyme*** hypothesis that earned G. W. Beadle, E. L. Tatum and J. Lederberg the 1958 Nobel Prize for Physiology and Medicine. When enzymes were later shown to be proteins, the hypothesis became ***one-gene-one protein***. When proteins were shown to be composed of one or more polypeptides, the final hypothesis became ***one-gene-one-polypeptide***.

However, this relationship between genes and polypeptides failed to shed any light on how DNA might be the genetic material. In fact, quite the contrary! As chains of up to 20 different amino acids, polypeptides and proteins had the potential for enough structural diversity to account for the growing number of heritable traits in a given organism. Thus, proteins seemed more likely candidates for the molecules of inheritance.

The experiments you will read about here began around the start of World War I and lasted until just after World War 2. During this time, we learned that DNA was no mere tetramer, but was in fact a long polymer. This led to some very clever experiments that eventually forced the scientific community to the conclusion that DNA, not protein, was the genetic molecule, despite being composed of just four monomeric units. Finally, we look at the classic work of Watson, Crick, Franklin and Wilkins that revealed the structure of the genetic molecule.

8.2.1 Griffith's Experiment

Fred Neufeld, a German bacteriologist studying pneumococcal bacteria in the early 1900s discovered three immunologically different strains of *Streptococcus pneumonia (*Types I, II and III*)*. The virulent strain (Type III) was responsible for much of the mortality during the **Spanish Flu** (influenza) *pandemic* of 1918-1920. This pandemic killed between 20 and 100 million people, many because the influenza viral infection weakened the immune system of infected individuals, making them susceptible to bacterial infection by *Streptococcus pneumonia*.

In the 1920s, Frederick Griffith was working with virulent ***wild type*** (*Type III*) and ***benign*** (*Type II*) strains of *S. pneumonia*. The two strains were easy to tell apart in petri dishes because the virulent strain grew as morphologically *smooth colonies*, while the benign strain formed *rough colonies*. For this reason, the two bacterial strains were called ***S*** and ***R***, respectively. As we now know, *S* cells are coated with a polysaccharide (mucus) capsule making their colonies appear smooth. In contrast, *R* cell colonies look rough (don't glisten) because they lack the polysaccharide coating. Griffith knew that injecting mice with *S* cells killed them within about a day! Injecting the non-lethal *R* cells on the other hand, caused no harm. Then, he surmised that

perhaps, the exposure of mice to the R strain of *S. pneumonia* would immunize them against lethal infection by subsequent exposure to S cells. His experimental protocol and results, published in 1928, are summarized in Fig. 8.1 (below).

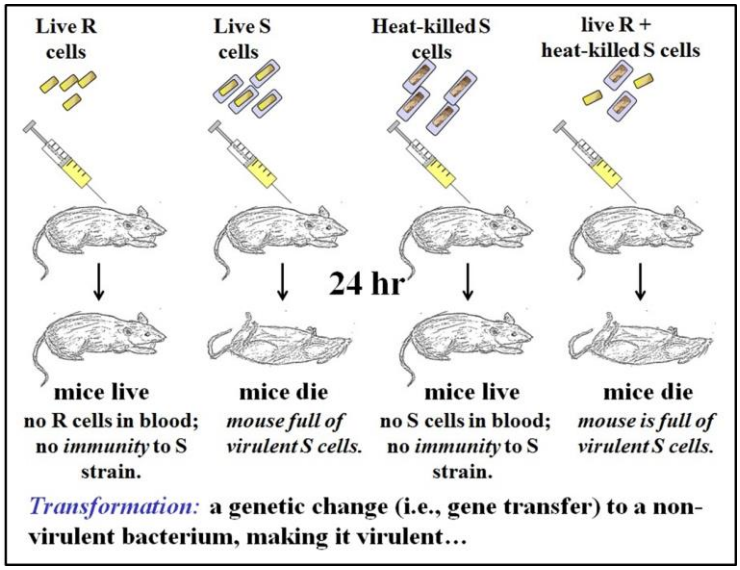


Fig. 8.1: The experiments of F. Griffith demonstrating the existence of a chemical that could transfer a genetic trait (i.e., virulence) between bacteria (see text for details).

To test his hypothesis, Griffith injected mice with R cells. Sometime later, he injected them with S cells. Unfortunately, the attempt to immunize the mice against S. *pneumonia* was unsuccessful! The control mice injected with S strain cells *and* the experimental mice that received the R strain cells first and then S cells, all died in short order! As expected, mice injected with R cells only survived.

Griffith also checked the blood of his mice for the presence of bacterial cells:

- Mice injected with benign **R (**rough) strain cells survived and after plating blood from the mice on nutrient medium, no bacterial cells grew.
- Many colonies of S cells grew from the blood of dead mice injected with S cells.

Griffith performed two other experiments, shown in the illustration:

- He injected mice with heat-killed S cells; these mice survived. Blood from these mice contained no bacterial cells. This was expected since heating the S cells should have the same effect as pasteurization has on bacteria in milk!

- Griffith also injected mice with a *mixture of live R cells and heat-killed S cell*s, in the hope that the combination might induce immunity in the mouse where injecting the R cells alone had failed. You can imagine his surprise when, far from being immunized against S-cell infection, the injected mice died and abundant S cells had accumulated in their blood.

Griffith realized that something important had happened in his experiments. In the mixture of live R cells and heat-killed S cells, something released from the dead S cells had transformed some R cells. Griffith named this "something" the **transforming principle**, a molecule present in the debris of dead S cells and sometimes acquired by a few live R cells, turning them into virulent S cells.

8.2.2 The Avery-MacLeod-McCarty Experiment

While Griffith didn't know the chemical identity of his *transforming principle*, his experiments led to studies that proved DNA to be ***the stuff of genes***. With improved molecular purification techniques developed in the 1930s, O. Avery, C. MacLeod, and M. McCarty transformed R cells *in vitro* (that is, without the help of a mouse!). They purified heat-killed S-cell components (DNA, proteins, carbohydrates, lipids…) and separately tested the transforming ability of each molecular component on R cells in a test tube. Since only the ***DNA fraction*** of the dead S cells could cause transformation, Avery et al. concluded that DNA must be the ***Transforming Principle***. Fig. 8.2 (below) summarizes the experiments of Avery et al. But despite the evidence, DNA was not readily accepted as the stuff of genes.

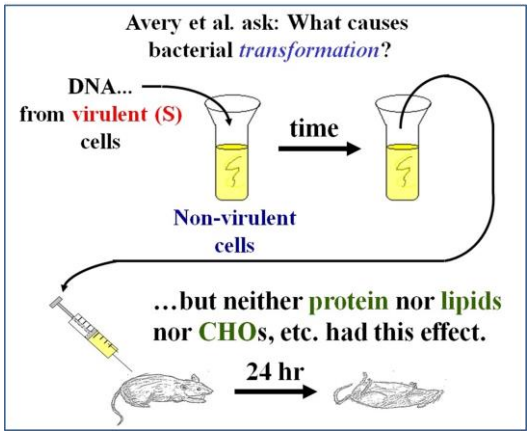


Fig. 8.2: The experiments of O. Avery et al. demonstrating for the first time that the chemical stuff of genes, of inheritance is DNA (see text for details).

The sticking point was that DNA was composed of only four nucleotides. Even though scientists knew that DNA was a large polymer, they still thought of DNA as that simple molecule, for example a polymer made up of repeating sequences of the four nucleotides, for example, **…AGCTAGCTAGCTAGCTAGCT…** Only the seemingly endless combinations of 20 amino acids in proteins promised the biological specificity necessary to account for an organism's many genetic traits. Lacking structural diversity, DNA was explained as a mere scaffold for protein genes. To adapt Marshal McLuhan's famous statement that *the medium is the message* (i.e., airwaves do not merely *convey*, but are the message), many still believed that proteins are the medium of genetic information *as well as* the functional message itself. The reluctance of influential scientists of the day to accept a DNA *transforming principle* deprived its discoverers of the Nobel Prize stature it deserved. After new evidence made further resistance to that acceptance untenable, even the Nobel Committee admitted that failure to award a Nobel Prize for the discoveries of Avery et al. was an error. The key experiments of Alfred Hershey and Martha Chase finally put to rest any notion that proteins were genes

167-2 Transformation In & Out of Mice-Griffith, McCarty et al.

8.2.3 The Hershey-Chase Experiment

Earlier we learned that viruses cannot live an independent life! Bacterial viruses, called bacteriophage or just phage for short, consist of DNA inside a protein capsule. The life cycle of bacterial viruses is shown below in Fig. 8.3 (below).

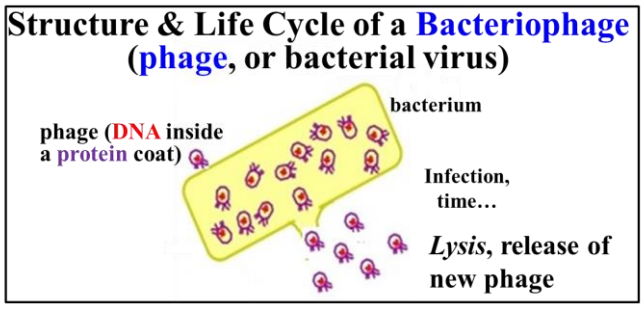


Fig. 8.3: Life cycle of a bacteriophage: The phage *coat* remains attached to a cell after infection, but the chemicals inside the phage enter the infected cell.

Phages are inert particles until they bind to and infect bacterial cells. Phage particles added to a bacterial culture could be seen attach to bacterial surfaces in an electron microscope. Investigators found that they could detach phage particles from bacteria by agitation in a blender (similar to one you might have in your kitchen). Centrifugation

then separated the bacterial cells in a pellet at the bottom of the centrifuge tube, leaving the detached phage particles in the supernatant. By adding phage to bacteria and then detaching the phage from the bacteria at different times, it was possible to determine how long it the phage had to remain attached before the bacteria become infected. It turned out that pelleted cells that had been attached to phage for short times would survive and reproduce when re-suspended in growth medium. But pelleted cells left attached to phage for longer times had become infected. When centrifugally separated from the detached phage and resuspended in fresh medium, these cells would go on and lyse, producing new phage. Therefore, the transfer of genetic information for virulence from virus to phage took some time. The viral genetic material responsible for infection and virulence was apparently no longer associated with the phage capsule, which could be recovered from the centrifugal supernatant.

Alfred Hershey and Martha Chase designed an experiment to determine whether the *DNA* enclosed by the viral *protein capsule* or the capsule protein itself caused phage to infect the bacterium. In the experiment, they separately grew *E. coli* cells infected with ***T2 bacteriophage*** in the presence of either **^{32}P** or **^{35}S** (*radioactive* isotopes of phosphorous and sulfur, respectively). The result was to generate phage that contained either radioactive DNA or radioactive proteins, but not both (recall that only DNA contains phosphorous and only proteins contain sulfur). They then separately infected fresh *E. coli* cells with either ^{32}P- or ^{35}S-labeled, radioactive phage. Their experiment is described in Fig. 8.4 (below).

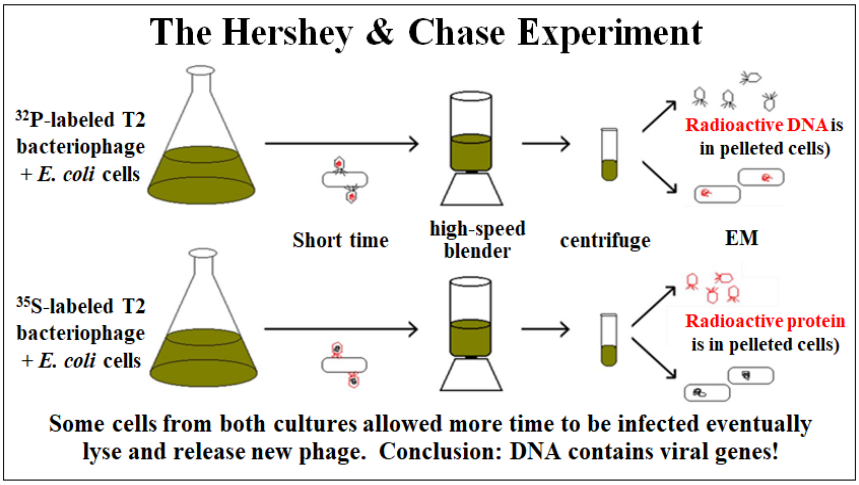


Fig. 8.4: The experiments of Alfred Hershey and Martha Chase demonstrating that the chemical stuff of viral genes is DNA (see text for details)

Phage and cells were incubated with either ^{32}P or ^{35}S just long enough to allow infection. Some of each culture was allowed to go on and lyse to prove that the cells were infected. The remainder of each mixture was sent to the blender. After centrifugation of each blend, the pellets and supernatants were examined to see where the radioactive proteins or DNA had gone. From the results, the ^{32}P always ended up in the pellet of bacterial cells while the ^{35}S was found in the phage remnant in the supernatant. Hershey and Chase concluded that the genetic material of bacterial viruses was DNA and not protein, just as Avery et al. had suggested that DNA was the bacterial transforming principle.

Given the earlier resistance to "simple" DNA being the genetic material, Hershey and Chase used cautious language in framing their conclusions. They need not have; all subsequent experiments confirmed that DNA was the genetic material. Concurrent with these confirmations were experiments demonstrating that DNA might not be (indeed, was not) such a simple, uncomplicated molecule! For their final contributions to pinning down DNA as the "stuff of genes", Alfred D. Hershey shared the 1969 Nobel Prize in Physiology or Medicine with Max Delbruck and Salvador E. Luria. So why didn't Martha Chase share in the recognition of the Nobel Prize, given that she was Hershey's sole co-author on the paper presenting their findings? You may well ask!

168 Hershey and Chase: Viral Genes are in Viral DNA

8.3 DNA Structure

By 1878, a substance in the pus of wounded soldiers derived from cell nuclei (called *nuclein*) was shown to be composed of 5 bases (the familiar ones of DNA and RNA). The four bases known to make up DNA (as part of nucleotides) were thought to be connected through the phosphate groups in short repeating chains of four nucleotides. By the 1940s, we knew that DNA was a long polymer. Nevertheless, it was still considered too simple to account for genes (see above). After the Hershey and Chase experiments, only a few holdouts would not accept DNA as the genetic material. So when DNA was accepted DNA as the *stuff of genes*, the next questions were

- Was what did DNA look like?
- How did its structure account for its ability to encode and reproduce life?

While the nature of DNA, its composition of 4 nucleotides had been known for some time, it became mandatory to explain how such a "simple molecule" could inform the thousands of proteins necessary for life. The answer to this question was to lie at least in part in an understanding of the physical structure of DNA, made possible by the advent of ***X-Ray Crystallography***.

8.3.1 X-Ray Crystallography and the Beginnings of Molecular Biology

If a substance can be crystallized, the crystal will diffract X-rays at angles revealing regular (repeating) structures of the crystal. William Astbury demonstrated that high molecular weight DNA had just such a regular structure. His ***crystallographs*** suggested DNA to be a linear polymer of stacked bases (nucleotides), each nucleotide separated from the next by 0.34 nm. Astbury is also remembered for coining the term "*molecular biology*" to describe his studies. The term now covers as all aspects of biomolecular structure, as well as molecular functions (e.g. replication, transcription, translation, gene regulation…).

In an irony of history, the Russian biologist Nikolai Koltsov had already intuited in 1927 that the basis of genetic transfer of traits would be a "giant hereditary molecule" made up of "two mirror strands that would replicate in a semi-conservative fashion using each strand as a template". A pretty fantastic inference if you think about it since it was proposed long before Watson and Crick and their colleagues worked out the structure of the DNA double-helix!

8.3.1 Wilkins, Franklin, Watson & Crick – DNA Structure Revealed

Maurice Wilkins, an English biochemist, was the first to isolate highly pure, high molecular weight DNA. Working in Wilkins laboratory, Rosalind Franklin was able to crystalize this DNA and produce very high-resolution X-Ray diffraction images of the DNA crystals. Franklin's most famous (and definitive) crystallograph was "Photo 51" (Fig. 8.5).

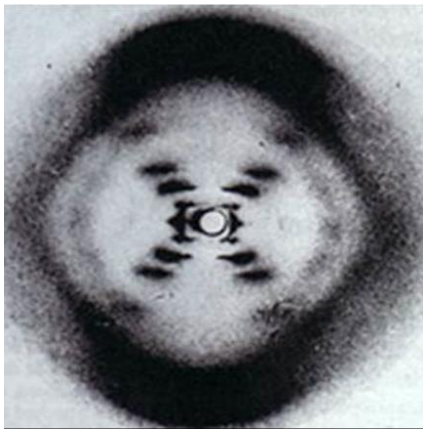


Fig. 8.5: *Photo 51*, the X-Ray crystallograph of DNA taken by Rosalind Franklin revealed 3 crucial molecular dimensions that ultimately led to the double helical DNA model.

This image confirmed Astbury's **0.34 nm** repeat dimension and revealed two more numbers, **3.4 nm** and **2 nm**, reflecting additional repeat structures in the DNA crystal. When James Watson and Francis Crick got hold of these numbers, they used them along with other data to build DNA models out of nuts, bolts and plumbing. Their models eventually revealed DNA to be a pair of **antiparallel complementary** strands of nucleic acid polymers…, shades of Koltsov's mirror-image macromolecules! Each strand is a string of nucleotides linked by *phosphodiester bonds*, the two strands held together in a *double helix* by complementary H-bond interactions. Let's look at the evidence for these conclusions. As we do, refer to the two illustrations of the double helix in Fig. 8.6.

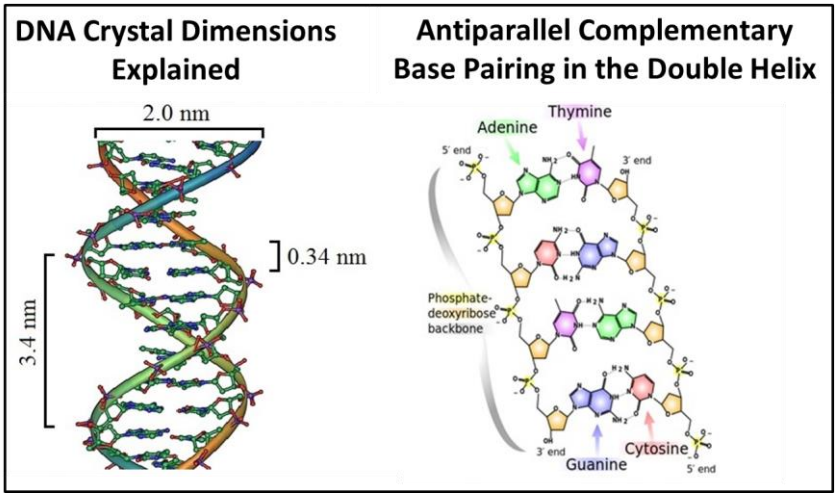


Fig. 8.6: The 3 molecular dimensions from Franklin's Photo 51 was best explained by proposing a DNA double helix (LEFT). Model building by Watson and Crick revealed the antiparallel structure of the double helix (RIGHT).

Recalling that Astbury's 0.34 nm dimension was the ***distance between successive nucleotides*** in a DNA strand, Watson and Crick surmised that the 3.4 nm repeat was a structurally meaningful 10-fold multiple of Astbury's number. When they began building their DNA models, they realized from the bond angles connecting the nucleotides that the strand was forming a helix, from which they concluded that the 3.4 nm repeat was the ***pitch*** of the helix, i.e., the length of one complete turn of the helix. This meant that there were 10 bases per turn of the helix. They further reasoned that the 2.0 nm number might reflect the diameter of helix. When their scale model of a single stranded DNA helix predicted a helical diameter much less than 2.0 nm, they

were able to model a ***double helix*** that more nearly met the 2.0 nm diameter requirement. In building their double helix, Watson and Crick realized that bases in opposing strands would come together to form H-bonds, holding the helix together. However, for their double helix to have a constant diameter of 2.0 nm, they also realized that the smaller **pyrimidine** bases, ***Thymine*** (**T**) and ***Cytosine*** (**C**), would have to H-bond to the larger **purine** bases, ***Adenine*** (**A**) and ***Guanosine*** (**G**)…, and that neither A-G nor C-T would form pairs in the double helix.

Now to the question of how a "simple" DNA molecule could have the structural diversity needed to encode thousands of different polypeptides and proteins. In early studies, purified *E. coli* DNA was chemically hydrolyzed down to nucleotide monomers. The hydrolysis products contained nearly equal amounts of each base, reinforcing the notion that DNA was that simple molecule that could not encode genes. But Watson and Crick had private access to revealing data from Erwin Chargaff. Chargaff had determined the base composition of DNA isolated from different species, including *E. coli*. He found that the base composition of DNA from different species was not always *equimolar*, meaning that for some species, the DNA was not composed of equal amounts of each of the four bases (see some of this data in Table 8.1).

Base Compositions of DNA from Different Organisms

base		*human*	*yeast*	*fly*
purine	A	27%	21%	15%
pyrimidine	C	23%	32%	35%
purine	G	23%	32%	35%
pyrimidine	T	27%	21%	15%

The mere fact that DNA from some species could have base compositions that deviated from equimolarity put to rest the argument that DNA had to be a very simple sequence. Finally it was safe to accept that to accept the obvious, namely that DNA sequences could vary almost infinitely and could indeed be *the stuff of genes*.

Chargaff's data also showed a unique pattern of base ratios. Although the base compositions could vary between species, the **A/T** and **G/C** *ratio* was always one, for every species; likewise, the ratio of **(A+C/(G+T)** and **(A+G)/(C+T)**. From this information, Watson and Crick inferred that **A** (a purine) would H-bond with **T (a pyrimidine)**, and **G** (a purine) would H-bond with **C** (a pyrimidine) in the double helix. When building their model with this new information, they also found H-bonding

between the complementary bases would be maximal only if the two DNA strands were **antiparallel**, leading to the most stable structure of the double helix. Watson and Crick published their conclusions about the structure of DNA in 1953 (Click here to read their seminal article: Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid.

Their article is also famous for predicting a semiconservative mechanism of replication, something that had also been predicted by Koltsov 26 years earlier, albeit based on intuition… and much less evidence! Watson, Crick and Wilkins shared a Nobel Prize in 1962 for their work on DNA structure. Unfortunately, Franklin died in 1958 and Nobel prizes were not awarded posthumously. There is still controversy about why Franklin did not get appropriate credit for her role in the work. But she has been getting well-deserved, long-delayed recognition, including a university in Chicago named in her honor!

169-2 Unraveling the Structure of DNA

8.3.3 Meselson and Stahl's Experiment – Replication is Semiconservative

Confirmation of Watson & Crick's suggestion of semiconservative replication came from Meselson and Stahl's very elegant experiment, which tested the three possible models of replication (Fig. 8.7)

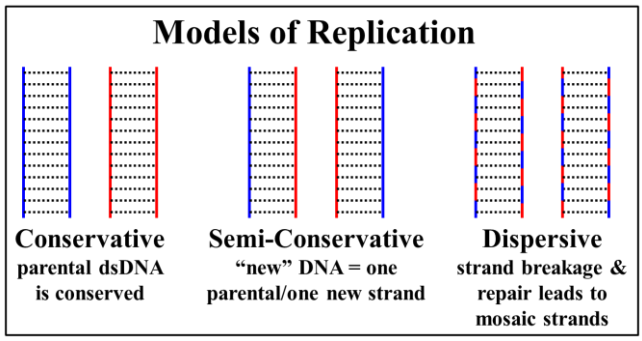


Fig. 8.7: Three possible modes of DNA replication could be imagined.

In their experiment, *E. coli* cells were grown in medium containing ^{15}N, a 'heavy' nitrogen isotope. After many generations, all of the DNA in the cells had become labeled with the heavy isotope. At that point, the ^{15}N-tagged cells were placed back

in medium containing the more common, 'light' ^{14}N isotope and allowed to grow for exactly one generation. Fig. 8.8 (below) shows Meselson and Stahl's predictions for their experiment. Meselson and Stahl knew that ^{14}N-labeled and ^{15}N-labeled DNA would **form separate** bands after centrifugation on *CsCl chloride density gradients*.

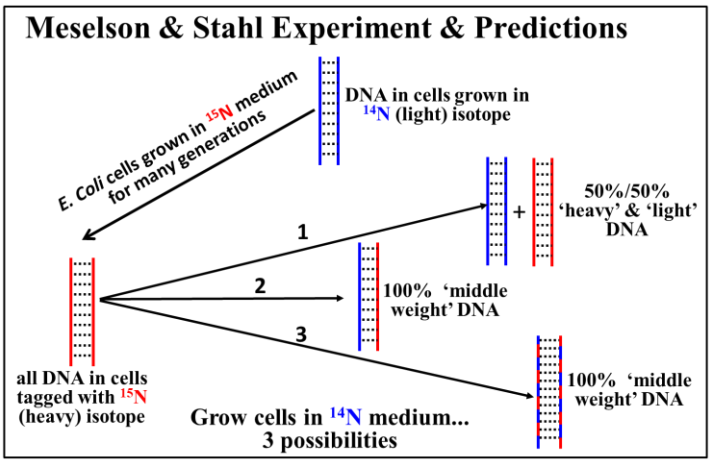


Fig. 8.8: Meselson and Stahl predict experimental outcomes based on 3 possible modes of DNA replication.

They tested their predictions by purifying and centrifuging the DNA from the ^{15}N-labeled cells grown in ^{14}N medium for one generation. They found that this DNA formed a single band with a density between that of ^{15}N-labeled DNA and ^{14}N-labeled DNA, eliminating the *conservative mode*l of DNA replication (possibility #1), as Watson and Crick had also predicted. That left two possibilities: replication was either semiconservative (possibility #2) or dispersive (possibility #3). The dispersive model was *eliminated* when DNA isolated from cells grown for a 2nd generation on ^{14}N were shown to contain two bands of DNA on the CsCl density gradients.

170 Replication is Semiconservative

8.4 Chromosomes

We knew from the start of the 20th century that eukaryotic chromosomes contain genes. Therefore, it became necessary to understand the relationship between chromosomes, chromatin, DNA and genes. As noted earlier, chromosomes are a specialized, *condensed* kind of chromatin with key structural features shown below in Fig 8.9.

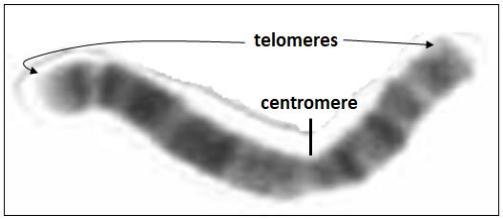


Fig. 8.9: Light micrograph of a human female chromosome stained for banding patters, showing the *centromere* constriction and defining *telomeres* (chromosome ends).

We now know that the compact structure of a chromosome prevents damage to the DNA during cell division. This damage can occur when forces on centromeres generated by mitotic or meiotic spindle fibers pull chromatids apart. As the nucleus breaks down during mitosis or meiosis, late 19th century microscopists saw chromosomes condense from the dispersed cytoplasmic background. These chromosomes remained visible as they separated, moving to opposite poles of the cell during cell division. Such observations of chromosome behavior during cell division pointed to their role in heredity. Check out the chromatids being separated during anaphase of mitosis in the computer-colorized micrograph at https://www.flickr.com/photos/185778834@N06/49363817053/.

It's possible to distinguish one chromosome from another by ***karyotyping***. Cells in metaphase of mitosis placed under pressure burst and the chromosomes spread out. Fig. 8.10 (below) shows such a chromosome spread.

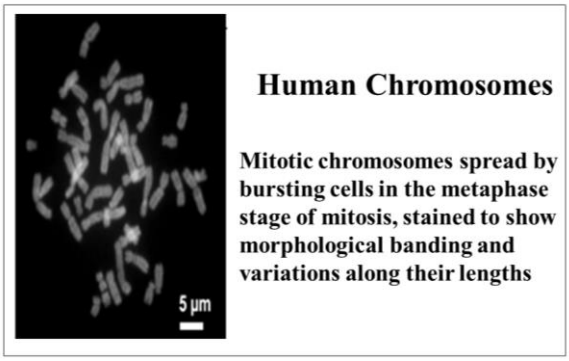


Fig. 8.10: Spread of human mitotic cell chromosomes.

By the early 1900s, the number, sizes and shapes of chromosomes were shown to be species-specific. What's more, a close look at chromosome spreads revealed that chromosomes came in morphologically matched pairs. This was so reminiscent of

Gregor Mendel's paired hereditary factors that chromosomes became widely accepted as the structural seat of genetic inheritance. Cutting apart micrographs like the one above (and below!) and pairing the chromosomes by their morphology generates a ***karyotype***. Paired human homologs are easily identified in the colorized micrograph (Fig. 8.11, below).

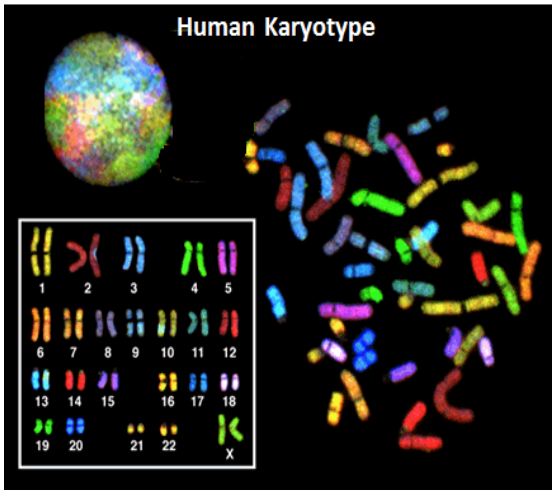


Fig. 8.11: Computer-colorized human *karyotype*.

Seen in cells in mitosis, all dividing human cells contain 23 pairs of homologous chromosomes. The ***karyotype*** is from a female; note the pair of homologous sex ("X") chromosomes (lower right of the inset). X and Y chromosomes in males are not truly homologous. Chromosomes in the original spread and in the aligned karyotype were stained with fluorescent antibodies against chromosome-specific DNA sequences to 'light up' the different chromosomes.

171-2 DNA, Chromosomes, Karyotypes & Gene Maps

8.5 Genes and Chromatin in Eukaryotes

Bacterial DNA (and prokaryotic DNA generally) is relatively 'naked' – not visibly associated with protein. Chromatin is an association of DNA with more or less protein seen in eukaryotic nuclei; eukaryotic chromosomes are a unique form of chromatin seen only in mitosis and meiosis.

The electron micrograph of an *interphase* cell nucleus (Fig. 8.12) reveals chromatin in two states of condensation.

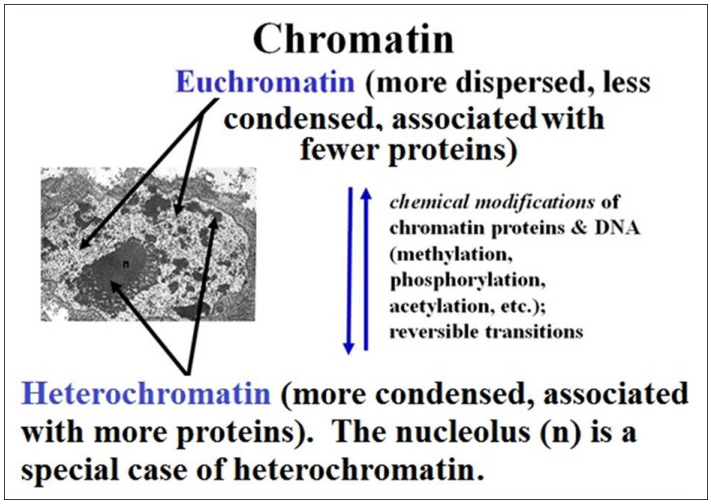


Fig. 8.12: Transmission micrograph of *euchromatin* and *heterochromatin* in the nucleus.

Chromatin is maximally condensed during mitosis to form chromosomes. During interphase, chromatin exists in more or less condensed forms, called ***heterochromatin*** and ***euchromatin*** respectively. Transition between these chromatin forms involve changes in the amounts and types of proteins bound to the chromatin that can occur during gene regulation, i.e., when genes are turned on or off. Experiments to be described later showed that active genes tend to be in the more dispersed euchromatin where enzymes of replication and transcription have easier access to the DNA. Transcriptionally *inactive* genes are heterochromatic, obscured by additional chromatin proteins present in heterochromatin.

We can define three levels of chromatin organization in general terms:
1. DNA wrapped around histone proteins (n*ucleosomes)* like "beads on a string".
2. Multiple nucleosomes coiled (condensed) into 30 nm fiber (solenoid) structures.
3. Higher-order packing of the 30 nm fiber to form the familiar metaphase chromosome.

These aspects of chromatin structure were determined by gentle disruption of the nuclear envelope of nuclei, followed by salt extraction of extracted chromatin. Salt extraction dissociates most of the proteins from the chromatin. The results of a low [salt] extraction are shown in Fig. 8.13 (below).

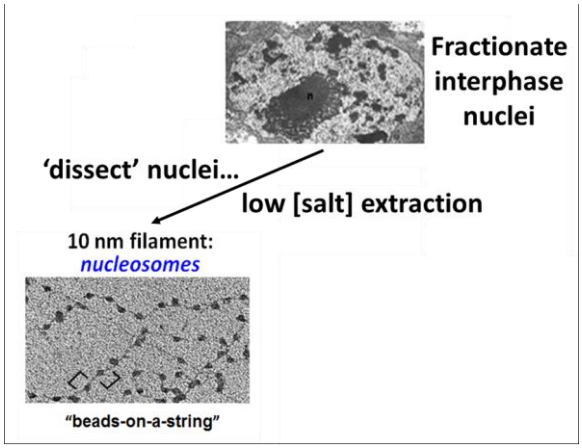


Fig. 8.13: Low salt fractionation of interphase nuclei yields 10nm nucleosome *beads on a string*.

When the low salt extract is centrifuged and the pellet resuspended, the remaining chromatin looks like *beads on a string*. DNA-wrapped *nucleosomes* are the beads, which are in turn linked by uniform lengths of the metaphorical DNA 'string'. After a high salt chromatin extraction, the structure visible in the electron microscope is the 30nm solenoid, the coil of nucleosomes modeled in Fig. 8.14 (below).

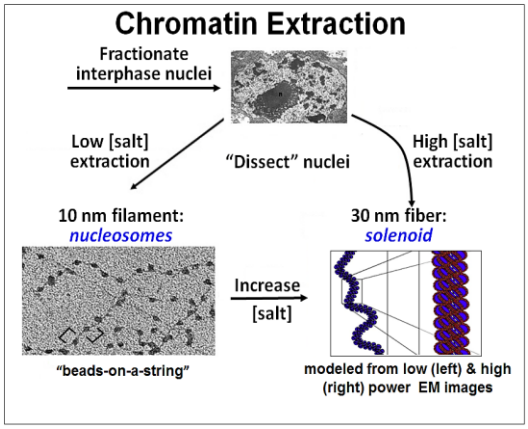


Fig. 8.14: High salt chromatin extraction from nuclei, or high salt treatment of 10 nm filaments yields 30 nm *solenoid* structures, essentially coils of 10 nm filaments.

As shown in the illustration, increasing the salt concentration of an already extracted nucleosome preparation will cause the 'necklace' to fold into the 30nm solenoid structure. In fact, there are at least the five levels (***orders***) of chromatin structure (Fig. 8.15 below). The first order is the *string of beads*, DNA-wrapped *nucleosomes* beads linked by uniform lengths of the metaphorical DNA 'string' (**# 1** in the illustration). The next *order* (**# 2**) is the high salt chromatin extract, the *30 nm fiber* (*solenoid, or* nucleosome coil). Other extraction protocols revealed other aspects of chromatin structure shown in #s **3** and **4**. Chromosomes seen in metaphase of mitosis are the 'highest *order*', most condensed form of chromatin.

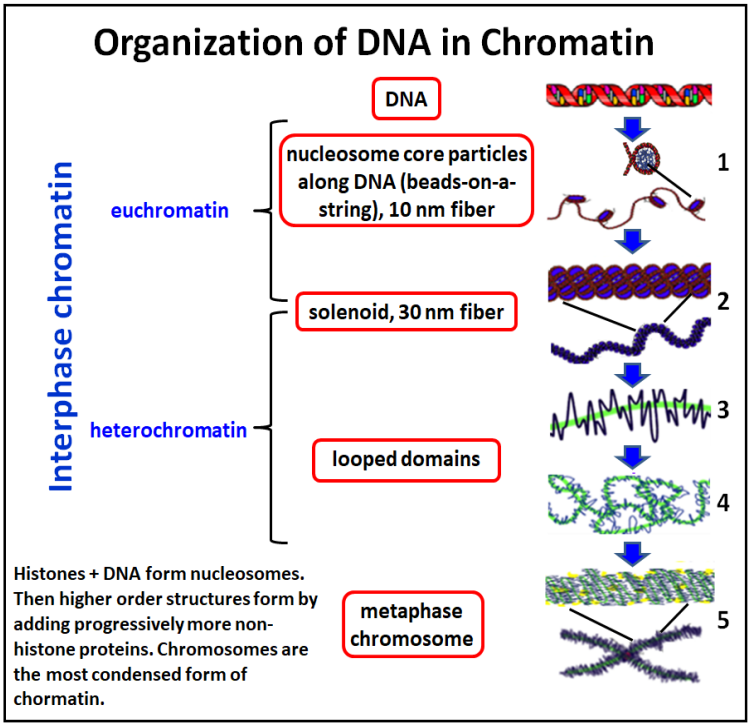


Fig. 8.15: Five different levels (orders) of chromatin structure (see text for deatiils).

Results of a DNA nuclease digestion of the beads-on-a-string are shown below in Fig. 8.16. A brief digestion of nucleosome 'necklaces' *deoxy-ribonuclease* I (**DNAse I**) degrades DNA between the 'beads' leaving behind shortened 10nm filaments of different lengths. After a longer digestion, single beads bound to small amounts of DNA remain.

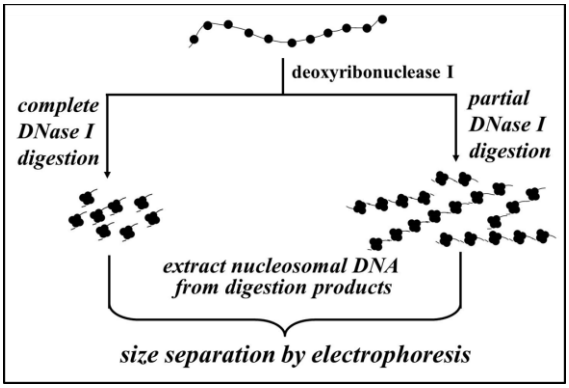


Fig. 8.16: Short times 10 nm filament digestion with DNase I leaves behind shortened *beads on a string*. A long time of digestion releases single nucleosomes associated with some DNA.

Roger Kornberg, one son of Nobel Laureate Arthur Kornberg (discoverer of the first DNA polymerase enzyme of replication-see the next chapter), participated in the discovery and characterization of nucleosomes while still a post-doc! He found that each nucleosome is associated with about 200 base pairs of DNA. Electrophoresis of DNA extracted from digests of nucleosome beads-on-a-string preparations revealed that *nucleosomes* are separated by a "linker" DNA stretch of about 80 base pairs. DNA extracted from the nucleosomes was about 147 base pairs long, a closer estimate of the DNA wrapped around the proteins of the nucleosome. It is appropriate to note here that Roger Kornberg earned the 2006 Nobel Prize for Chemistry for his work on eukaryotic transcription and the structure of RNA polymerase (among other things), topics we cover in a later chapter!

Five ***histone*** proteins could be isolated from nucleosomes and were separated by electrophoresis; the results are illustrated in Fig. 8.17 (below).

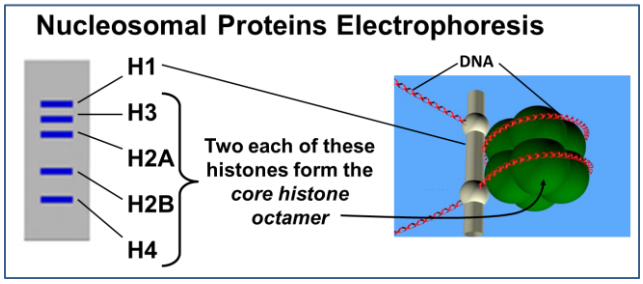


Fig. 8.17: Electrophoretic separation of five histones extracted from 10 nm filaments.

Histones are basic proteins containing many *lysine* and *arginine* amino acids. Their positively charged side chains enable these amino acids bind to the acidic, negatively charged *phosphodiester backbone* of double helical DNA. The DNA wraps around an octamer of histones (2 each of 4 of the histone proteins) to form the *nucleosome*. About a gram of histones is associated with each gram of DNA.

172-2 Nucleosomes-DNA and Protein

173-2 Chromatin Structure-Dissecting Chromatin

As you might guess, an acidic extraction of chromatin should selectively remove the basic histone proteins, leaving behind an association of DNA with non-histone proteins. This proves to be the case. An electron micrograph of the chromatin remnant after an acid extraction of metaphase chromosomes is shown below in Fig. 8.18. DNA freed of the regularly spaced histone-based nucleosomes, loops out, away from the long axis of the chromatin. Dark material along this axis is a protein scaffolding that makes up what's left after histone extraction. Much of this protein is *topoisomerase*, an enzyme that prevents DNA from breaking apart under the strain of replication (to be detailed in a later chapter).

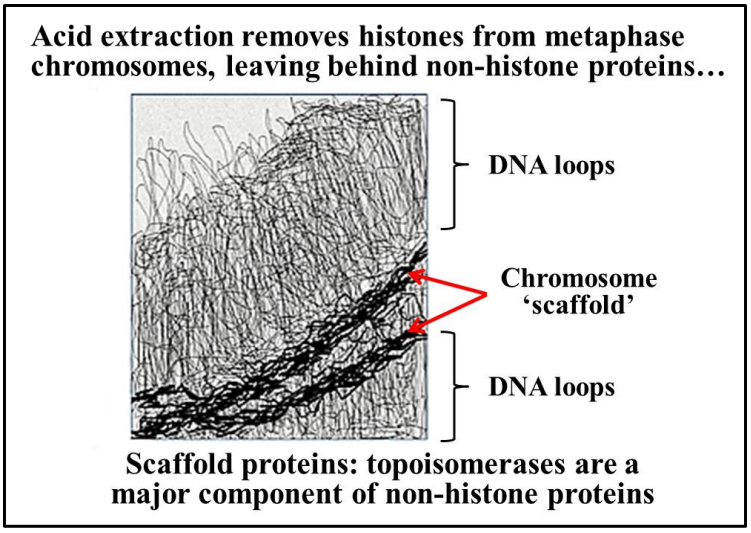


Fig. 8.18: Acid extraction of chromatin removes histones, leaving non-histone proteins behind. (see original micrograph at https://www.flickr.com/photos/185778834@N06/49368658231/

8.6 Structure, Organization of Bacterial DNA… and Bacterial Sex

Sexual reproduction allows compatible genders (think *male* and *female*) to share genes, a strategy that increases species diversity. It turns out that bacteria and other single celled organisms can also share genes… and spread diversity. We will close this chapter with a look at sex (*E. coli* style!), and gene-mapping experiments showing linearly arranged genes on a circular bacterial DNA molecule (the bacterial 'chromosome').

E. coli sex begins when F^+ and F^- cells meet. These "opposite" mating type cells can share DNA during ***conjugation***. F^+ cells contain the ***fertility***, or ***F plasmid***, a small circular DNA molecule that is separate from the *E. coli* chromosome. The *F plasmid* has genes that encode ***sex pili*** on F^+ cells as well as factors needed to form a mating bridge, or ***conjugation tube***. Fig. 8.19 illustrates the behavior of the F plasmid during conjugation.

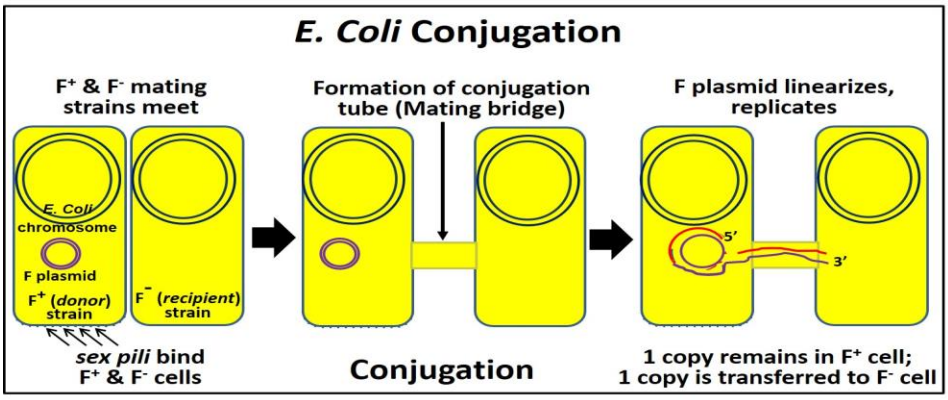


Fig. 8.19: Bacterial *conjugation* (sex in bacteria): The *F plasmid (*Fertility plasmid) can transfer bacterial chromosomal DNA from the F+ mating strain of *E. coli* to an opposite, F^- mating strain, leading to a measure of genetic diversity.

When an F^+ (*donor*) cell encounters an F^- (*recipient*) cell, sex pili on the donor cell initiate recognition. Next, the conjugation tube forms, linking the cytoplasms of the two cells. After nicking one strand of the F plasmid DNA, the nicked strand begins to roll across the conjugation tube into the recipient F^- cell. The DNA strand entering the recipient cell replicates, as does the intact circle remaining in the donor cell (replicating DNA is shown in red in the illustration). *E. coli* conjugation can have different outcomes:

1. One outcome is that one of two semi-conservatively replicated F plasmids remains in the donor cell and another is now in the recipient cell. In this case, the recipient cell becomes a new F^+ *donor* cell!

2. The other outcome is integration of the F plasmid into recipient cell chromosomal DNA. Insertion is typically at specific sites in the DNA where there is sufficient sequence similarity between the plasmid and chromosomal DNA to allow insertion by *recombination*. The result is that the recipient cell becomes an ***Hfr*** (*High-frequency recombination*) cell. This cell will produce ***Hfr strain*** progeny cells.

Note that in both cases, the outcome is to change the mating type of the recipient F^- cell into a strain that can initiate mating with other F^- cells. These two possible results of conjugation in *E. coli* are shown in Fig. 8.20 (below).

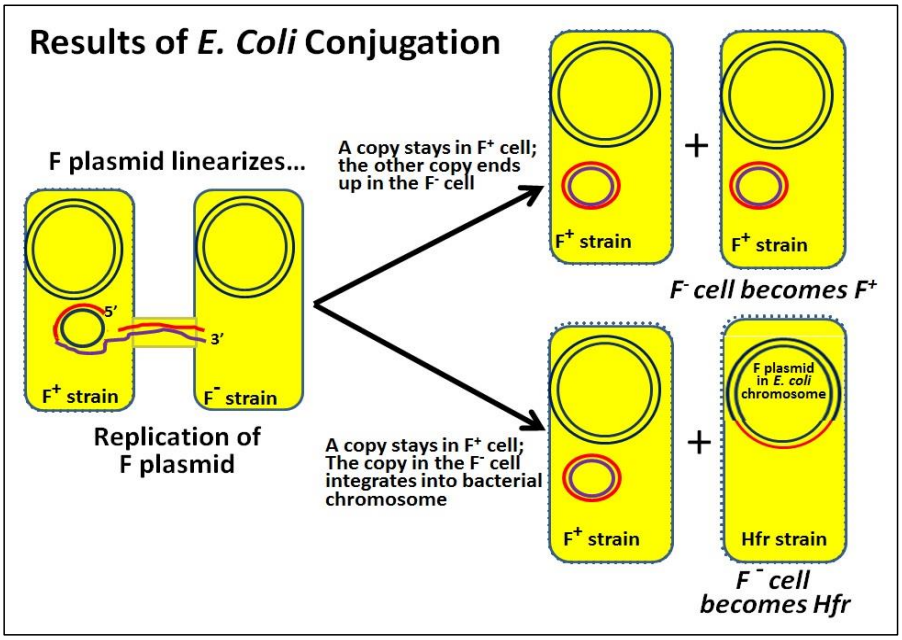


Fig. 8.20: Possible results of conjugation *in E. coli*. The F plasmid transferred from an F^+ donor cell may or may not integrate into the F^- (recipient) cell. If integration occurs, the recipient cell becomes *Hfr*, with a high frequency of conjugation.

Hfr cells readily express their integrated F plasmid genes, and like F+ cells, develop sex pili and form a conjugation tube with an F^- cell. One strand of the bacterial chromosomal DNA will be nicked at the original insertion site of the F plasmid. The next events parallel the replicative transfer of an F plasmid during F^+/F^- conjugation, except that only part of the Hfr donor chromosomal DNA is transferred (Fig. 8.21, below). In this illustration, the F

plasmid has inserted *in front of* an A gene so that when it enters the conjugation tube, it brings along several *E. coli* chromosomal genes Because of the size of the bacterial chromosome, only a few bacterial genes enter the recipient gene before transfer is aborted. But in the brief time of DNA transfer, at least some genes did get in to the recipient F- strain where they can be expressed.

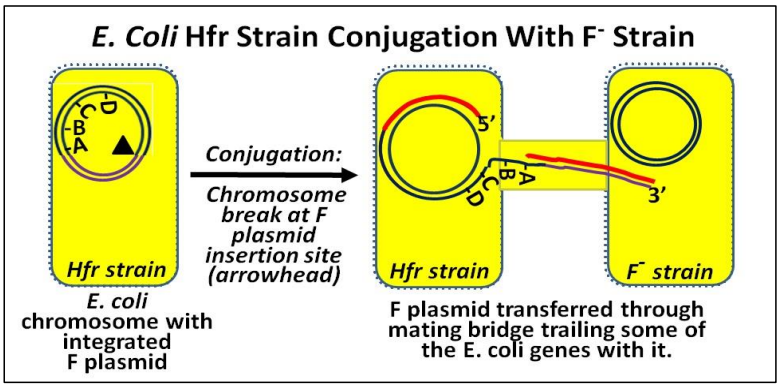


Fig. 8.21: During conjugation with *Hfr donor* strain cells, portions of donor chromosomal DNA follow the excised plasmid across the conjugation bridge into the F- recipient cell.

Here is the outline of an experiment that allowed mapping bacterial genes on a circular DNA chromosome:

1. Hfr cells containing functional **A, B, C or D** genes were mated with recipient cells containing mutants of either the A, the B, the C or the D gene.
2. Conjugation was mechanically disrupted at different times after the formation of a conjugation tube.
3. Recipient cells from each of the disrupted conjugations were then grown in culture and analyzed for specific gene function.

In this hypothetical example, the results were that a recipient cell with a *mutant A* gene acquired a wild type A gene (and therefore A-gene function) after a short time before conjugation disruption. Progressively longer times of conjugation (measured in separate experiments) were required to transfer gens B, C and D (respectively) to the recipient cell. Thus, the order of these genes on the bacterial chromosome was **-A-B-C-D-**

The timing of conjugation that led to F- mutants acquiring a functional gene from the Hfr strain was so refined that not only could the gene locus be determined, but even the size (length) of the genes! Thus, the time to transfer a complete gene to an F- cell reflects the size (length) of the gene. The other important conclusion is that genes are arranged linearly on bacterial DNA. Recall that genes already mapped along the length of

eukaryotic chromosomes implied a linear order of the genes. However, little was known about eukaryotic chromosome structure at the time, and the role of DNA as the 'stuff of genes' was not appreciated. These bacterial mating experiments demonstrated for the first time that genes are linearly arranged not just along a chromosome, but also along the DNA molecule.

Over time, many bacterial genes were mapped all along the *E. coli* chromosome by isolating many different Hfr strains in which an F plasmid had inserted into different sites around the DNA circle. These Hfr strains were mated to F- bacteria, each with mutations in one or another known bacterial gene. As in the original 'ABCD' experiment, the order of many genes was determined and shown to be linked at a greater or lesser distance to those ABCD genes and each other. Using the different Hfr strains (numbered in the diagram) in conjugation experiments, it was shown that in fact, the different Hfr cells transferred different genes into the recipient cells in the order implied by the chromosome map illustrated Fig. 8.22 below.

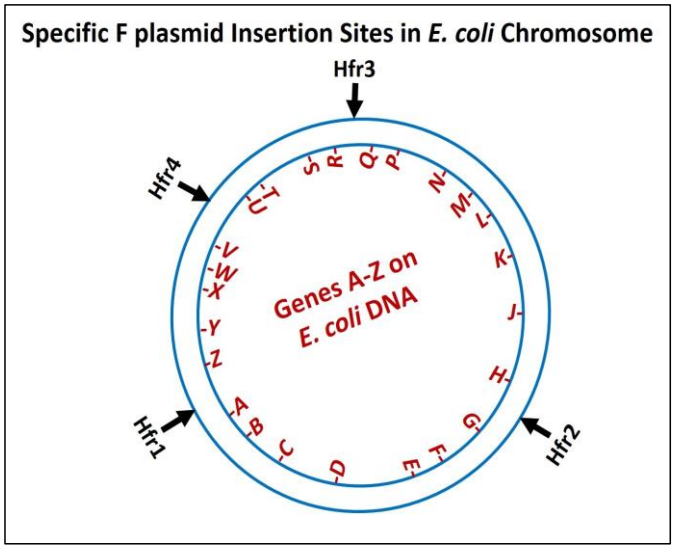


Fig. 8.22: Map of the *E. coli* chromosome based on conjugation of F^- recipient cells with different strains of Hfr donor cells indicated as Hfr 1, 2 , etc. The different Hfr donors are cells in which the Hfr plasmid integrated at different place on a recipient cell chromosome. When these different Hfr strains transfer DNA to new F^- cells, they bring along different regions of the donor cell chromosome (and thus different genes) into their conjugated F^- cells.

What's more, when the experiment was done with *Hfr4* (in this generic diagram), the order of genes transferred after longer times of conjugation was found to be **-V-W-X-Y-Z-A-B….** The obvious conclusion from experiments like these was that the *E. coli* DNA

molecule (its 'chromosome') is a closed circle! We will see visual evidence of circular *E. coli* chromosomes in the next chapter, with some discussion of how this evidence informed our understanding of DNA replication.

8.7 Phage Can Integrate Their DNA Into the Bacterial Chromosome

We have seen how phage can infect bacterial cells and coopt the host bacterial metabolic machinery for their own reproduction, eventually lysing the bacterium and releasing ne phage. This *lytic* activity of infection is one of two alternate phage life cycles.

Lysogeny is another, alternate life-cycle pathway for bacteriophage. In lysogeny, instead of immediately reproducing, the phage integrate their DNA into the bacterial chromosome. The lysogenic phage life cycle infection is illustrated below in Fig. 8.23.

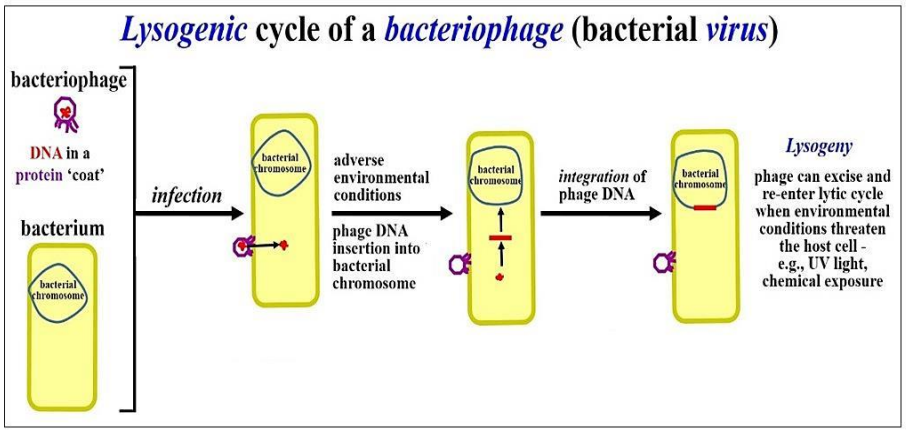


Fig. 8.23: The lysogenic life cycle of phage (see text for details).

Lysogeny typically begins when the phage DNA in a host bacterium is incorporated (i.e., is spliced into) into the bacterial genome. There it will replicate along with the hoist chromosome during rounds of *binary fission*. Upon excision, usually when the bacteria are under stress (e.g., nutrient deprivation), the phage DNA can re-enter the lytic pathway, reproducing and lysing the host cell.

During excision, the phage DNA can pick up bacterial DNA (i.e., genes) that will then be transferred to a new host cell in a subsequent infection. The transfer of genes from one bacterial cell to another in this way is called ***bacterial transduction***. Esther and Joshua Lederberg studied the lysogenic activity of lambda phage (**λ phage**) in *E. coli*, showing that like conjugation, bacterial transduction by λ phage resulted in ***lateral gene transfer***

between bacteria. Lateral gene transfer in general increases genetic diversity in bacteria…, in fact in many prokaryotic and eukaryotic organisms. We will see more examples of lateral gene transfer in later chapters.

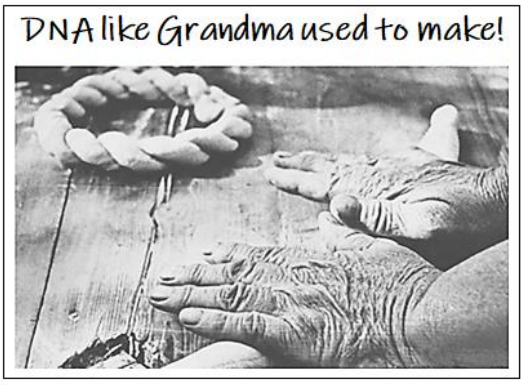
Recall that Joshua Lederberg shared a 1958 Nobel Prize with G. W. Beadle and E. Tatum Prize for proposing the one gene-one enzyme hypothesis (later shown to be the one gene-one polypeptide relationship). Esther Lederberg had studied earlier with Beadle, Tatum and her husband at Stanford University in1944 and many credit her with helping her mentors win their 1958 Nobel prize. In her own right, held adjunct or directorial positions at the University of Wisconsin-Madison and Stanford University, where her other seminal research included discovery of lambda phage (1950), invention of the ***replica plating*** technique (1951), demonstration of lambda phage transduction in *E. coli* and the discovery of the ***F (fertility) factor*** in *E. coli.* To explore why she was never a faculty member or Nobel Laureate, click https://www.whatisbiotechnology.org/Lederberg_Esther

Some iText & VOP Key Words and Terms

10 nm fiber	**double helix**	**mutations**
30 nm fiber	**euchromatin**	**non-histone proteins**
5' -to-3' replication	**F and Hfr plasmids**	**nuclear proteins**
antiparallel DNA strands	**F- strain**	**nucleosomes**
bacterial conjugation	**F+ strain**	**recipient cell**
base ratios	**fertility plasmid**	**replication**
beads-on-a-string	**heterochromatin**	***S. pneumonia* type III-S**
chromatin	**Hfr strain**	***S. pneumonia* type II-R**
chromosomes	**histone octamer**	**semi-conservative replication**
conjugation tube	**histone proteins**	**sex pili**
conservative replication	**influenza**	**solenoid fiber**
deoxyribonuclease	**karyotype**	**spindle fibers**
discontinuous replication	**levels of chromatin packing**	**transforming principle**
dispersive replication	**mating bridge**	**X & Y chromosomes**
DNA	**metaphase chromatin**	**X-ray crystallography**
donor cell	**mitosis & meiosis**	**X-ray diffraction**

Chapter 9: Details of DNA Replication and Repair

Replicons, Replication Origins and Forks; Bidirectional Replication; The Many Enzymes of Replication; Okazaki Fragments; Mechanisms, Actions and Enzymes of DNA Repair; When DNA Repair Fails



9.1 Introduction

Replication begins at one or more ***origins of replication*** along DNA, where ***helicase enzymes*** catalyze unwinding of the double helix. DNA unwinding creates replicating bubbles, or ***replicons***, with ***replication forks*** at either end. Making a new DNA strand starts with making an RNA ***primer*** with RNA nucleotides and ***primase*** enzymes. DNA nucleotides are then added to the 3'-ends of primers by a ***DNA polymerase***. Later, other DNA polymerases catalyze removal of the RNA primers and replacement of the hydrolyzed ribonucleotides with new deoxyribonucleotides. Finally, ***DNA ligases*** stitch together the fragments of new DNA synthesized at the replication forks. This complex mechanism is common to the replication of 'naked' prokaryotic DNA and of chromatin-encased eukaryotic DNA, and must therefore have arisen early in the evolution of replication biochemistry. In this chapter, we look at the details of replication as well as differences in detail between prokaryotic and eukaryotic replication that arise because of differences in DNA packing. As with any complex process with many moving parts, replication is error-prone. Therefore, we will also look at how the overall ***fidelity*** of replication relies mechanisms of ***DNA repair*** that target specific kinds of replication mistakes, i.e., ***mutations***. At the same time, lest we think that uncorrected errors in replication are always a bad thing, they usually *do not* have bad outcomes. We will see instead that they leave behind the mutations necessary for *natural selecti*on and the *evolution of diversity*.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain how Cairns interpreted his theta (θ) images.
2. compare and contrast the activities of enzymes required for replication.
3. describe the order of events at an origin of replication and at each *replication fork*.
4. compare *unidirectional* and *bidirectional* DNA synthesis from an origin of replication.
5. outline the basic synthesis and proofreading functions of a *DNA polymerase*.
6. identify the major players and their roles in the initiation of replication.
7. explain how Okazaki's experimental results were not entirely consistent with how both strands of DNA replicate.
8. list the major molecular players (enzymes, etc.) that elongate a growing DNA strand.
9. list the non-enzymatic players in replication and describe their functions.
10. describe how the structure of *telomerase* enables proper replication.
11. compare the activities of topoisomerases 1 and 2.
12. explain the reasoning behind the hypothesis of *processive replication*.
13. compare and contrast the impacts of germline and somatic mutations.
14. describe common forms of DNA damage.
15. list enzymes of replication that were adapted to tasks of DNA repair.
16. explain why a DNA glycosylase is useful in DNA repair.
17. explain the connection between 'breast cancer genes' and DNA repair.

9.2 DNA Replication

As we've seen, DNA strands have directionality, with a 5' nucleotide-phosphate end and a 3' deoxyribose hydroxyl end. This is even true for circular bacterial chromosomes…, if the circle is broken! Because the strands of the double helix are *antiparallel*, the 5' end of one strand aligns with the 3'end of the other at both ends of the double helix. In presenting the complementary pairing of bases in the double helix, Watson and Crick immediately realized that the base sequence of one strand of DNA can be used as a template to make a new complementary strand. As we'll see, this structure of DNA puzzlement surrounding how replication proceeds begins with experiments that visualize replicating DNA.

9.2.1 Visualizing Replication and Replication Forks

Recall the phenomenon of bacterial conjugation that allowed the demonstration that bacterial chromosomes were circular. In 1963, John Cairns confirmed this fact by direct visualization of bacterial DNA. He cultured *E. coli* cells for long periods on ^{3}H-thymidine (^{3}H-T) to make all of their cellular DNA radioactive. He then disrupted the cells gently to minimize damage to the DNA. The DNA released was allowed to settle and adhere to membranes. A sensitive film was placed over the membrane and time

was allowed for the radiation to expose the film. After Cairns developed the films (***autoradiographs***), he examined them in the electron microscope. He saw tracks of silver grains in the autoradiographs (the same kind of silver grains that create an image on film in old-fashioned photography). Look at the two drawings of Cairn's autoradiographs in Fig. 9.1 below. Cairns measured the length of the "silver" tracks, which usually consisted of three possible closed loops, or circles. The circumferences of two of these circles were always equal, their length closely predicted by the DNA content of a single, non-dividing cell. Cairns therefore interpreted these images to be bacterial DNA in the process of replication.

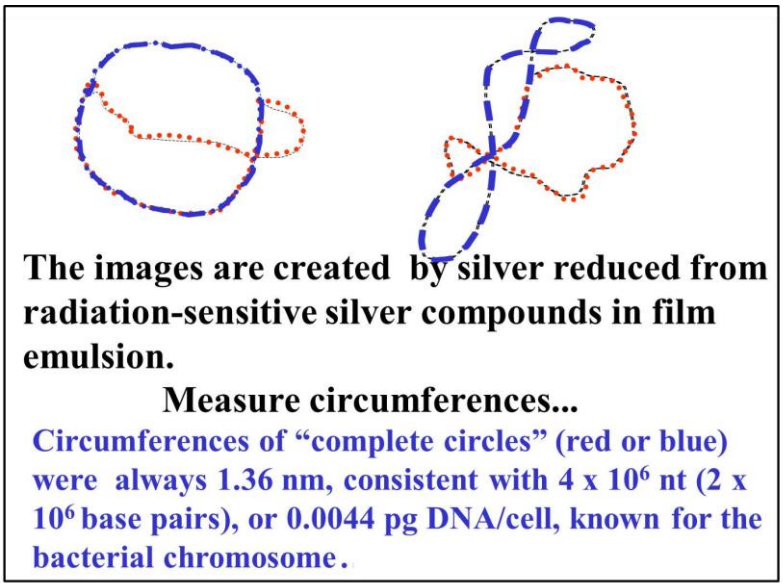


Fig. 9.1: Illustration of J. Cairns' autoradiographs of DNA extracted from *E. coli* cells allowed to grow on ^{3}H- thymidine for more than one generation of cells so that all cellular DNA would become radioactive. Silver tracks represented in the figure are exposures of bacterial chromosomal DNA.

Cairns called these replicating chromosomes ***theta images*** because they resembled the Greek letter theta (**θ**). From his many autoradiographs, he arranged a sequence of his θ images (shown in Fig. 9.2, below) to illustrate his inference that replication starts at a single ***origin of replication*** on the bacterial chromosome, proceeding around the circle to completion.

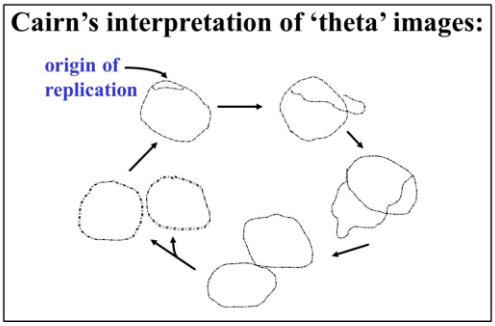


Fig. 9.2: An ordering of Cairns' autoradiograph images to suggest the progress of replication of the *E. coli* circular chromosome.

175 Seeing *E. coli* Chromosomes

9.2.2 Visualizing Bidirectional Replication

David Prescott demonstrated ***bidirectional replication***. Replication indeed begins at an origin of replication, but that double helix then unwinds in *opposite directions,* replicating DNA *both* ways away from the origin from two ***replication forks*** (Fig. 9.3).

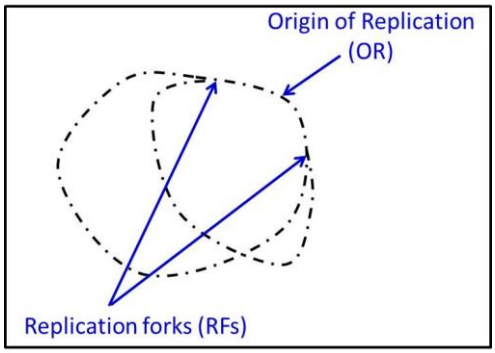


Fig. 9.3: Image of an *E. coli* circular chromosome with two *replication forks* (RFs) unwinding and replicating DNA in both directions from an *origin of replication* (OR); the *E. coli* OR is a defined sequence.

176 Semiconservative Bidirectional Replication From Two RFs

Bacterial cells can divide every hour (or even less); the rate of bacterial DNA synthesis is about 2 X 10^6 base pairs per hour. This is in fact about the size of the *E. coli* genome! A typical eukaryotic cell nucleus contains thousands of times as much DNA as a bacterium, and typical eukaryotic cells double every 15-20 hours. Even a small chromosome can contain hundreds or thousands of times as much DNA as a bacterium. It appeared that eukaryotic cells could not afford to double their DNA at a bacterial rate of replication! Eukaryotes solved this problem *not* by evolving a faster biochemistry of replication, but by using multiple origins of replication from which DNA synthesis proceeds in both directions. This results in the creation of multiple ***replicons***. Each replicon enlarges, eventually meeting other growing replicons on either side to replicate most of each linear chromosome, as suggested in Fig. 9.4.

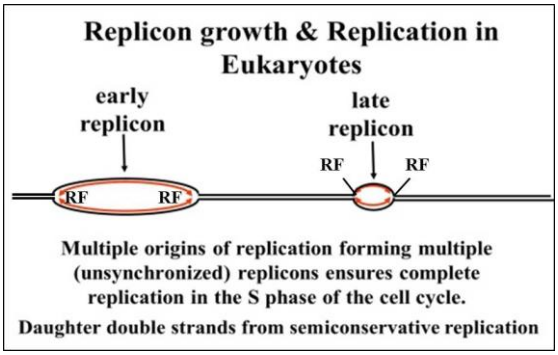


Fig. 9.4: Eukaryotic replication enlarges replicons that eventually merge.

177 Multiple Replicons in Eukaryotes

Before we consider the biochemical events at replication forks in detail, let's look at the role of DNA polymerase enzymes in the process.

9.3 DNA Polymerases Catalyze Replication

Before we consider what happens at replication forks in detail, let's look at the role of DNA polymerase enzymes in the process. The first DNA polymerase enzyme was discovered in *E. coli* by Arthur Kornberg, for which he received the 1959 Nobel Prize in Chemistry. However the rate of catalysis of new DNA, at least *in vitro*, was too slow to account for the *in vivo* rate of *E.coli* replication. It was Thomas Kornberg, one of Arthur Kornberg's sons, who later found two more faster-acting DNA polymerases (we already met the older Kornberg brother Roger!).

All DNA polymerases require a template strand against which to synthesize a new complementary strand. They all grow new DNA by adding to the 3' end of the growing DNA chain in successive condensation reactions. And finally, all DNA polymerases also have the odd property that they can only add to a pre-existing strand of nucleic acid, raising the question of where the 'pre-existing' strand comes from! DNA polymerases catalyze the formation of a phosphodiester linkage between the end of a growing strand and the incoming nucleotide complementary to the template strand. The energy for the formation of the phosphodiester linkage comes in part from the hydrolysis of two phosphates (*pyrophosphate*) from the incoming nucleotide during the reaction. While replication requires the participation of many nuclear proteins in both prokaryotes and eukaryotes, DNA polymerases perform the basic steps of replication (Fig. 9.5, below).

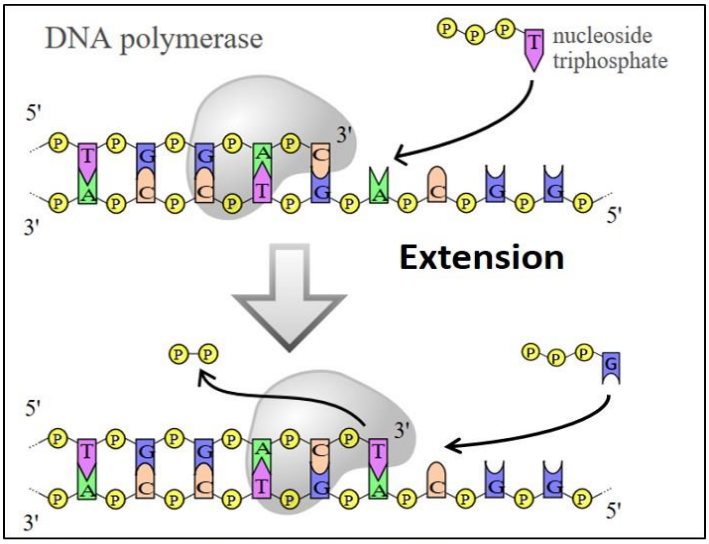


Fig. 9.5: *DNA polymerase* activity grow s a DNA strand from the 5' to 3' direction.

178 DNA Polymerases & Their Activities

Although DNA polymerases replicate DNA with high fidelity with as little as one error per 10^7 nucleotides, mistakes do occur. The proofreading ability of some DNA polymerases corrects many of these mistakes. The polymerase can sense a mismatched base pair, slow down and then catalyze repeated hydrolyses of nucleotides until it reaches the mismatched base pair. Fig. 9.6 (below) illustrates this basic proofreading by DNA polymerase.

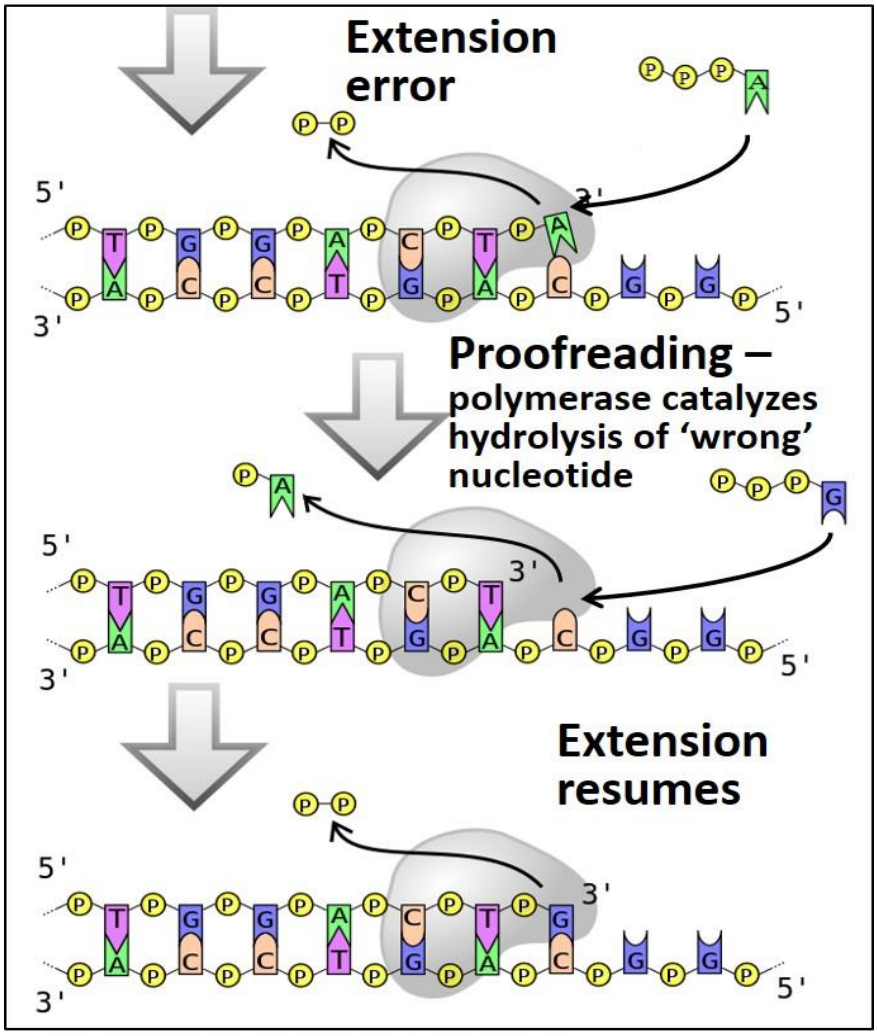


Fig. 9.6: Detection of replication errors and correction by DNA polymerase proofreading.

After mismatch repair, the DNA polymerase resumes its forward movement. Of course, not all mistakes are caught by this or other repair mechanisms (see *DNA Repair*, below). Mutations in the eukaryotic germ line cells that elude correction can cause genetic diseases. However, most of these are the mutations that fuel evolution.

Without mutations in germ line cells (egg and sperm), there would be no mutations and no evolution, and without evolution, life itself would have reached a quick dead end! Other replication mistakes can generate mutations somatic cells. If these somatic mutations escape correction, they can have serious consequences, including the generation of tumors and cancers.

9.4 The Process of Replication

As noted, DNA replication is a sequence of repeated condensation (dehydration synthesis) reactions linking nucleotide monomers into a DNA polymer. Like all biological polymerizations, replication proceeds in three enzymatically catalyzed and coordinated steps: ***initiation***, ***elongation*** and ***termination***.

9.4.1 Initiation

As we have seen, DNA synthesis starts at one or more origins or replication. These are DNA sequences targeted by *initiator proteins* in *E. coli* (Fig. 9.7).

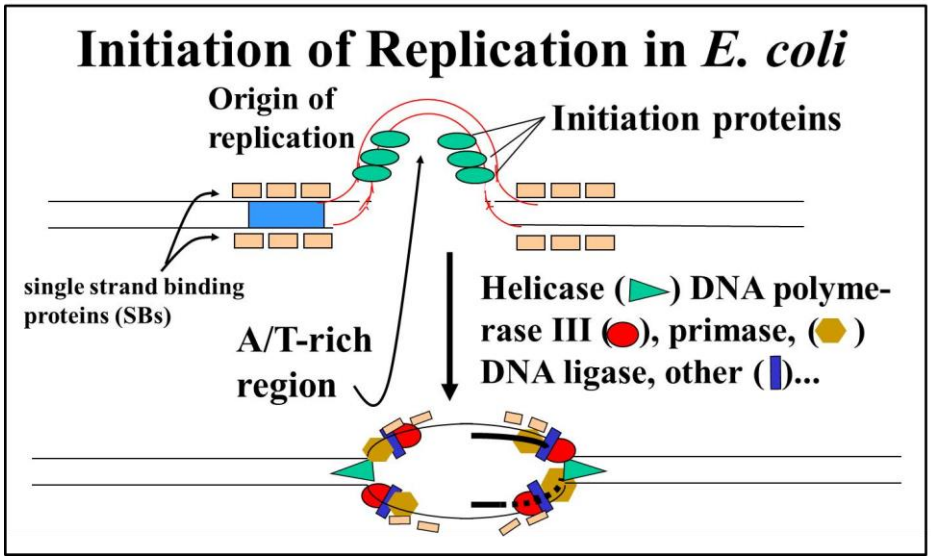


Fig. 9.7: Initiation proteins recognize and begin unwinding the double helix by bending *E. coli* DNA at the OR. Helicase then continues to unwind the DNA. SSBs (single stranded binding proteins) stabilize unwound DNA as DNA polymerase and other enzymes begin replication in both directions away from the OR.

After breaking hydrogen bonds at the origin of replication, the DNA double helix is progressively unzipped in both directions (i.e., by *bidirectional replication*). The separated DNA strands serve as templates for new DNA synthesis. Sequences at replication origins that bind to initiation proteins tend to be rich in adenine and thymine bases. This is because A-T base pairs have two hydrogen (H-) bonds that require less energy to break than the three H-bonds holding G-C pairs together. Once initiation proteins loosen H-bonds at a replication origin, ***DNA helicase*** uses the energy of ATP hydrolysis to further unwind the double helix. DNA polymerase III is the main enzyme that then elongates new DNA. Once initiated, a replication bubble (replicon) forms as repeated cycles of elongation proceed at opposite replication forks.

179 Replication Initiation in *E. coli*

Recall that new nucleotides are only added to the free 3' hydroxyl group of a pre-existing nucleic acid strand. Since no known DNA polymerase can start synthesizing new DNA strands from scratch, this is a problem!

If DNA polymerases require a ***primer***, a nucleic acid strand to which to add nucleotides, then what is the primer and where does it come from? Since *RNA polymerases*, enzymes that catalyze RNA synthesis) are the only polymerases that grow a new nucleic acid strand from scratch (i.e., from the first base) against a DNA template, it was suggested that the primer might be RNA. After synthesizing a short RNA primer, new *deoxynucleotides* would be added at its 3' end by DNA polymerase. Discovery of short stretches of RNA at the 5' end of Okazaki fragments confirmed the notion of RNA primers.

We now know that cells use ***primase***, a special RNA polymerase used during replication, to make RNA primers against DNA templates before a DNA polymerase can grow the DNA strands at replication forks. We'll see below that the requirement for RNA primers is nowhere more in evidence in events at a replication fork.

9.4.2 Elongation

Looking at elongation at one replication fork we see another problem. One of the two new DNA strands can grow continuously towards the replication fork as the double helix unwinds. But what about the other strand? Either this other strand must grow in pieces in the opposite direction or it must wait to begin synthesis until the double helix is fully unwound. The problem is illustrated in Fig. 9.8 (below).

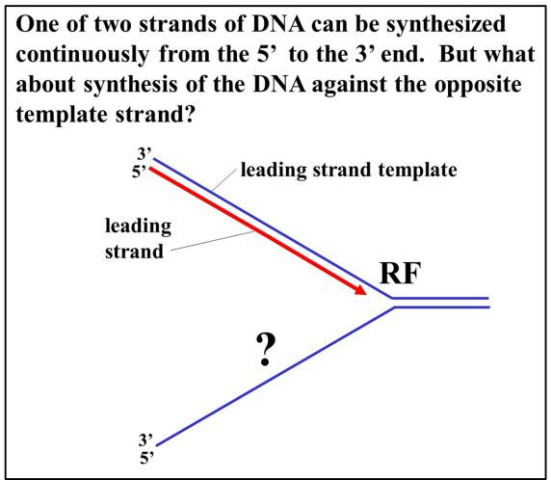


Fig. 9.8: 5'-to-3' replication creates the problem at the replication fork shown here.

If one strand of DNA must be replicated in fragments, then those fragments would have to be stitched (i.e., ligated) together, as suggested in Fig. 9.9 below.

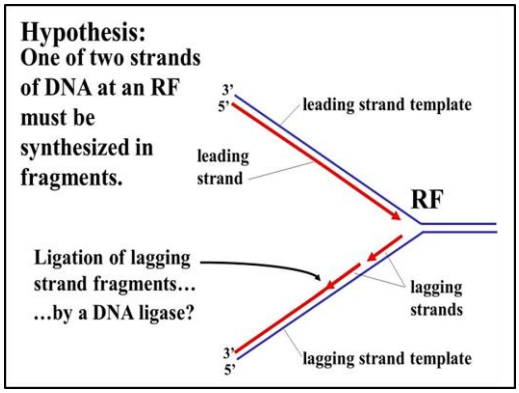


Fig. 9.9: Hypothesis proposing that at least one DNA strand at a replication fork (the *lagging strand*) is synthesized in pieces, each starting with an RNA primer, that must be later correctly stitched together.

According to this hypothesis, a new ***leading strand*** of DNA grows (is lengthened) continuously by sequential addition of nucleotides to its 3' end against its ***leading strand template***. The other strand however, would be made in pieces that would be

joined in phosphodiester linkages in a subsequent reaction. Because joining these new DNA fragments should take extra time, this new DNA is called the ***lagging strand***, making its template the ***lagging strand template***.

Reiji Okazaki and his colleagues were studying infections of slow-growing mutants of T4 phage in *E. coli* host cells. They compared the growth rates of wild-type and mutant T4 phage and demonstrated that slow growth of the mutant phage was due to a deficient ***DNA ligase*** enzyme. (Fig. 9.10 below).

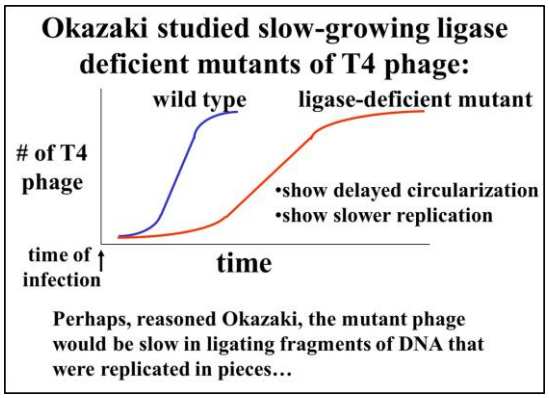


Fig. 9.10: Growth curves for wild type and a mutant T4 bacteriophage phage that synthesized a slow-acting DNA ligase enzyme. This suggested that slower growth might be due to inefficient ligation of lagging strand DNA fragments made during replication.

DNA ligase was already known to catalyze the circularization of linear phage DNA molecules being replicated in infected host cells. Okazaki's hypothesis was that the deficient DNA ligase in the mutant phage not only slowed down circularization of replicating T4 phage DNA, but would also be slow at joining phage DNA fragments replicated against at least one of the two template DNA strands. When the hypothesis was tested, the Okazakis found that short DNA fragments did indeed accumulate in *E. coli* cells infected with ligase-deficient mutants, but not in cells infected with wild type phage. The lagging strand fragments are now called ***Okazaki fragments***. You can check out Okazaki's original research at ncbi.nlm.nih.gov/pmc/Okazaki article.

180-2 Okazaki Experiments-Solving a Problem at an RF

181 Okazaki Fragments are Made Beginning with RNA Primers

Each Okazaki fragment would have to begin with a 5' RNA primer, creating yet another dilemma! The RNA primer must be replaced with deoxynucleotides before stitching the fragments together. This process in fact happens (Fig. 9.11, below). Removal of RNA primer nucleotides from Okazaki fragments requires the action of ***DNA polymerase I***. This is the slow-acting DNA polymerase first characterized by Arthur Kornberg. DNA polymerase I has the unique ability to catalyze hydrolysis of the phosphodiester bonds between the RNA (or DNA) nucleotides from the 5'-end of a nucleic acid strand.

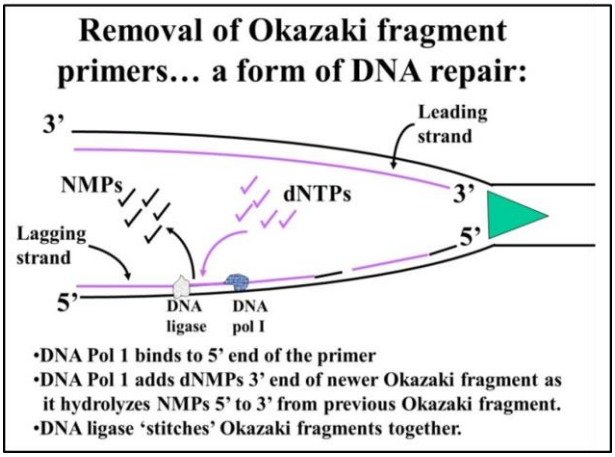


Fig. 9.11: Steps in the synthesis of DNA against the lagging template strand (see text for details).

Flap Endonuclease 1 (***FEN 1***) also plays a role in removing 'flaps' of nucleic acid from the 5' ends of the fragments often displaced by polymerase as it replaces the replication primer. At the same time as the RNA nucleotides are removed, DNA polymerase I catalyzes their replacement by the appropriate deoxynucleotides.

Finally, when a fragment is entirely DNA, ***DNA ligase*** links it to the rest of the already assembled lagging strand DNA. Because of its 5' *exonuclease* activity (not found in other DNA polymerases), DNA polymerase 1 also plays unique roles in DNA repair (discussed further below).

As Cairn's suggested and others demonstrated, replication proceeds in two directions from the origin to form a replicon with its two replication forks (RFs). Each RF has a primase associated with the replication of Okazaki fragments along lagging strand templates. Fig. 9.12below illustrates the requirement for primases at replication forks.

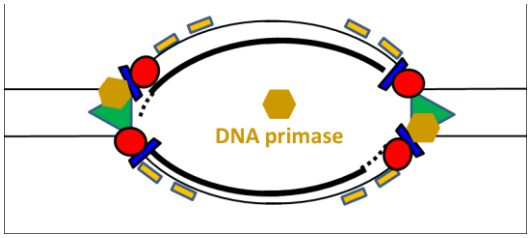


Fig. 9.12: A pair of replication forks (RFs), each assembling proteins and enzymes required for replication, including DNA primases.

Now we can ask what happens when replicons reach the ends of linear chromosomes in eukaryotes.

182 Replication Elongation in *E. coli*

9.4.3 Termination

In prokaryotes, replication is complete when two replication forks meet after replicating their portion of the circular DNA molecule. In eukaryotes, many replicons fuse to become larger replicons, eventually reaching the ends of the chromosomes. And now… there is still another problem, illustrated below in Fig. 9.13!

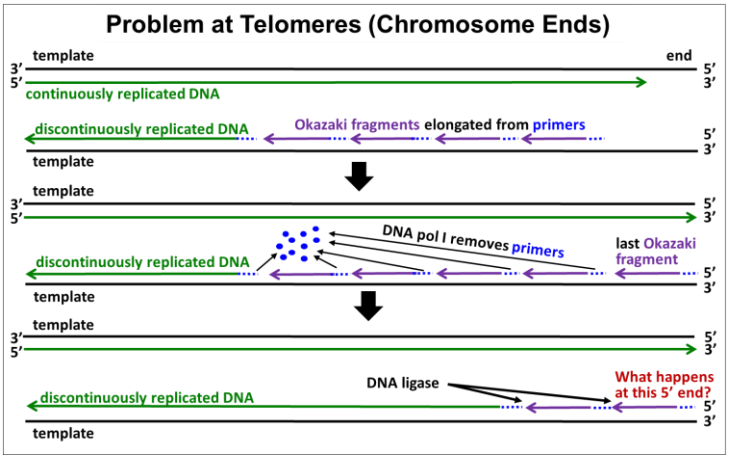


Fig. 9.13: In linear chromosomes, the lagging strand at the end (telomere) of a double helix can't be primed and thus cannot be replicated, causing chromosome shortening at each cell division.

When a replicon nears the end of a double-stranded DNA molecule, i.e., at the end of a chromosome, the strand synthesized continuously stops when it reaches the 5' end of its template DNA. In theory, synthesis of a last Okazaki fragment can be primed from the 3' end of the lagging template strand. The illustration above implies removal of a primer from the penultimate Okazaki fragment and DNA polymerase-catalyzed replacement with DNA nucleotides. But what about that last Okazaki fragment? Would its primer be hydrolyzed? Moreover, without a free 3' end to add to, how are those RNA nucleotides replaced with DNA nucleotides? The problem here is that every time a cell replicates, one strand of new DNA (likely both) would get shorter and shorter. Of course, this would not do…, and does not happen! Eukaryotic replication undergoes a *termination* process involving extending the length of one of the two strands by the enzyme ***telomerase***, as illustrated in Fig. 9.14 below.

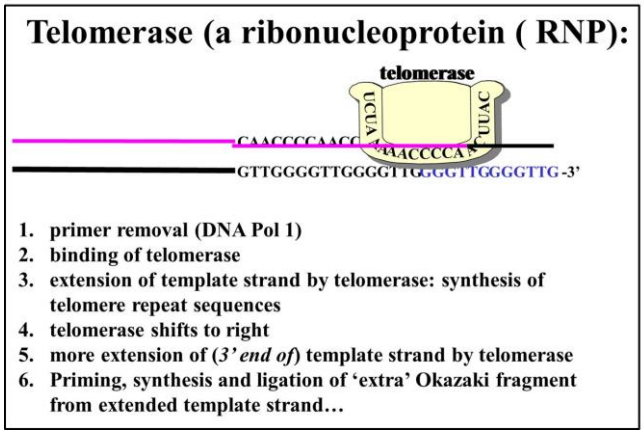


Fig. 9.14: The *ribonucleoprotein* enzyme *telomerase* resolves the dangers of chromosome shortening by using its RNA sequence to generate repeats on the telomeric end of lagging strand DNAs (see text and illustration for details).

Telomerase consists of several proteins and an RNA. From the drawing, the RNA component serves as a template for 5'-> 3' extension of the problematic DNA strand. The protein with the requisite reverse transcriptase activity is called ***Te****lomerase* ***R****everse* ***T****ranscriptase*, or ***TERT***. The ***Te****lomerase* ***R****NA* ***C****omponent* is called ***TERC***. Carol Greider, Jack Szostak and Elizabeth Blackburn shared the 2009 Nobel Prize in Physiology or Medicine for discovering telomerase.

183 Telomere Replication Prevents Chromosome Shortening

We know now that differentiated, non-dividing cells no longer produce the telomerase enzyme, while telomerase genes are active in dividing cells (e.g., stem cells) and cancer cells, which contain abundant telomerase.

CHALLENGE: Why the difference in telomerase activities here!?

9.4.4 Is Replication *Processive*?

Drawings of replicons and replication forks suggest separate events on each DNA strand. Yet events at replication forks seem to be coordinated. Thus replication may be ***processive***, meaning both new DNA strands are replicated in the same direction at the same time, smoothing out the process. How might this be possible? The drawing in Fig. 9.15 shows lagging strand template DNA bending, so that it faces in the same direction as the leading strand at the replication fork.

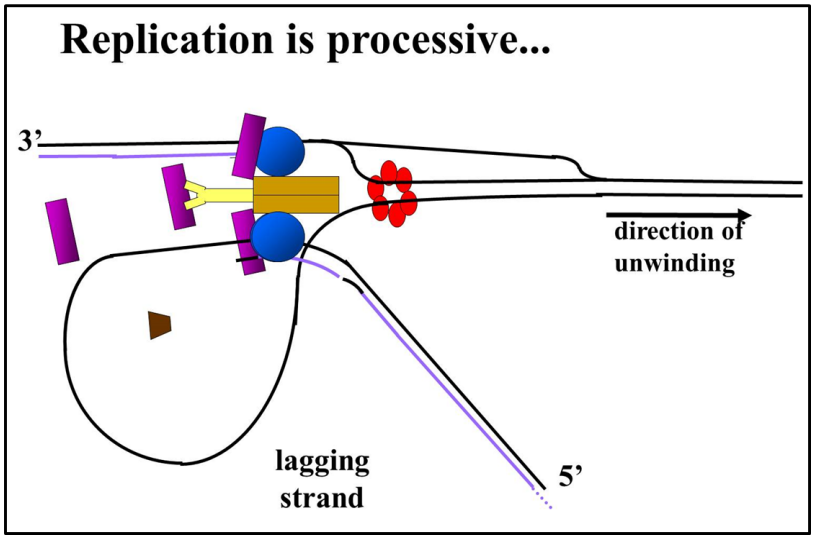


Fig. 9.15: The *Processive Replication* hypothesis unites all components needed to replicate both strands of DNA in a *replisome*, so the both strands are replicated in the same direction.

184 Processive Replication

The ***replisome*** structure cartooned at the replication fork consists of *clamp proteins*, primase, helicase, DNA polymerase and single-stranded binding proteins among others.

Newer techniques of visualizing replication by real-time fluorescence videography have called the processive model into question, suggesting that the replication process is anything but smooth! Are lagging and leading strand replication not in fact coordinated? Alternatively, is the jerky movement of DNA elongation in the video an artifact, so that the model of smooth, coordinated replication integrated at a replisome still valid? Or is coordination defined and achieved in some other way? Check out the video yourself in the article at 2017 Real-Time Fluorescent Replication Video.

9.4.5 One more problem with replication

Cairns recorded many images of *E. coli* of the sort shown in Fig. 9.16.

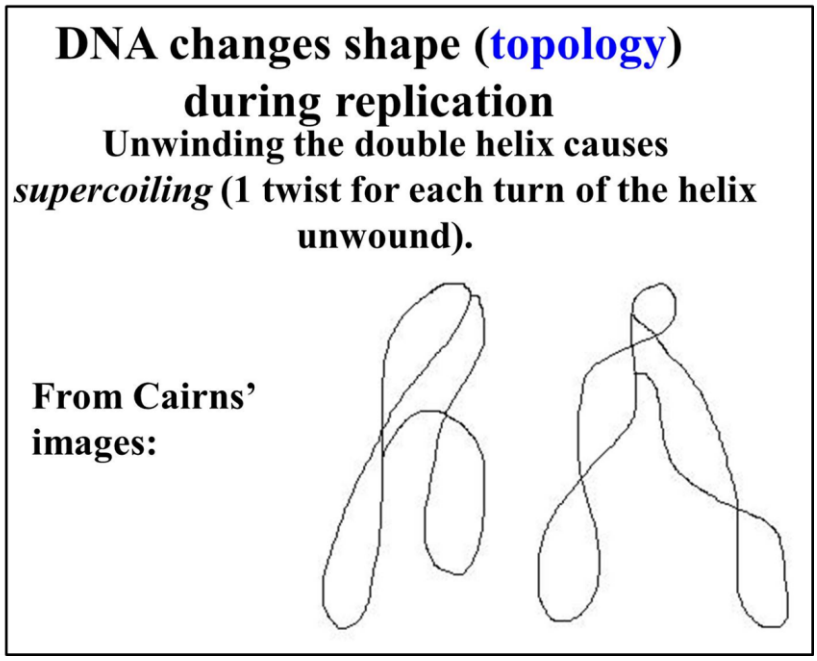


Fig. 9.16: Unwinding a circular DNA molecule (or any double helix that is rigidly associated with chromosomal proteins wherever it is not replicating) causes the DNA to twist and coil on itself, a phenomenon called *supercoiling*. Cairns' saw such supercoils in *E. coli* chromosomes.

The coiled, twisted appearance of the replicating circles were interpreted to be a natural consequence of trying to pull apart helically intertwined strands of DNA… or intertwined strands of any material! As the strands continued to unwind, the DNA should twist into a ***supercoil*** of DNA. Increased DNA unwinding would cause the phosphodiester bonds in the DNA to rupture, fragmenting the DNA. Obviously, this does not happen. Experiments were devised to demonstrate supercoiling, and to test hypotheses explaining how cells would *relax* the supercoils during replication. Testing these hypotheses revealed the ***topoisomerase*** enzymes. These enzymes bind and hold on to DNA, catalyze hydrolysis of phosphodiester bonds, control unwinding of the double helix, and finally catalyze the re-formation of the phosphodiester linkages. It is important to note that the topoisomerases are not part of a replisome, but can act far from a replication fork, probably responding to the tensions anywhere in the supercoiled DNA. Recall that topoisomerases comprise much of the protein lying along eukaryotic chromatin.

185-2 Topoisomerases Relieve Supercoiling During Replication

We have considered most of the molecular players in replication. Below is a list of the key replication proteins and their functions (Table 9.2, from DNA Replication in Wikipedia):

Enzyme	Function in DNA replication
DNA Helicase	Also known as helix destabilizing enzyme. Unwinds the DNA double helix at the Replication Fork.
DNA Polymerase	Builds a new duplex DNA strand by adding nucleotides in the 5' to 3' direction. Also performs proof-reading and error correction.
DNA clamp	A protein which prevents DNA polymerase III from dissociating from the DNA parent strand.
Single-Strand Binding (SSB) Proteins	Bind to ssDNA and prevent the DNA double helix from re-annealing after DNA helicase unwinds it thus maintaining the strand separation.
Topoisomerase	Relaxes the DNA from its super-coiled nature.
DNA Gyrase	Relieves strain of unwinding by DNA helicase; this is a specific type of topisomerase
DNA Ligase	Re-anneals the semi-conservative strands and joins Okazaki Fragments of the lagging strand.
Primase	Provides a starting point of RNA (or DNA) for DNA polymerase to begin synthesis of the new DNA strand.
Telomerase	Lengthens telomeric DNA by adding repetitive nucleotide sequences to the ends of **eukaryotic chromosomes**.

DNA Repair

We generally accept the notion that replication faithfully duplicates the genetic material, occasional making mistakes (mutating). At the same time, evolution would not be possible without mutation, and mutation is not possible without at least some adverse consequences. Because we see the results of mutation as disease, the word *mutation* in common parlance (and even among scientists) anticipates dire consequences. But mutation (changes in DNA sequence) is a f*act of life.* In fact, mutations occur frequently between generations. Most are inconsequential, and many are corrected by one or another mechanism of DNA repair.

9.5.1 Germline vs. Somatic Mutations; A Balance Between Mutation and Evolution

Germline mutations are heritable. When present in one, but especially in both alleles of a gene, such mutations can result in genetic disease (e.g., Tay-Sach's disease, cystic fibrosis, hemophilia, sickle-cell anemia, etc.). Rather than causing disease, some germline mutations may increase the *likelihood* of becoming ill (e.g., mutations of the *BRCA2* gene greatly increase a woman's odds of getting breast cancer). ***Somatic mutations*** in actively dividing cells might result in benign "cysts" or malignant tumors (i.e., cancer). Other somatic mutations may play a role in dementia (Alzheimer's disease) or in some neuropathologies e.g., along the autism spectrum.

Since the complex chemistry of replication is subject to an inherent high rate of error, cells have evolved systems of DNA repair to survive high mutation rates. As we saw, DNA polymerases themselves have proofreading ability so that incorrectly inserted bases can be quickly removed and replaced. Beyond this, multiple mechanisms have evolved to repair mismatched base pairs and other kinds of damaged DNA that escape early detection. How often and where DNA damage occurs is random, as is which damage will be repaired and which will escape to become a mutation. For those suffering the awful consequences of unrepaired mutation, the balance between retained and repaired DNA damage is to say the least, imperfect. However, evolution and the continuance of life itself rely on this balance.

9.5.2 What Causes DNA Damage?

The simplest damage to DNA during replication is the point mutation, the accidental insertion of a 'wrong' nucleotide into a growing DNA strand. Other mutations, equally accidental, include DNA deletions, duplications, inversions, etc., any of which might escape repair. The causes of DNA damage can be chemical or physical, and include spontaneous intracellular events (e.g., oxidative reactions) and environmental factors (radiation, exogenous chemicals, etc.). Based on studies of different kinds of DNA

damage, Thomas Lindahl estimated that DNA damaging events might be occurring at the rate of 10,000 per day! Lindahl realized that there must be some "fundamental DNA repair mechanisms" at work to protect cells against such a high rate of DNA damage. The discovery of the ***base excision repair*** mechanism earned Thomas Lindahl a share in the 2015 Nobel Prize in Chemistry.

Specific environmental factors that can damage DNA include UV light, X-rays and other radiation, as well as chemicals (e.g., toxins, carcinogens, and even drugs, etc.). Both germline and somatic cells can be affected. While mutations can and do cause often debilitating diseases, it is instructive to keep the impact of mutations in perspective. Most mutations are actually *silent*; they do not cause disease. And among mutations that could cause disease, much of the DNA damage is repaired. Cells correct more than 99.9% of mistaken base changes before they have a chance to become mutations. That is why we think of replication as a "faithful" process. Let's look at some common types of DNA damage that are usually repaired:

- ***Pyrimidine dimers***, typical of adjacent thymines (less often cytosines) in a single DNA strand, caused by UV exposure
- ***Depurination***; the *hydrolytic* removal of guanine or adenine from the #1 C (carbon) of deoxyribose in a DNA strand
- ***Deamination***: hydrolytic removal of amino ($-NH_2$) groups from guanine (most common), cytosine or adenine
- ***Oxidative damage*** of deoxyribose with any base, but most commonly purines
- Inappropriate ***methylation*** of any bases, but most commonly purines
- ***DNA strand breakage*** during replication or from radiation or chemical exposure

9.6 Molecular Consequences of Uncorrected DNA Damage

While bacteria suffer DNA damage, we will focus here on eukaryotes since they have evolved the most sophisticated repair mechanisms. Remember that unrepaired DNA damage ***will*** be passed on to daughter cells in mitosis, or ***might*** be passed on to the next generation if the mutation occurs in a germline cell.

9.6.1 Depurination

This is the spontaneous *hydrolytic* removal of guanine or adenine from the #1 carbon (C#1) of deoxyribose in a DNA strand. Its frequency of 5000 depurinations per cell per day emphases the high rate of DNA damage that demands a fix! If not repaired, depurination results in a single base-pair deletion in the DNA of one chromosome after replication, leaving the DNA in the same region of the other chromosome unchanged. Fig 9.17 (below) shows the effects of depurination.

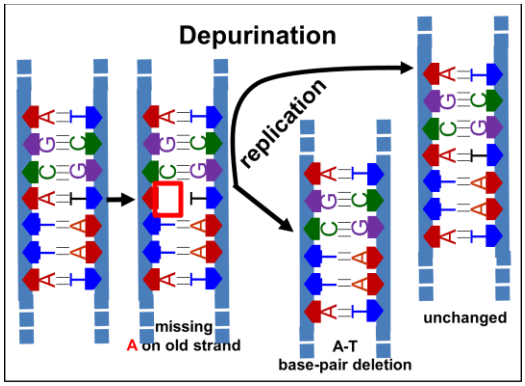


Fig. 9.17: Spontaneous depurination results in hydrolytic removal of a guanine or adenine from a nucleotide, resulting in a nucleotide deletion at that site in the DNA during replication.

The replisome ignores the depurinated nucleotide (an A in this example), jumping to the C in the template DNA. Unrepaired, one new double-stranded DNA will have a deletion, leaving the other new one with no mutation.

9.6.2 Pyrimidine Dimerization

Exposure of DNA to UV light can cause adjacent pyrimidines (commonly thymines; less often, cytosines) on a DNA strand to dimerize. Pyrimidine dimers form at a rate of a bit less than 100 per cell per day! Uncorrected dimerization results in 2-base deletion in one chromosome while the other is unchanged (Fig. 9.18, below).

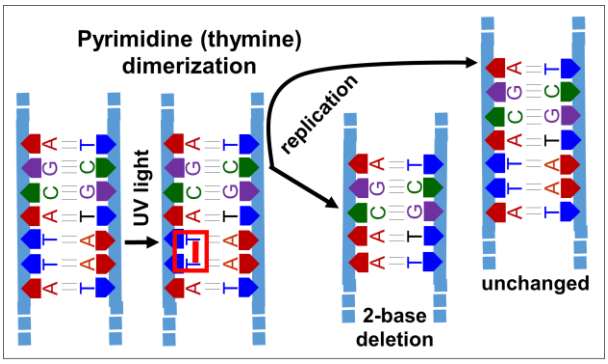


Fig. 9.18: Exposure of DNA to UV light can cause adjacent thymines to dimerize, resulting in deletion of two nucleotides at that site in the DNA during replication.

You can predict that correction of this radiation-induced damage will either involve disrupting the dimers (in this case thymine dimers), or removal and replacement of the dimerized bases by monomeric bases.

9.6.3 Deamination

Fig. 9.19 shows the consequences of deamination to a DNA base sequence.

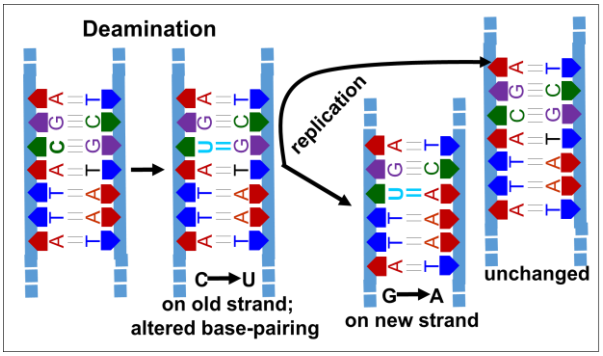


Fig. 9.19: -NH_2 removal (deamination) of a base in one DNA strand results in a base substitution during replication.

Deamination is the hydrolytic removal of amino (-NH_2) groups from guanine (most common), cytosine or adenine, at a rate of 100 per cell per day. Deamination does not affect thymine (because it has no amino groups!). Uncorrected deamination results in a *base substitution* on one chromosome (actually, a T-A pair substitution for the original C-G in this example) and no change on the other. Deamination of adenine or guanine results in unnatural bases (hypoxanthine and xanthine, respectively). These are easily recognized and corrected by DNA repair systems. The U-A base pair remains occasionally un-repaired.

9.7 DNA Repair Mechanisms

Many enzymes and proteins are involved in DNA repair. Some of these function in normal replication, mitosis and meiosis, but were co-opted for DNA repair activities. These molecular co-optations are so vital to normal cell function that some repair activities and molecular players are highly conserved in evolution. Among the DNA repair pathways that have been identified, we will look at ***Base Excision Repair***, ***Nucleotide Excision Repair***, ***Transcription Coupled Repair***, ***Non-homologous End-Joining***, and ***Homologous Recombination*** (of these, the last is perhaps the most complex).

9.7.1 Base Excision Repair

On detection of an incorrect. e.g., oxidized, open-ring, deaminated bases or bases containing saturated C=C bonds, ***DNA glycosylases*** catalyze hydrolysis of the damaged base from their deoxyribose in the DNA. For more on these enzymes click https://en.wikipedia.org/wiki/DNA_glycosylase Fig. 9.20 shows ***base excision repair***.

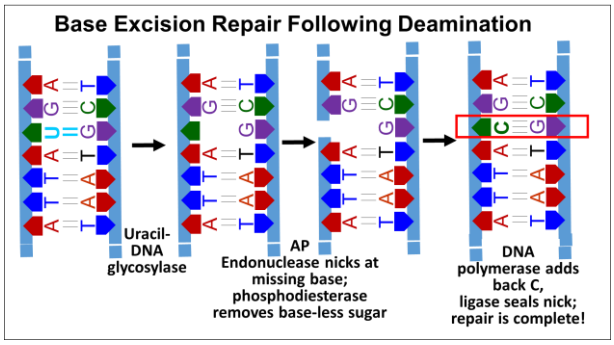


Fig. 9.20: Base Excision Repair mechanisms can detect and fix e.g., a deamination prior to replication (see text for details).

9.7.2 Nucleotide Excision Repair

The discovery of ***nucleotide excision repair*** earned Aziz Sancar a share in the 2015 Nobel Prize in Chemistry. Fig 9.21 illustrates ***nucleotide excision repair*** for a pyrimidine dimer.

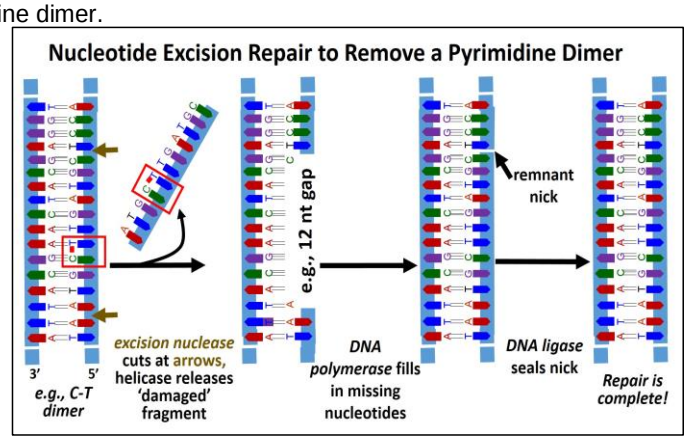


Fig. 9.21: Nucleotide Excision Repair mechanisms can detect and fix e.g., a pyrimidine dimer prior to replication (see text for details).

In this example, an ***Excision Nuclease*** recognizes a pyrimidine dimer and hydrolyzes phosphodiester bonds between nucleotides several bases away from either side of the dimer. A ***DNA helicase*** then unwinds and separates the DNA fragment containing the dimerized bases from the damaged DNA strand. Finally, ***DNA polymerase*** acts 5'-3' to fill in the gap and ***DNA ligase*** seals the remaining nick to complete the repair.

9.7.3 Mismatch repair

DNA Mismatch Repair occurs when DNA polymerase proofreading misses an incorrect base insertion into a new DNA strand. This repair mechanism relies on the fact that double-stranded DNA shows a specific pattern of methylation. The discovery of the *mismatch repair* mechanism earned Paul Modrich *a* share in the 2015 Nobel Prize in Chemistry. These methylation patterns are related to epigenetic patterns of gene activity and chromosome structure that are expected to be inherited by daughter cells. When DNA replicates, the methyl groups on the template DNA strands remain, but the newly synthesized DNA is un-methylated. In fact, it will take some time for methylation enzymes to locate and methylate the appropriate nucleotides in the new DNA. In the intervening time, several proteins and enzymes can detect inappropriate base pairing (the mismatches) and initiate mismatch repair. The basic process is illustrated in Fig. 9.22.

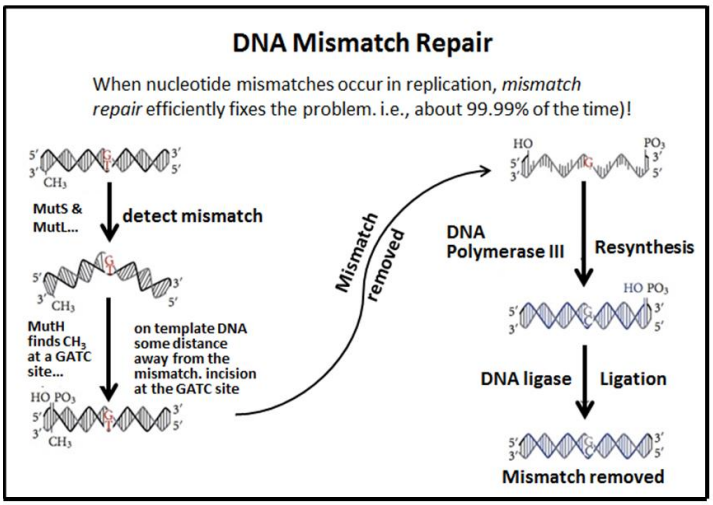


Fig. 9.22: DNA Mismatch Repair can detect and fix post-replication errors (i.e., mismatched base pairing (see text for details).

9.7.4 Transcription Coupled Repair (in Eukaryotes)

If an RNA polymerase reading a template DNA encounters a nicked template or one with an unusual base substitution, it might stall transcription and "not know what to do next". Thus at a loss, a normal transcript would not be made and the cell might not survive.

No big deal in a tissue comprised of thousands if not millions of cells, right? Still, ***Transcription Coupled Repair*** exists! In this repair pathway, if ***RNA polymerase*** encounters a DNA lesion (i.e., damaged DNA) while transcribing a template strand, it will indeed stall. This allows time for ***coupling proteins*** to reach a stalled polymerase and enable repair machinery (e.g., by base, or nucleotide excision) to effect the repair. Once the repair is complete, the ***RNA polymerase*** 'backs up' along the template strand with the help of other factors, and resumes transcription of the corrected template.

9.7.5 Non-homologous End-Joining

DNA replication errors can cause ***double stranded breaks***, as can environmental factors (ionizing radiation, oxidation, etc.). Repair by ***non-homologous end-joining*** deletes damaged and adjacent DNA and rejoins 'cut' ends (Fig. 9.23).

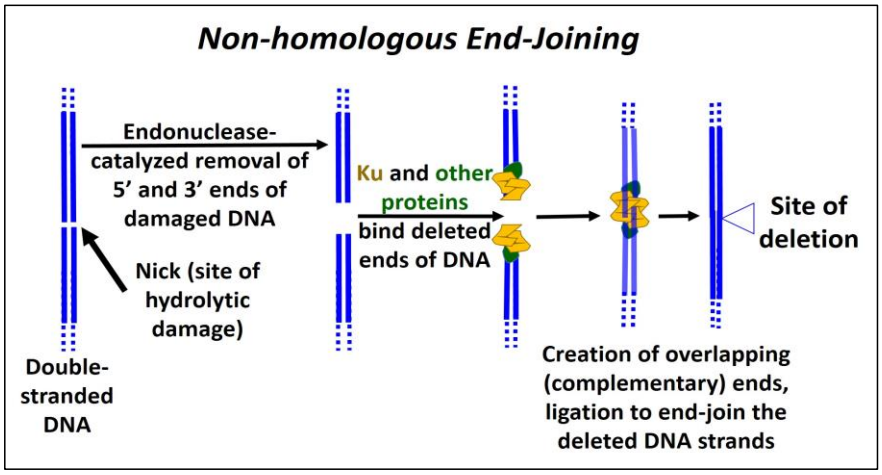


Fig. 9.23: Non-Homologous End-Joining detects and repairs double-stranded breaks in DNA, but can leave nucleotide deletions (see text for details).

Once the site of a double-stranded break is recognized, nucleotides ***hydrolyzed*** from the ends of both strands at the break-site leave '*blunt ends*'. Next, several proteins (***Ku*** among others) bring DNA strands together and further hydrolyze single DNA strands to create staggered (overlapping, or ***complementary***) ends. The overlapping ends of these DNA strands form H-bonds. Finally, ***DNA ligase*** seals the H-bonded overlapping ends of DNA strands, leaving a repair with deleted bases.

In older people, there is evidence of more than 2000 'footprints' of this kind of repair per cell. How is this possible? This *quick-fix* repair often works with no ill effects because most of the eukaryotic genome does not encode genes or even regulatory DNA (whose damage would otherwise be more serious).

9.7.6 Homologous Recombination

Homologous recombination is a complex but normal and frequent part of meiosis in eukaryotes. You may recall that ***homologous recombination*** occurs in ***synapsis*** in the first cell division of ***meiosis*** (Meiosis I). Alignment of homologous chromosomes during synapsis may lead to DNA breakage, an *exchange* of alleles, and ligation to reseal the now recombinant DNA molecules. Novel recombinations of variant ***alleles*** in the chromosomes of sperm and eggs ensure ***genetic diversity*** in species. The key point is that breakage of DNA is required to exchange alleles between ***homologous chromosomes***. Consult the genetics chapter in an introductory biology textbook, or the recombination chapter in a genetics text to be reminded of these events.

Cells use the same machinery to reseal DNA breaks during normal recombination and to repair DNA damaged by single or double stranded breakage. A single DNA strand that is nicked during replication can be repaired by recombination with strands of homologous DNA that are being replicated on the other strand. A double stranded break can be repaired using the same recombination machinery that operates on sister chromatids in meiosis. In both cases, the process accurately repairs damaged DNA ***without any deletions***. These mechanisms are conserved in the cells of all species. This further demonstrates an evolutionary imperative of accurate repair to the survival of species, no less than the imperative to maintain genetic diversity of species.

9.7.6.a Repair of a Single-Stranded Break

A specific example of homologous recombination repair is the re-establishment of a replication fork damaged when a replisome reaches a break in one of the two strands of replicating DNA (Fig. 9.24, below).

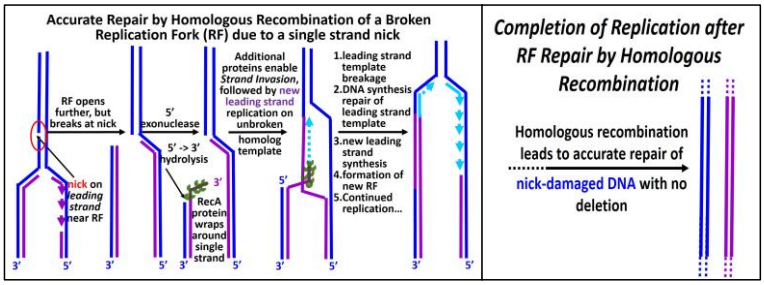


Fig. 9.24: Single-Stranded Break Repair by *Homologous Recombination* can fix a break in one DNA strand when the break is detected at a replication fork. The fix leaves an accurate repair with no deletion or base substitution (see text for details).

Such a break may have occurred prior to replication itself, and repair begins when the replication fork (RF) reaches the lesion. In the first step, a ***5'-3' exonuclease*** trims template DNA back along its newly synthesized complement. Next, ***RecA*** protein monomers (each with multiple DNA binding sites) bind to the single-stranded DNA to form a ***nucleoprotein filament***. With the help of additional proteins, the 3' end of the 'filament' scans the 'other' replicating strand for homologous sequences. When such sequences are found, the RecA-DNA filament binds to the homologous sequences and the filament of new DNA 'invades' the homologous (i.e., opposite) double stranded DNA, separating its template and newly replicated DNA. After ***strand invasion***, replication of a leading strand continues from the 3' end of the invading strand. A new RF is established as the leading strand template is broken and re-ligated to the original break; New lagging strand replication then resumes at the new (re-built) RF. The result is an accurate repair of the original damage, with no deletions or insertions of DNA.

RecA is a bacterial protein, an example of another of those evolutionarily conserved proteins. Its homolog in Archaea is called ***RadA***. In Eukaryotes, the homolog is called ***Rad51***, where it initiates ***synapsis*** during meiosis. Thus, it seems that a role for ***RecA*** and its conserved homologs in DNA repair predated its use in synapsis and crossing over in eukaryotes! For more about the functions of RecA protein and its homologs, click The functions of RecA.

9.7.6.b Repair of a Double-Stranded Break

Homologous recombination can also repair a double-stranded DNA break with the aid of a number of enzymes and other proteins. Alternate repair pathways are summarized in Fig. 9.25 (below).

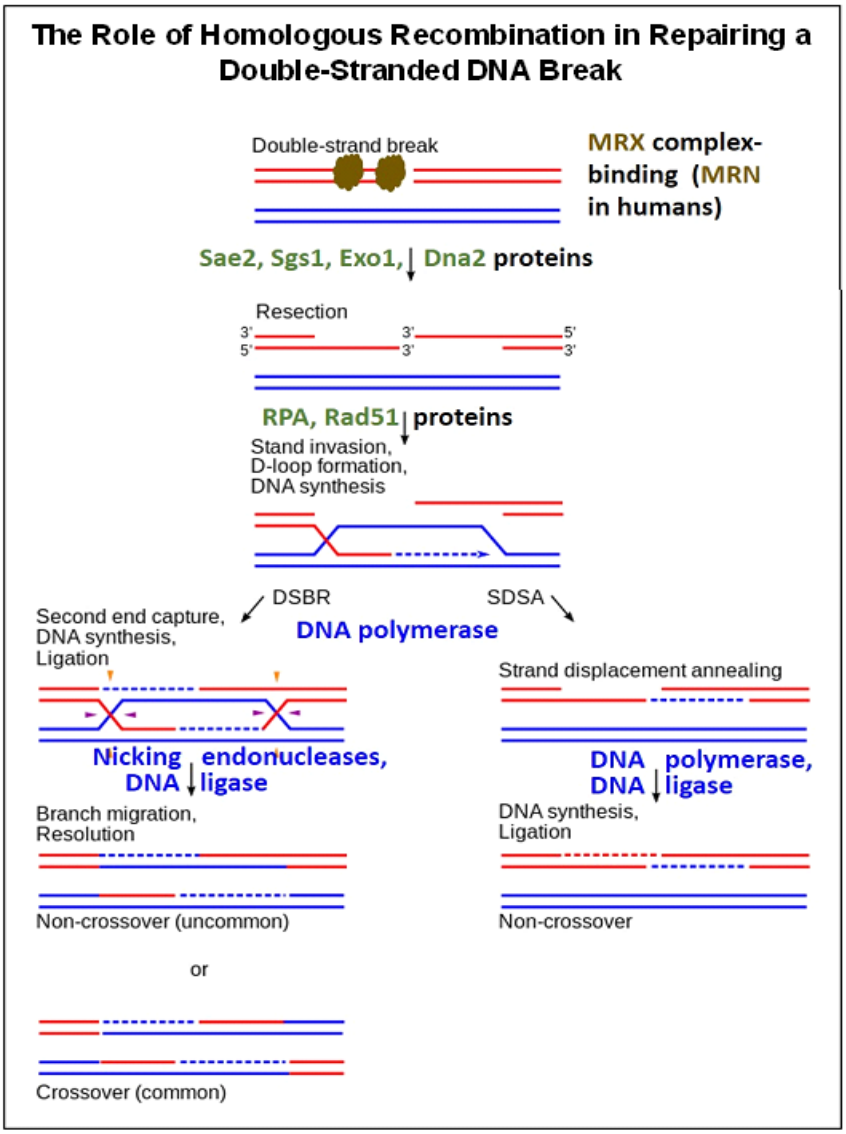


Fig. 9.25: Double-Stranded Break Repair by *Homologous Recombination* can detect and fix a break in double stranded breaks in DNA (see text for details).

Here is a list of proteins involved in these homologous recombination pathways:

- ***MRX, MRN***: bind at double-stranded break; recruit other factors.
- ***Sae2***: an endonuclease (active when phosphorylated).
- ***Sgs1***: a helicase.
- ***Exo1, Dna2***: single strand exonucleases.
- ***RPA, Rad51, DMC1***: proteins that bind to overhanging DNA to form a nucleoprotein filament and initiate *strand invasion* at similar sequences.

The activities of other enzymes in the drawing are identified. Not shown in the illustration are two gene products that interact with some of the proteins that mediate the repair pathway. These are products of the BRCA1 and BRCA2 genes (the same ones that when mutated, increase the likelihood of a woman getting breast cancer). Expressed mainly in breast tissue, their wild-type (normal) gene products participate in homologous recombination repair of double-stranded DNA breaks. They do this by binding to Rad51 (the human RecA homolog!).

When mutated, the BRCA proteins function poorly and DNA in the affected cells is not efficiently repaired. This is the likely basis of the increased chance of getting breast cancer. It doesn't help matters that the normal BRCA1 protein also plays a role in mismatch repair… and that the mutated protein can't! To end this chapter, here is a bit of *weird science*! Read all about the genome of a critter, nearly 17% of which is comprised of foreign DNA, possibly the result of Extreme DNA Repair of the Spaced-Out Tardigrade Genome

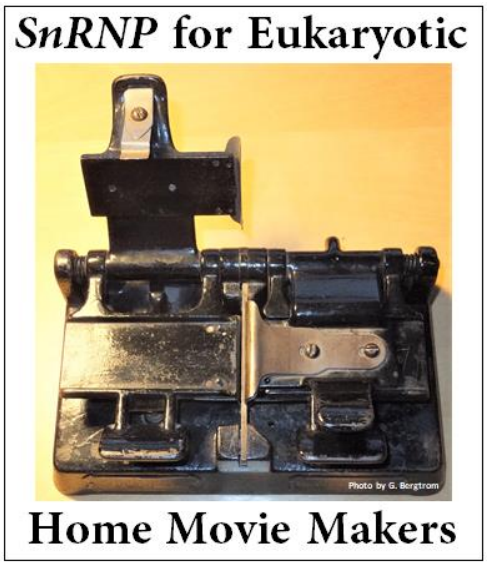
Some iText & VOP Key Words and Terms

base excision repair	**initiation**	**replicons**
bidirectional replication	**initiator proteins**	**replisome**
Central Dogma	**lagging strand**	**S phase of the cell cycle**
clamp proteins	**leading strand**	**satellite DNA**
condensation reactions	**methylation**	**single-strand binding proteins**
deamination	**mutations**	**siRNA**
density gradient centrifugation	**nucleotide excision repair**	**supercoiling**
depurination	**Okazaki fragments**	**T2 phage**
direct repeats	**origin of replication**	**tardigrade**
discontinuous replication	**phosphate backbone**	**telomerase**

DNA ligase	**phosphodiester linkage**	**telomeres**
DNA mismatch repair	**primase**	**theta images**
DNA polymerase I, II and III	**primer**	**topoisomerases**
DNA repair	**processive replication**	**transcription-coupled repair**
DNA sequence phylogeny	**proofreading**	**transposase**
DNA strand breakage	**pyrimidine dimers**	**triplets genetic code**
DNA topology	**pyrophosphate**	**VLP**
elongation	**RadA protein**	**VNTRs**
env	**RecA protein**	
helicase	**replication**	
high-speed blender	**replication fork**	

Chapter 10: Transcription and RNA Processing

RNA Transcription, RNA Polymerases, Initiation, Elongation, Termination, Processing



10.1 Introduction

Transcription, the synthesis of RNA based on a DNA template, is the central step of the *Central Dogma* proposed by Crick in 1958. The basic steps of transcription are the same as for replication: ***initiation***, ***elongation*** *and* ***termination***. The differences between transcription in prokaryotes and eukaryotes are in the details. Here are some:

- *E. coli* uses a single ***RNA polymerase*** enzyme to transcribe all kinds of RNAs while eukaryotic cells use different RNA polymerases to catalyze the syntheses of ***ribosomal RNA*** (*rRNA*), ***transfer RNA*** (*tRNA*) and ***messenger RNA*** (*mRNA*).
- In contrast to eukaryotes, some bacterial genes are part of ***operons*** whose mRNAs encode multiple polypeptides.
- Most RNA transcripts in prokaryotes emerge from transcription ready to use
- Eukaryotic transcripts synthesized as longer precursors undergo ***processing*** by ***trimming***, ***splicing*** or both!
- DNA in bacteria is virtually 'naked' in the cytoplasm while eukaryotic DNA is wrapped up in chromatin proteins in a nucleus.

- In bacterial cells the association of ribosomes with mRNA and the translation of a polypeptide can begin even before the transcript is finished. This is because these cells have no nucleus. In eukaryotic cells, RNAs must exit the nucleus before they encounter *ribosomes* in the cytoplasm.

In this chapter, you will meet bacterial ***polycistronic*** mRNAs (transcripts of ***operons*** that encode more than one polypeptide) and the ***split genes*** of eukaryotes (with their ***introns*** and ***exons***. We will look at some details of transcription of the three major classes of RNA and then at how eukaryotes process precursor transcripts into mature, functional RNAs. Along the way, we will see one example of how protein structure has evolved to interact with DNA.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. discriminate between the three *steps of transcription* in pro- and eukaryotes, and the *factors involved* in each.
2. state an hypothesis for why eukaryotes evolved complex *RNA processing* steps.
3. speculate on why any cell in its right mind would have genes containing *introns* and *exons* so that their transcripts would have to be processed by *splicing.*
4. articulate the differences between *RNA and DNA structure.*
5. explain the need for *sigma factors* in bacteria.
6. speculate on *why eukaryotes do not have operons.*
7. list structural features of proteins that bind and *recognize specific DNA sequences.*
8. explain how proteins that *do not* bind specific DNA sequences *can* still bind to specific regions of the genome.
9. formulate an hypothesis for why bacteria do not polyadenylate their mRNAs as much as eukaryotes do.
10. formulate an hypothesis for why bacteria do not cap their mRNAs.

10.2 Overview of Transcription

Transcription is the first step of the "central dogma" of information transfer from DNA to protein in which genetic information in genes is transcribed into RNA. As we will see, some RNAs are translated into polypeptides while others serve functional and even enzymatic roles in the cell. We begin with a look at the main kinds of RNA in cells.

10.2.1 The Major Types of Cellular RNA

All cells make three main kinds of RNA: ribosomal RNA (***rRNA***), transfer RNA (***tRNA***) and messenger RNA (***mRNA***). *rRNA* is a structural as well as enzymatic component of ribosomes, the protein-synthesizing machine in the cell. Quantitatively, rRNAs are

by far the most abundant RNAs in the cell and mRNAs, the least. Three **rRNAs** and about 50 ribosomal proteins make up the two subunits of a bacterial ribosome, as illustrated in Fig. 10.1 below.

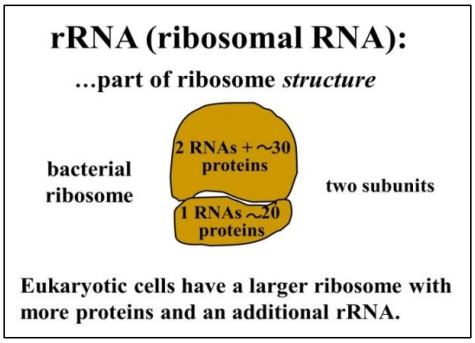


Fig. 10.1: Ribosomal RNAs and proteins in a bacterial ribosome.

tRNAs attached to amino acids bind to ribosomes based on codon-anticodon recognition (Fig. 10.2).

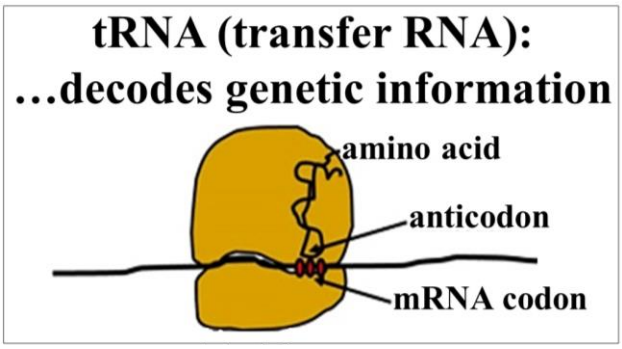


Fig. 10.2: Transfer RNA (tRNA) associated with a ribosome, held in place by codon/anticodon complementarity as well as tRNA-ribosomal forces.

186 Transcription Overview: Ribosomes and Ribosomal RNAs

187 Transcription Overview: Demonstrating the Major RNAs

In 2009, Venkatraman Ramakrishnan, Thomas A. Steitz and Ada Yonath received the Nobel Prize in Chemistry for their studies on the structure and molecular biology of the ribosome.

The fact that genes are inside the eukaryotic nucleus and that the synthesis of polypeptides encoded by those genes happens in the cytoplasm led to the proposal that there must be an mRNA. Sidney Brenner eventually confirmed the existence of mRNAs. Check out his classic experiment in Brenner S. (1961, *An unstable intermediate carrying information from genes to ribosomes for protein synthesis.* Nature 190:576-581).

Before we look at details of transcription, recall for future reference that multiple ribosomes can load an mRNA and move along the same mRNA as *polyribosomes (polysomes),* translating multiple copies of the same polypeptide (Fig. 10.3).

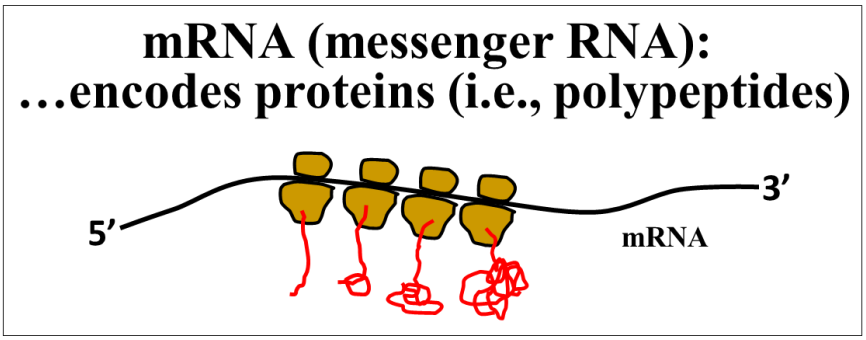


Fig. 10.3: *Polysomes* form along an mRNA. Multiple ribosomes can assemble at the 5' end of an mRNA and then sequentially translate multiple polypeptides

10.2.2 Key Steps of Transcription

In transcription, an *RNA polymerase* uses the template DNA strand of a gene to catalyze synthesis of a complementary, antiparallel RNA strand. RNA polymerases use ribose nucleotide triphosphate (NTP) precursors, in contrast to the DNA polymerases, which use *deoxyribose nucleotide* (dNTP) precursors. In addition, RNAs incorporate *uracil* (U) nucleotides into RNA strands instead of the thymine (T) nucleotides that end up in new DNA. Another contrast to replication that we have already seen is that RNA synthesis does not require a primer. With the help of *transcription* ***initiation factors***, RNA polymerase locates the ***transcription start site*** of a gene and begins synthesis of a new RNA strand from scratch. Finally, like replication, transcription is error-prone.

We can identify several of the DNA sequences that characterize a gene in the summary of the basic steps of transcription in Fig. 10.4 below. The *promoter* is the binding site for RNA polymerase. It usually lies 5' to, or *upstream* of the transcription start site (the bent arrow). Binding of the RNA polymerase positions the enzyme near the transcription start site, where it will start unwinding the double helix and begin synthesizing new RNA.

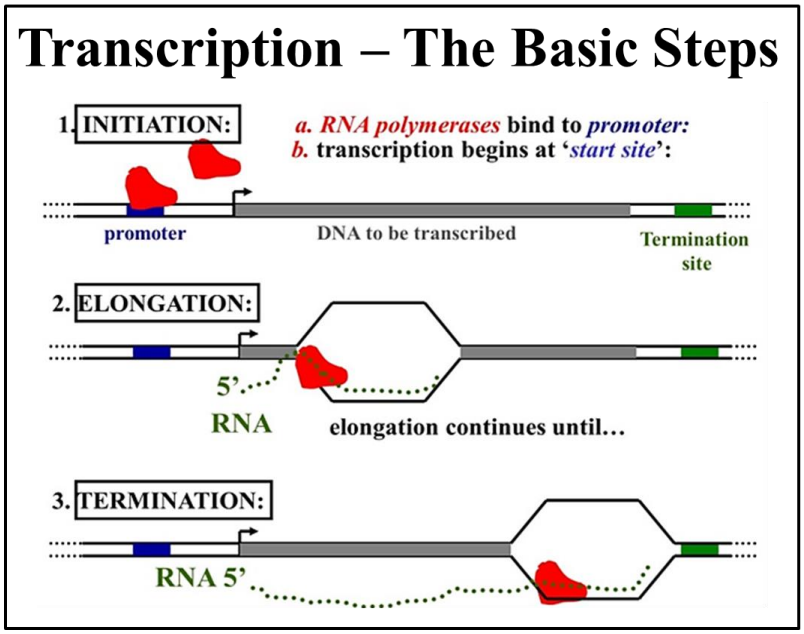


Fig. 10.4: Transcription - The basic Steps: *Initiation* is the recognition of a promoter sequence near the transcription start site by *RNA polymerase*. *Elongation* is the successive addition of nucleotides to a growing RNA strand. *Termination* occurs at the end of the gene, releasing the newly made RNA.

The transcribed grey DNA region in each of the three panels is the ***transcription unit*** of the gene. Termination sites are typically 3' to, or *downstream* from the transcribed region of the gene. By convention, *upstream* refers to DNA *5'* to a given reference point on the DNA (e.g., the transcription start-site of a gene). *Downstream* then, refers to DNA 3' to a given reference point on the DNA.

188-2 Transcription Overview- The Basics of RNA Synthesis

Some bacterial transcription units encode more than one mf RNA. Bacterial *operons* are an example of this phenomenon. The resulting mRNAs can be translated into multiple polypeptides at the same time. RNA polymerase is transcribing an ***operon*** into a single mRNA molecule encoding three separate polypeptides in Fig. 10.5.

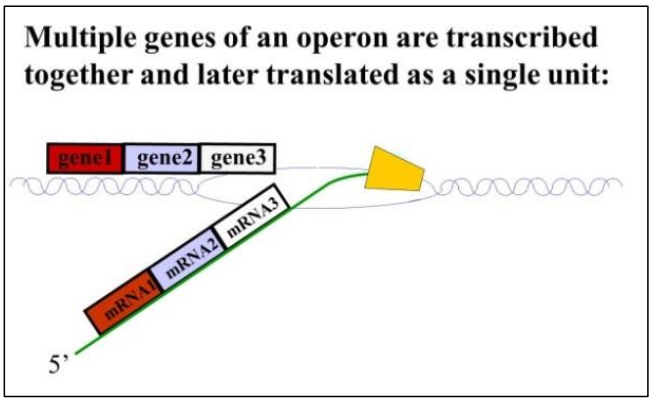


Fig. 10.5: An *Operon* is a contiguous group of 2 or more genes that are transcribed as a single messenger RNA that will be translated into two or more polypeptides.

Transcription of the different bacterial RNAs requires only one RNA polymerase. Different RNA polymerases catalyze rRNA, mRNA and tRNA transcription in eukaryotes. We already noted that Roger Kornberg received the Nobel Prize in Medicine in 2006 for his discovery of the role of ***RNA polymerase II*** and other proteins involved in eukaryotic messenger RNA transcription.

189 RNA Polymerases in Prokaryotes and Eukaryotes

While mRNAs, rRNAs and tRNAs are most of what cells transcribe, a growing number of other RNAs (e.g., *siRNAs*, *miRNAs, lncRNAs*…) are also transcribed. Some functions of these transcripts (including control of gene expression or other transcript use) are discussed in an upcoming chapter.

10.2.3 RNAs are Extensively Processed After Transcription in Eukaryotes

Eukaryotic RNAs are processed (trimmed, chemically modified) from large precursor RNAs to mature, functional RNAs. These precursor RNAs (pre-RNAs, or *primary transcripts*) contain in their sequences the information necessary for their function in the cell. Fig. 10.6 below is an overview of transcription and processing of the three major types of transcripts in eukaryotes.

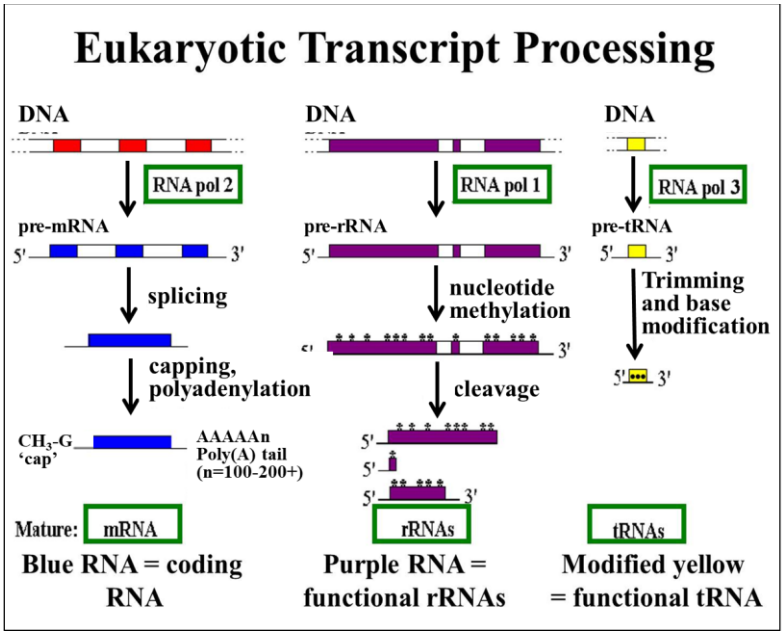


Fig. 10.6: Eukaryotic mRNAs, rRNAs and tRNAs are transcribed by different RNA polymerases and are processed by different mechanisms to yield usable, mature RNAs.

To summarize the illustration:

- Many eukaryotic genes are 'split' into coding regions (***exons***) and non-coding intervening regions (***introns***). Transcription of split genes generates a primary mRNA (pre-mRNA) transcript. Primary mRNA (pre-mRNA) transcripts are ***spliced*** to remove the ***introns*** from the ***exons***; exons are then ligated into a continuous mRNA. In some cases, the same pre-mRNA is spliced into alternate mRNAs encoding related but not identical polypeptides!
- Pre-rRNA is cleaved and/or trimmed (not spliced!) to make shorter mature rRNAs.
- Pre-tRNAs are trimmed, some bases within the transcript are modified and 3 bases (not encoded by the tRNA gene) are enzymatically added to the 3'-end.

190 Post Transcriptional Processing Overview

The details of transcription and processing differ substantially in prokaryotes and eukaryotes. Let's focus first on details of transcription itself and then RNA processing.

10.3 Details of Transcription

Find a well-written summary of transcription in prokaryotes and eukaryotes at an NIH website (Transcription in Prokaryotes and Eukaryotes). Here (and at this link), you will encounter proteins that bind DNA. Some proteins bind DNA to regulate transcription, *inducing* or *silencing* transcription of a gene. We will discuss their role in the regulation of gene expression later. Other proteins interact with DNA simply to allow transcription. These include one or more that, along with RNA polymerase itself, that must bind to the gene promoter to initiate transcription. We will look at bacterial transcription first.

10.3.1 Details of Transcription in Prokaryotes

In *E. coli*, a single RNA polymerase transcribes all kinds of RNA, associating with one of several sigma factor proteins (***σ–factors***) to initiate transcription. It turns out that different promoter sequences and corresponding σ–factors play roles in the transcription of different genes (Fig. 10.7, below).

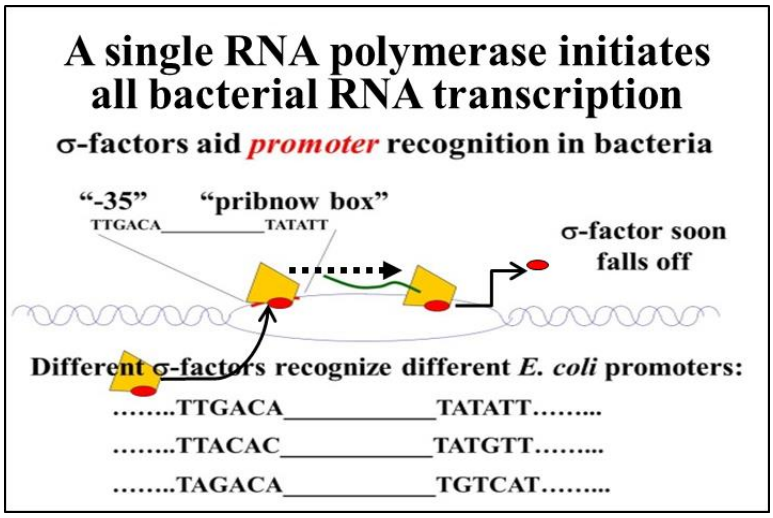


Fig. 10.7: Bacterial transcription requires initiation factors (i.e. σ–factors) to help the RNA polymerase find and bind to a gene promoter to begin transcription (see text for details).

In the absence of ***σ***–factor, *E. coli* RNA polymerase transcribes RNA, but does so at a high rate, and from random sequences in the chromosome. With the ***σ***–factor bound to the RNA polymerase, the complex seems to scan the DNA, recognize and bind to the

promoter sequence of a gene. The overall transcription rate is slower, but only genes are transcribed, rather than random bits of the bacterial genome! The *Pribnow box*, named for its discoverer, was the first promoter sequence characterized.

One way that bacteria regulate which genes are expressed is to selectively control the cellular concentrations of different σ–factors. A recent example may be *sigma 54*, a protein produced under stress (e.g., higher temperatures, antibiotic attack…). *Sigma 54*-bound RNA polymerase finds and binds to the promoters of genes that the bacterium express to mount a defense against the stress (for more details, check out Sigma 54 - a bacterial stress protein that alters transcription). We shall see more modes of prokaryotic gene regulation in the next chapter.

Soon after transcription is initiated, the *σ–factor* falls off the RNA polymerase, which then continues unwinding the double helix and elongating the transcript (Fig. 10.8).

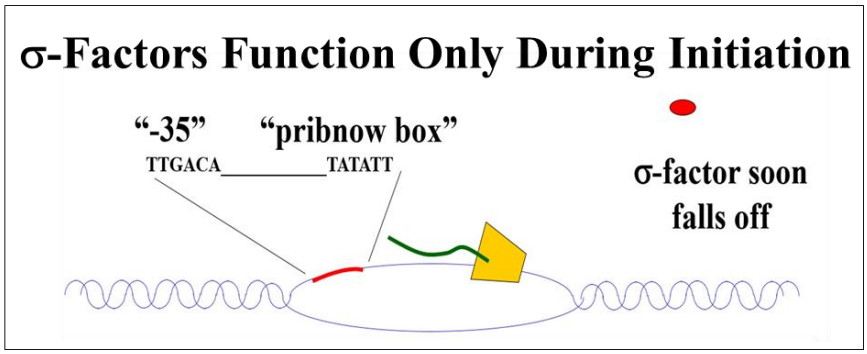


Fig. 10.8: After transcription has been initiated, σ factors soon fall away as elongation continues.

Elongation is the successive addition of nucleotides complementary to their DNA templates, forming phosphodiester linkages. The enzymatic condensation reactions of elongation are similar to DNA polymerase-catalyzed elongation during replication.

There are two ways that bacterial RNA polymerase 'knows' when it has reached the end of a transcription unit. In one case, as the RNA polymerase nears the 3' end of the nascent transcript, it transcribes a 72 base, C-rich region. At this point, a *termination factor* called the ***rho*** protein binds to the nascent RNA strand. *rho* is an ATP-dependent helicase that breaks the H-bonds between the RNA and the template DNA strand, thereby preventing further transcription. Fig. 10.9 illustrates ***rho-dependent*** termination (below).

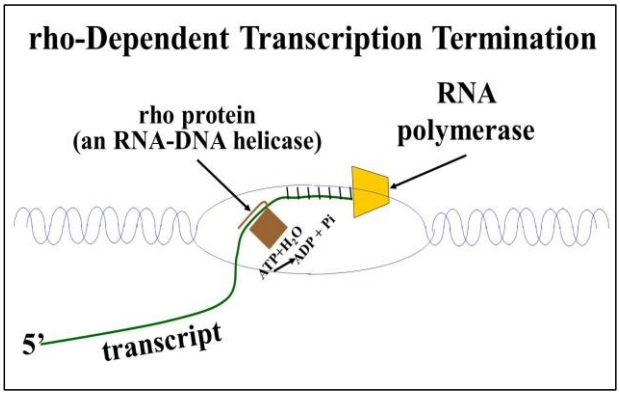


Fig. 10.9: *rho-dependent termination* of bacterial transcription involves recognition of sequences at the end of the gene and transcript

In the other mechanism of termination, a sequence of the RNA forming near the 3' end of the transcript folds into a secondary structure ***hairpin loop*** that serves as a termination signal, causing the dissociation of the RNA polymerase, template DNA and the new RNA transcript. The role of the hairpin loop in ***rho-independent termination*** is shown in Fig. 10.10 (below).

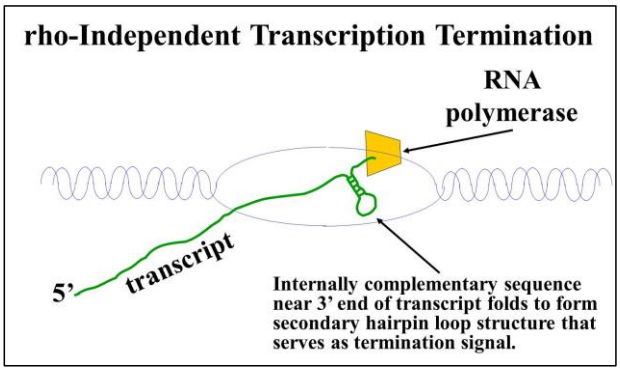


Fig. 10.10: *rho-independent termination* of bacterial transcription involves complementary bases near the end of the transcript that form a stem/loop, or *hairpin loop* structure that serves as a termination signal.

191 Details of Prokaryotic Transcription

10.3.2 Details of Transcription in Eukaryotes

Eukaryotes use three RNA polymerases to synthesize the three major RNAs (Fig. 10.11).

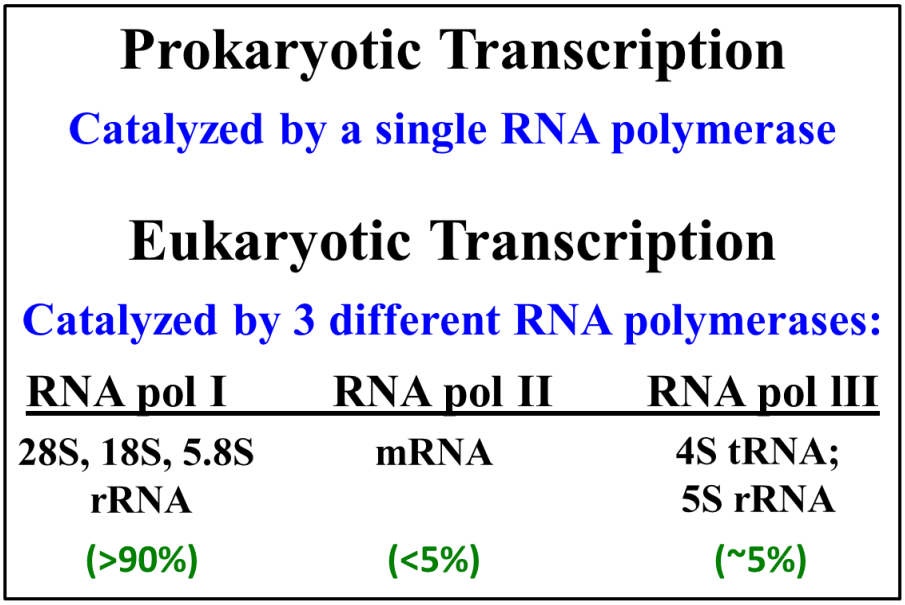


Fig. 10.11: Comparison of transcription in prokaryotes and eukaryotes; the roles of three RNA polymerases in Eukaryotes.

Note that catalysis of the synthesis of most of the RNA in a eukaryotic cell (rRNAs) is by RNA polymerase I. With the help of initiation proteins, each RNA polymerase forms an *initiation complex* consisting of several ***transcription factors*** (*TFs*) and one of the RNA Polymerases. Once the DNA at the start site of transcription unwinds, RNA polymerases catalyze the successive formation of phosphodiester bonds to elongate the transcript. These condensation reactions add ribose nucleotides to the free 3' end of a growing RNA molecule in reactions that are similar to the elongation of DNA strands. Unfortunately, the details of the termination of transcription in eukaryotes are not as well understood as they are in bacteria. Here, we focus on initiation, followed by discussion of the processing of different eukaryotic RNAs into ready-to-use molecules.

10.3.2.a Eukaryotic mRNA Transcription

Fig. 10.12 shows the multiple steps of eukaryotic mRNA transcription initiation.

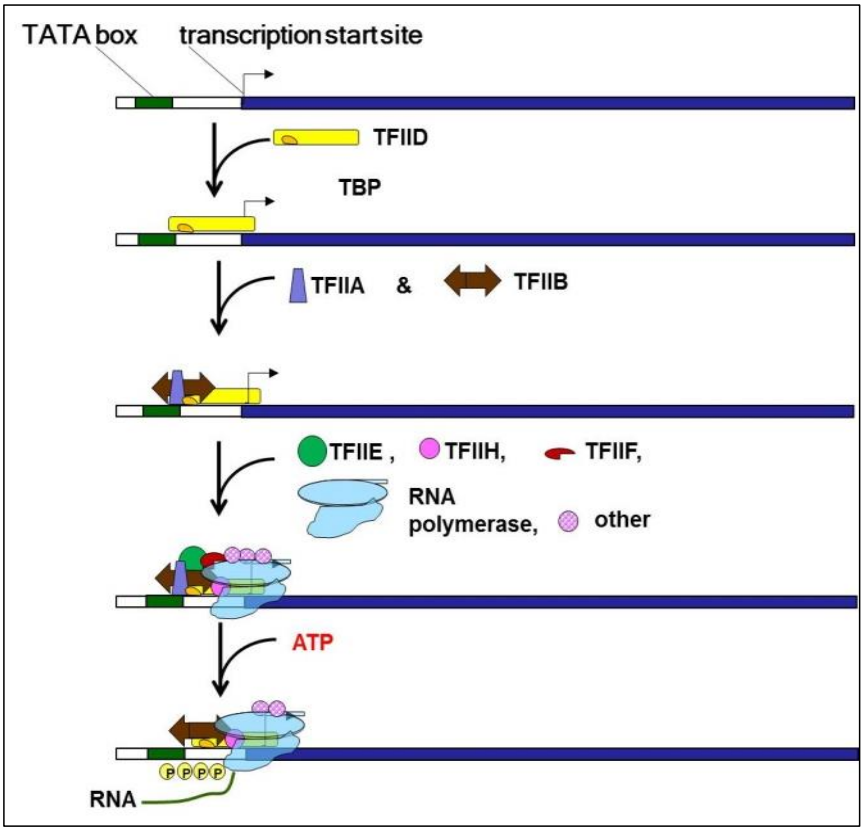


Fig. 10.12: Eukaryotic mRNA transcription initiation starts with recognition of a promoter *TATA box* by initiation factor TFIID. The association of a series of initiation factors follows. RNA polymerase II binds last. Its phosphorylation starts transcription.

Transcription of eukaryotic mRNAs by *RNA polymerase II* begins with sequential assembly of a eukaryotic *initiation complex* at a gene promoter. The typical promoter for a protein-encoding gene contains a TATA *sequence motif*, or ***TATA box*** and other short upstream sequences that recruit components of the initiation complex.

TATA-binding protein (*TBP*) first binds to the *TATA box* along with *TFIID* (*transcription factor IID*). This intermediate in turn recruits *TFIIA* and *TFIIB*. *TFIIE*, *TFIIF* and *TFIIH* are added to form the ***core initiation complex***. Finally, several other initiation factors and *RNA polymerase II* bind to complete the mRNA transcription ***initiation complex***. Phosphorylation adds several phosphates to the amino-terminus of the RNA polymerase, after which some of the TF's dissociate from the initiation complex. The remaining RNA polymerase-transcription factor complex can now start making the mRNA.

The first task of the complete initiation complex is to unwind the template DNA strands at the start site of transcription. Unlike prokaryotic RNA polymerase, eukaryotic RNA Polymerase II does not have an inherent DNA helicase activity. For this, eukaryotic gene transcription relies on the multi-subunit TFIIH protein, two of whose subunits have an ATP-dependent helicase activity. This helicase is not required for elongation of the RNA strand. Consistent with the closer relationship of *archaea* to eukaryotes (rather than to prokaryotes), archaeal mRNA transcription initiation resembles that of eukaryotes, albeit requiring fewer initiation factors during formation of an initiation complex.

192-2 Eukaryotic mRNA Transcription

A significant difference between prokaryotic and eukaryotic transcription is that RNA polymerases and other proteins involved at a eukaryotic gene promoter do not see naked DNA. Instead, they must recognize specific DNA sequences behind a coat of chromatin proteins. On the other hand, all proteins that interact with DNA have in common a need to recognize the DNA sequences to which they must bind…, within the double helix. In other words, they must see the bases within the helix, and not on its uniformly electronegative phosphate backbone surface. To this end, they must penetrate the DNA, usually through the ***major groove*** of the double helix. We will see that DNA regulatory proteins face the same problems in achieving specific shape-based interactions!

193-2 Recognition of Transcription Factors at Promoters

10.3.2.b Eukaryotic tRNA and 5S rRNA Transcription

Transcription of 5S rRNA and tRNAs by *RNA Polymerase III* is unusual in that the promoter sequence to which it binds (with the help of initiation factors) is ***not*** upstream of the transcribed sequence, but lies within the transcribed sequence.

RNA polymerase III and structural relatives of the *core* RNA polymerase II TFs (TBP, TFIIB, TFIIE, TFIIF…) form a core initiation complex at 5S RNA and tRNA gene promoters. Other gene-specific TFs add to this core complex. The completed initiation complex allows re-positioning the RNA polymerase III to transcribe the RNA from the transcription start site. As a result, the final transcript actually contains the promoter sequence! Fig. 10.13 illustrates the transcription of 5S rRNA (or tRNA) by RNA polymerase III.

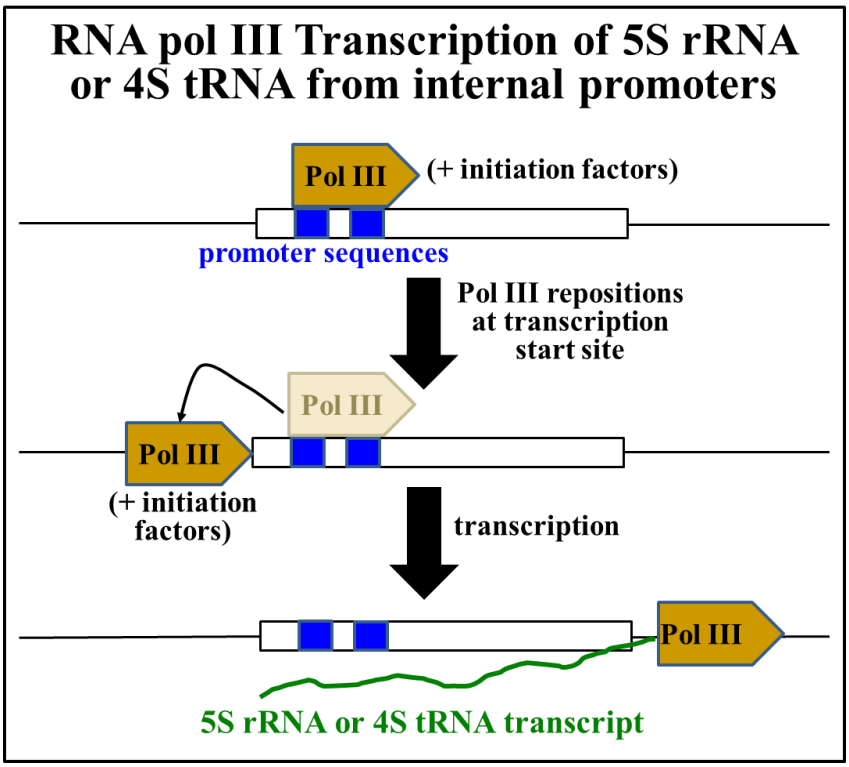


Fig. 10.13: 5S rRNA transcription in eukaryotes is initiated from an *internal promoter* by *RNA polymerase III*. After binding to the promoter, the polymerase re-positions itself near the transcription start-site.

RNA polymerase III-dependent transcription initiation does not seem to require an independent DNA helicase.

10.3.2.c Transcription of the Other Eukaryotic rRNAs

The production of the other eukaryotic rRNAs requires RNA polymerase I. It begins with transcription of a 45S RNA. This pre-rRNA is processed into 28S, 18S and 5.8S rRNAs (discussed in detail below). As with the transcription of 5S rRNA, tRNAs and mRNAs, RNA polymerase I and the 'core' TFs form a *core initiation complex* at promoters upstream of the 45S genes. Addition of several 45S rRNA gene--specific TFs completes assembly of the initiation complex that then unwinds the promoter DNA, again without the need for a separate helicase. More information about the structure and function of eukaryotic transcription initiation complexes can be had at Three Transcription Initiation Complexes.

As already noted, transcription termination is not as well understood in eukaryotes as in prokaryotes. Coupled termination and polyadenylation steps common to most prokaryotic mRNAs are discussed in more detail below, with a useful summary at the NIH-NCBI website Eukaryotic Transcription Termination.

10.4 Details of Eukaryotic mRNA Processing

Eukaryotic mRNA primary transcripts undergo extensive processing, including *splicing*, *capping and polyadenylation*. The steps described here are considered in order of (sometimes overlapping!) occurrence. We begin with splicing, an mRNA phenomenon.

10.4.1 Spliceosomal Introns

Bacterial gene coding regions are continuous. The discovery of eukaryotic split genes with introns and exons came as quite a surprise. Not only did it seem incongruous for evolution to have stuck irrelevant DNA in the middle of coding DNA, no one could have dreamt up such a thing! For their discovery of split genes, by Richard J. Roberts and Phillip A. Sharp shared the Nobel Prize for Physiology in 1993. In fact, all but a few eukaryotic genes are split, and some have one, two (or more than 30-50!) ***introns*** separating bits of coding DNA, the ***exons***. Fig. 10.14 below summarizes ***splicing*** to remove introns in pre-mRNAs and the slicing of exons to make mRNAs. The following illustration (Fig. 10.15) highlights the role of *small ribonuclear proteins* (***snRNPs***) splicing.

snRNPs are particles composed of RNA and proteins. They bind to specific sites in an mRNA and then direct a sequential series of cuts and ligations (the *splicing*) necessary to process the mRNAs. The process was reminiscent of the way in which movies were edited (see the photo of an early splicing 'machine' at the top of this chapter), hence the term *splicing* to describe mRNA processing.

In Fig. 10.14, RNA polymerase II transcribes an often very long pre-mRNA that is spliced.

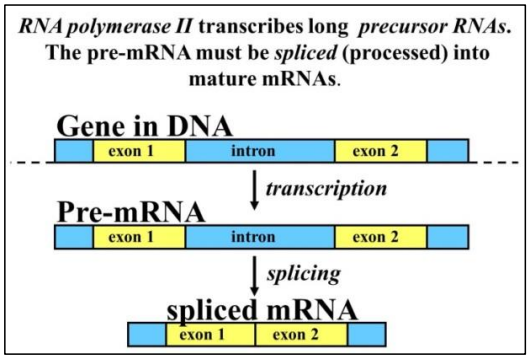


Fig. 10.14: Transcription and processing of eukaryotic mRNA. Many mRNAs in eukaryotes are encoded by split genes containing coding regions (exons) and non-coding regions (introns). These genes are transcribed as precursor (pre-) mRNAs that must be processed by splicing before they can be used in translation.

The roles of snRNPs in initiating splicing of mRNA are illustrated in Fig. 10.15.

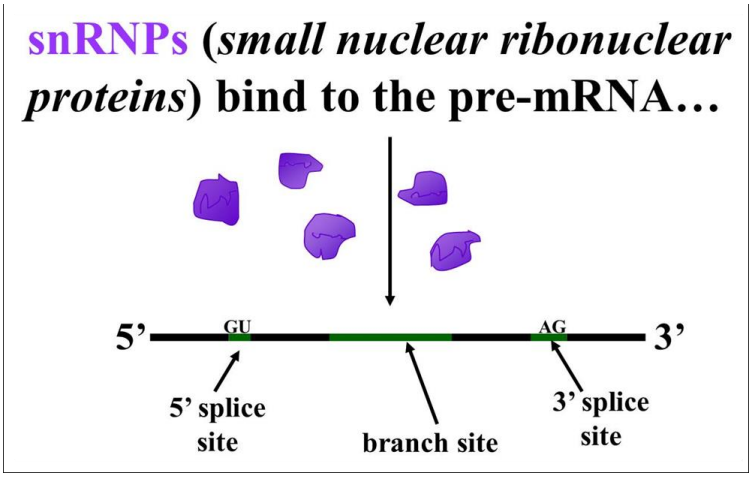


Fig. 10.15: Splicing of a eukaryotic pre-mRNA involves association of the primary transcript (*pre-mRNA*) with *snRNPs*, small ribonuclear proteins that catalyze cleavage of the pre-RNA at 5' and 3' splice sites followed by ligation of the 3' to 5' exon ends.

When snRNPs bind to a pair of splice sites flanking an intron in a pre-mRNA, they form the ***spliceosome*** that completes the splicing, including removal of the intermediate ***lariat*** structure of the intron. The last step is to ligate exons into a continuous mRNA with all its codons intact and in the right order, an impressive trick for some pre-mRNAs with as many as 50 introns!

Spliceosome action is summarized in Fig. 10.16 and in the animated in the ***mRNA splicing*** link below. Imagine this happening as many as 30 times in just a single pre-actin mRNA!

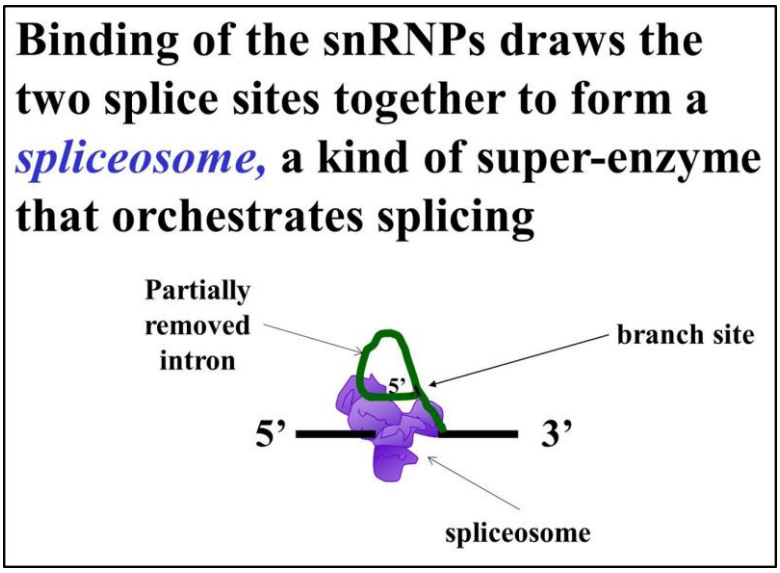


Fig. 10.16: Formation of a spliceosome by the binding of snRNPs to an mRNA leads to hydrolysis of splice sites and the formation of a lariat structure from the intron remnants.

194-2 The Discovery of Split Genes

195 mRNA Splicing

10.4.2 Specific Nuclear Body Proteins Facilitate SnRNP Assembly and Function

Recall the organization of nuclei facilitated by nuclear bodies. Nuclear ***speckles*** are associated regions of high mRNA transcription, processing and splicing (check out the animation at http://www.caltech.edu/news/cartography-nucleus-82442 for a 3-dimensional localization of markers of nuclear body activity.

Cajal bodies (**CB**s) and ***Gems*** are nuclear bodies that are similar in size and have related functions in assembling spliceosomal SnRNPs. Some splicing defects correlate with mutations in the ***coil*** protein that associate with Cajal bodies; others correlate with mutations in ***SMN*** proteins normally associated with Gems. One hypothesis was that CBs and Gems interact in SnRNP and spliceosome assembly…, but how? Consider the results of an experiment in which antibodies to ***coilin*** and the **SMN** protein were localized in undifferentiated and differentiated *neuroblastoma* cells (Fig. 10.17, below).

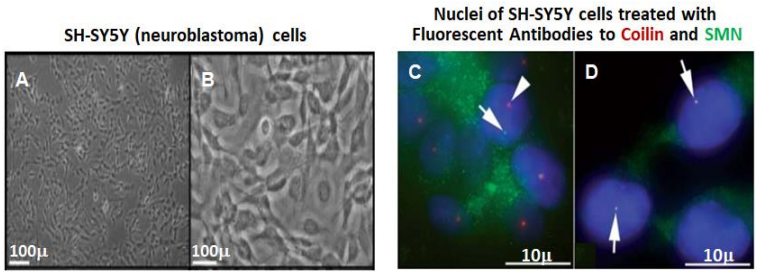


Fig. 10.17: Immunofluorescence staining with antibodies to coilin and SMN protein show that *Cajal bodies* and *Gems* aggregate when undifferentiated cells (panels A and C) are stimulated to differentiate (panels B and D). Since mutations in *coilin* and *SMN* proteins are associated with splicing defects, co-localization of Cajal bodies and Gems suggests their co-involvement of snRNP function.

A and C in Fig. 10.17 are undifferentiated cells in culture; B and D are cells that were stimulated to differentiate. In the fluorescence micrographs at the right, arrows point to fluorescent nuclear bodies. We would expect that fluorescent antibodies to coilin (green) to localize to CBs and antibodies to SMN protein (red) to bind to Gems. This is what happens in the nuclei of undifferentiated cells (panel C). But in panel D, the two antibodies co-localize, suggesting that the CBs and Gems aggregate in the differentiated cells. This would explain the need for both coilin and SMN protein to produce functional SnRNPs. The CBs and Gems may be aggregating in differentiated cells due to an observed increase in expression of the SMN protein. This could lead to Gems more able to associate with the CBs. This and similar experiments demonstrate that different nuclear bodies do have specific functions. They are not random structural artifacts but have evolved to organize nuclear activities in time and space in ways that are essential to the cell.

10.4.3 Group I and Group II *Self-Splicing* Introns

While Eukaryotic *Spliceosomal introns* are spliced using snRNPs as described above, *Group I* or *Group II* introns are removed by different mechanisms. ***Group I introns*** interrupt mRNA and tRNA genes in bacteria and in mitochondrial and chloroplast

genes. They are occasionally found in bacteriophage genes, but rarely in nuclear genes, and then only in lower eukaryotes. Group I introns are *self-splicing*! Thus, they are *ribozymes* that do not require snRNPs or other proteins for splicing activity. Instead, they fold into a secondary stem-loop structure that positions catalytic nucleotides at appropriate splice sites to excise themselves, and also re-ligate the exons. ***Group II introns*** in chloroplast and mitochondrial rRNA, mRNA, tRNA and some bacterial mRNAs can be quite long. They form complex stem-loop tertiary structures and self-splice, at least in a test tube! However, *Group II introns* encode proteins required for their own splicing *in vivo*. Like spliceosomal introns, they form a lariat structure at an *A* residue branch site. All this suggests that the mechanism of spliceosomal intron splicing evolved from that of *Group II* introns.

10.4.4 So, Why Splicing?

The puzzle implied by this question is of course, why do higher organisms have split genes in the first place? While the following discussion can apply to all splicing, it will reference mainly spliceosomal introns. Here are some answers to the question "Why splicing?"

- Introns in nuclear genes are typically longer (often much longer!) than exons. Since they are non-coding, they are large targets for mutation. In effect, non-coding DNA, including *introns* can buffer the ill effects of random mutations.

- You may recall that gene duplication on one chromosome (and loss of a copy from its homolog) arise from unequal recombination (non-homologous crossing over). It occurs when similar DNA sequences align during synapsis of meiosis. In an organism that inherits a chromosome with both gene copies, the duplicate can accumulate mutations as long as the other retains original function. The diverging gene then becomes part of a pool of selectable DNA, the grist of evolution. Descendants of organisms that inherit the duplicated genes have diversified the gene pool, again increasing the potential for evolution and species diversity.

- Unequal recombination can also occur between similar sequences (e.g., in introns) in the same or different genes, resulting in a sharing of exons between genes. After unequal recombination between introns flanking an exon, one gene will acquire another exon while the other will lose it. Once again, as long as an organism retains a copy of the participating genes with original function, the organism can make the required protein and survive. Meanwhile, the gene with the extra exon may produce the same protein, but one with a new structural domain and function. Like a complete duplicate gene, one with a new exon that adds a new function to an old gene has been entered in the pool of selectable

DNA. Thus, this phenomenon of ***exon shuffling*** increases species diversity! *Exon shuffling* has occurred, creating proteins with different overall functions that nonetheless share at least one domain and one common function.

An example discussed earlier involves calcium-binding proteins that regulate many cellular processes. Structurally related *calcium* (Ca^{++}) *binding domains* are common to many otherwise structurally and functionally unrelated proteins. Consider exon shuffling in the unequal crossover (non-homologous recombination) shown in Fig. 10.18).

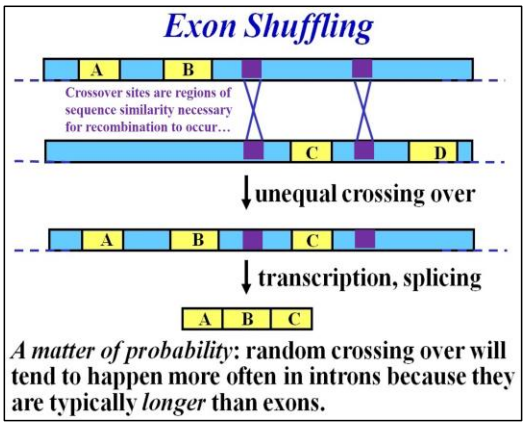


Fig. 10.18: *Exon shuffling* transfers/copies an exon from one gene to another by unequal recombination (cross--over) between different genes based on regions of sequence similarity within introns.

In this example, regions of strong similarity in different (non-homologous) introns in the same gene align during synapsis of meiosis. Unequal crossing over between the genes inserts exon C in one of the genes. The other gene loses the exon (not shown in the illustration).

In sum, introns are *buffers against deleterious mutations*, and equally valuable, are potential targets for gene duplication and exon shuffling. This makes introns key players in creating *genetic diversity*, the hallmark of evolution.

196-2 Origin of Introns

197 Intron Evolution-What was selected here?

10.4.5 5-Prime Capping

A ***methyl guanosine cap*** added 5'-to-5' to an mRNA functions in part to help mRNAs leave the nucleus and associate with ribosomes. The cap is added to an exposed 5' end, even as transcription and splicing are still in progress. A *capping enzyme* places a methylated guanosine residue at the 5'-end of the mature mRNA. Fig. 10.19 shows the 5' cap structure (check marks are 5'-3' linked nucleotides).

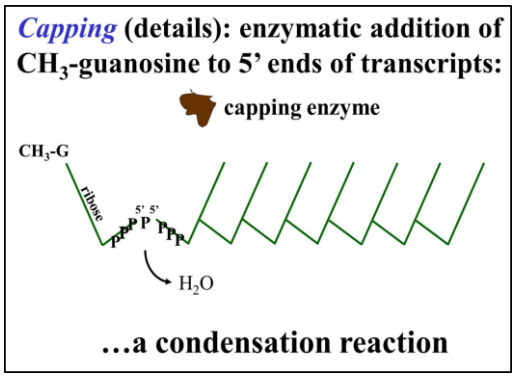


Fig. 10.19: mRNA *capping* results from a 5'–to–5' condensation reaction linking the 5' end of an mRNA to a methylated guanidine triphosphate (CH_3-GTP) .

10.4.6 3-Prime Polyadenylation

Polyadenylation occurs after transcription termination (Fig. 10.20, below).

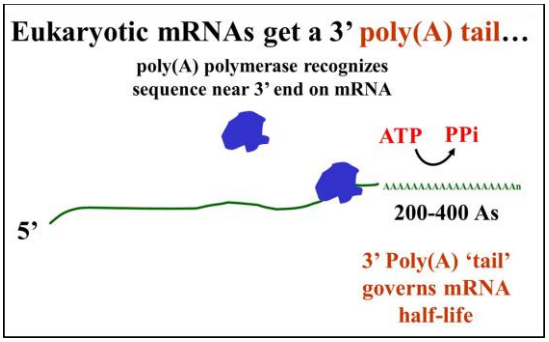


Fig. 10.20: mRNA *polyadenylation*; this is the addition of multiple adenine nucleotides to the 3' end of an mRNA, catalyzed by the enzyme *poly(A) polymerase*. Other than histone mRNAs, most eukaryotic mRNAs are polyadenylated.

The enzyme ***poly(A) polymerase*** catalyzes the addition of adenine monophosphates (AMPs) to the 3' end of most eukaryotic mRNAs, even before any splicing is complete. Polyadenylation requires ATP and can add several hundred AMPs to the 3' terminus of an mRNA. The enzyme binds to an **AAUAA** sequence near the 3' end of an mRNA to start adding the AMPs. The result of *polyadenylation* is a 3' ***poly (A) tail*** whose functions include assisting in the transit of mRNAs from the nucleus and regulating the half-life of mRNAs in the cytoplasm. The poly (A) tail shortens each time a ribosome completes translating the mRNA.

198 mRNA 5' Capping and 3' Polyadenylation

10.5 Ribosomal RNA Processing in Eukaryotic Nuclei

In most eukaryotes, a large rRNA gene transcribes a 45S precursor transcript containing (from shortest to longest) 5.8S rRNA, 18SrRNA and 28S rRNA. The 'S' stands for Svedberg, the biochemist who developed the *sedimentation velocity ultra-centrifugation* technique to separate molecules like RNA by size. The higher the S value, the larger the molecule and therefore the faster it moves through the viscous sugar gradient during centrifugation. RNA Polymerase I transcribes 45S precursor rRNAs (pre-RNAs) from multiple large *transcription units* in the genome. One such *unit* is shown below in Fig. 10.21.

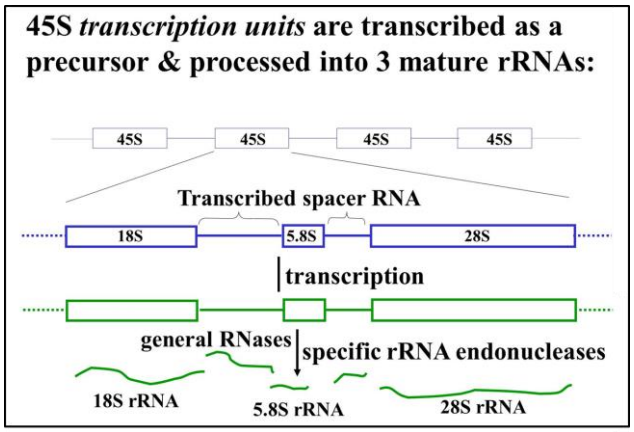


Fig. 10.21: Post-transcriptional processing of the 45S rRNA encoding three of the four eukaryotic rRNAs by hydrolytic cleavage. Cleavage is catalyzed by endonucleases that recognize differential methylation patterns of rRNA regions and transcribed spacer regions of the 45S precursor RNA.

The 45S pre-rRNA is processed by cleavage. The many copies (200-400!) of the 45S gene in eukaryotic cells might be expected, since making proteins (and therefore ribosomes) will be an all-consuming cellular activity. In humans, 45S genes (45S rDNA) are distributed among five *acrocentric* chromosomes (those that have a centromere very near one end of the chromosome). The 45S rDNA in chromosomes is packed in the ***nucleolus*** inside nuclei. Because these genes are present in so many copies and organized into a specific region of chromatin, it is possible to visualize 45S transcription in progress in electron micrographs such as the ones in Fig. 10.22 (below).

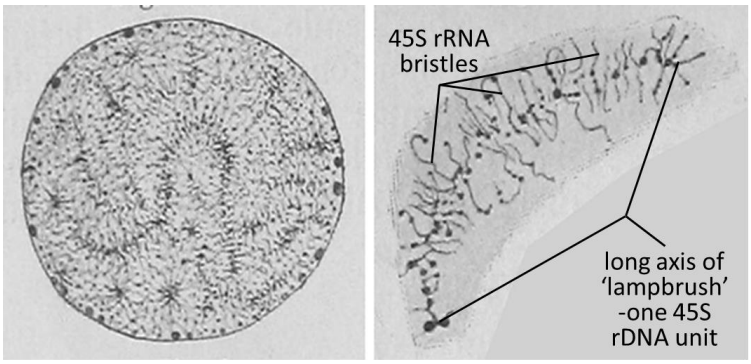


Fig. 10.22: Early (1906) low power (left) and high power (right) light micrographs of stained amphibian (salamander) oocyte nucleolar DNA in amphibian oocytes showing the "chromatic fibers" that (resemble) a bottle brush , now called *lampbrush chromosomes* because they were also reminiscent of brushes used to clean the chimneys of old-fashioned oil lamp chimneys. We now know that the bristles are nascent strands of rRNA.

The term *lampbrush* comes from the shape of the 45S genes in undergoing transcription; the RNAs extending from the DNA template look like an old-fashioned brush used to clean the chimney of a kerosene lamp. For a high resolution transmission electron micrograph, see https://www.flickr.com/photos/185778834@N06/49363712358/

Multiple gene copies encode 5S rRNAs. However, unlike the 45S rRNA genes, 5S rRNA gene may be spread among many chromosomes (seven in *Neurospora crassa*, the bread mold). Or in the case of humans, 5S RNA gene copies are found distributed along chromosome 1. The 5S rRNA genes are transcribed by RNA polymerase III with minimal post-transcriptional processing. As we noted, the promoters of the 5S genes are within the transcribed part of the genes, rather than upstream of their 5S transcription units.

199-2 rRNA Transcription & Processing

10.6 tRNA Processing in Eukaryotic Nuclei

RNA polymerase III also transcribes tRNA genes from internal promoters, but unlike the 5S rRNA genes, tRNA genes tend to cluster in the genome (Fig. 10.23).

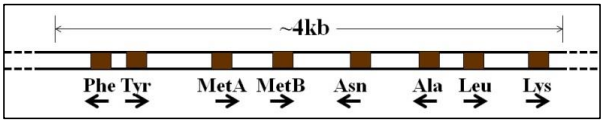


Fig. 10.23: Characteristic clustering of eukaryotic tRNA genes.

And also unlike 5S rRNA genes, tRNA primary transcripts are processed by

- trimming,
- enzymatic addition of a -C-C-A base triplet at the 3' end, and
- the modification of bases internal to the molecule

A yeast tRNA showing these modifications is illustrated in Fig. 10.24.

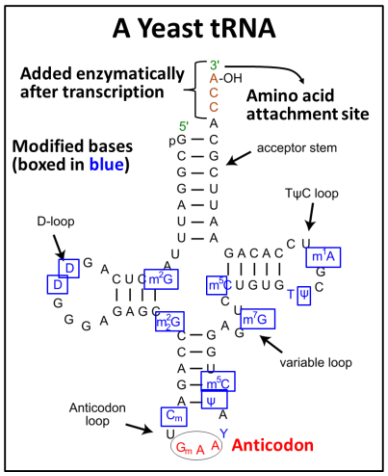


Fig. 10.24: Structure of a yeast tRNA.

The tRNA folds into several *hairpin loops* based on internal H-bond formation between complementary bases in the molecule. The 3'-terminal A residue of the **–C-C-**A add-on to this (and every) tRNA will bind to an amino acid specific for the tRNA.

200-2 tRNA Transcription and Processing

10.7 mRNA and Ribosome Export from the Nucleus

The synthesis and processing of rRNAs are coincident with the assembly of the ribosomal subunits (Fig. 10.25).

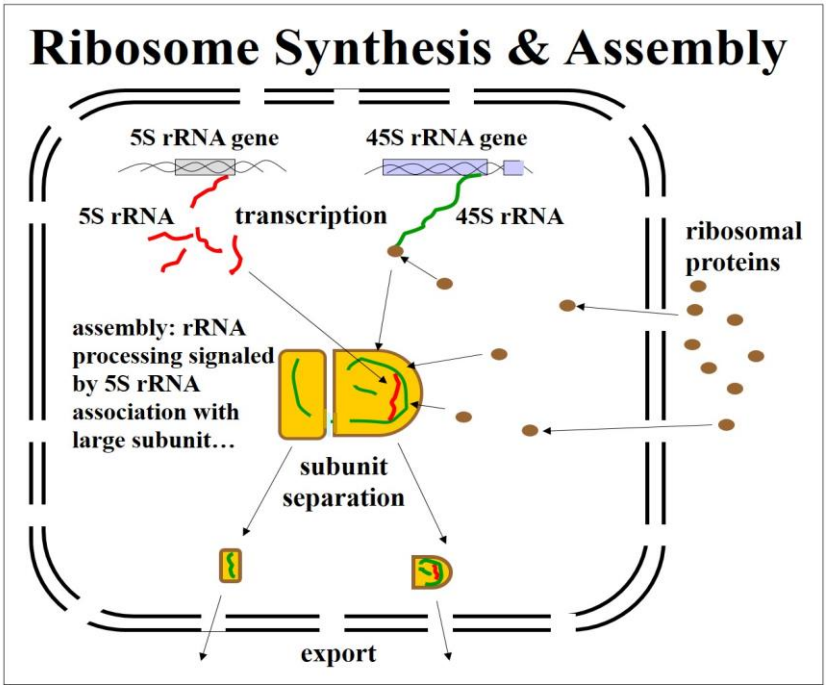


Fig. 10.25: The assembly of ribosomal subunits and their export to the cytoplasm is coordinated with the synthesis and association with the rRNAs and their ribosomal proteins.

The 45S pre-rRNAs initially bind to ribosomal proteins in the nucleolus (that big nuclear body!) to initiate assembly and then and serve as a scaffold for the continued addition of ribosomal proteins to both the small and large ribosomal subunits. After the 5S rRNA is added to the nascent large ribosomal subunit, processing (cleavage) of 45S rRNA is completed and the subunits are separated. The separated ribosomal subunits exit the nucleus to the cytoplasm where they will associate with mRNAs to translate new proteins. To better understand what is going on, try summarizing what you see here in the correct order of steps. You can also see this process animated at Ribosome Assembly and Transport from the Nucleus.

Messenger RNAs are independently exported from the nucleus. Their 5' methyl guanosine caps and poly(A) tails collaborate to facilitate transport into the cytoplasm. We now understand that proteins in the nucleus participate in the export process. A *nuclear transport receptor* binds along the mature (or maturing) mRNA, a *poly-A-binding protein* binds along the poly-A tail of the message, and another protein binds at or near the methyl guanosine CAP itself. These interactions enable transport of the mRNA through nuclear pores. After the mRNA is in the cytoplasm, the nuclear transport receptor re-cycles back into the nucleus while a *translation initiation factor* replaces the protein bound to the CAP. Fig. 10.26 summarizes the nuclear transport process for mRNAs.

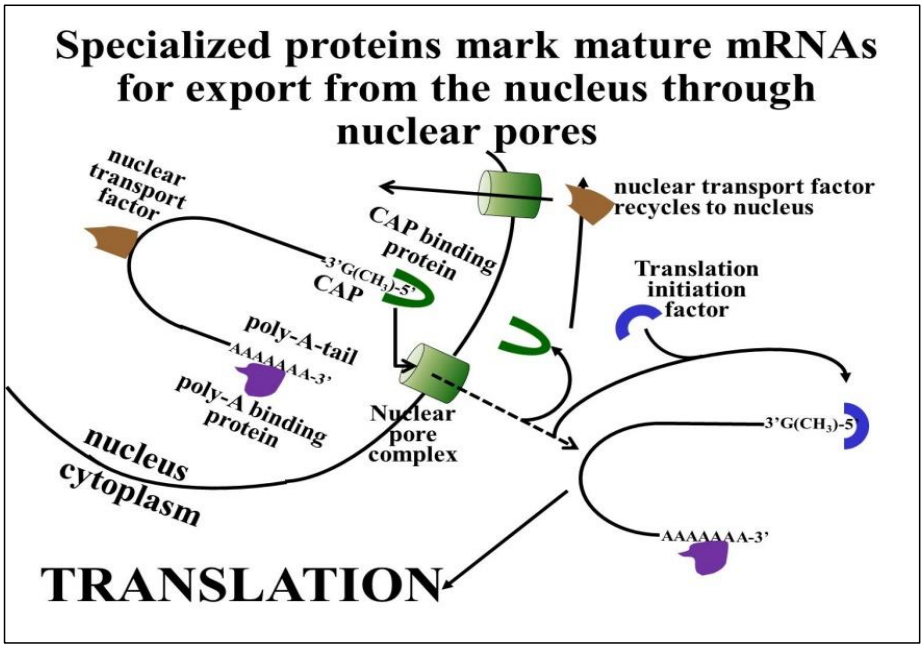


Fig. 10.26: Role of CAP and poly-A binding proteins and a nuclear transport factor in the export of an mRNA to the cytoplasm.

See a more detailed description of mRNA transport from the nucleus at this link: mRNA Export from the Nucleus . The mature mRNA, now in the cytoplasm, is ready for translation. Translation is the process of protein synthesis mediated by ribosomes and a host of translation factors (including the initiation factor in Fig. 10.25. The genetic code directs polypeptide synthesis during translation. Details of translation will be discussed shortly.

Some iText & VOP Key Words and Terms

16S rRNA	internal promoters	rRNA
18S rRNA	introns	rRNA cleavage
23S rRNA	lariat	rRNA endonucleases
28SrRNA	mature RNA transcript	σ-factor
45S pre-rRNA	mRNA	SINEs
45S rRNA methylation	mRNA capping	snRNP
4S rRNA	mRNA polyadenylation	spacer RNA
5'-methyl guanosine capping	mRNA splicing	splice sites
5S rRNA	operons	spliceosome
8S rRNA	poly (A) polymerase	Svedberg unit
adenine	poly(A) tail	TATA binding protein
Alu	polycistronic RNA	TBP
branch sites	Pribnow box	termination
crossing over	promoter	TFIIB, TFGIIE, TFIIF, TFIIH
cytosine	recombination	transcription
DNA binding proteins	regulatory DNA sequence	transcription start site
E. coli RNA polymerase	regulatory factor	transcription unit
elongation	rho termination factor	translation
eukaryotic RNA polymerases	rho-independent termination	transposition
exon shuffling	ribonucleoproteins	transposons
exons	RNA polymerase I	tRNA
guanine	RNA polymerase II	tRNA processing
helitrons	RNA polymerase III	upstream v. downstream
helix-turn-helix motif	RNA processing	uracil
initiation	RNA secondary structure	

Chapter 11: The Genetic Code and Translation

The Genetic Code, tRNA (Adapter) Molecules, Translation (Protein Synthesis)

Captain Midnight, assisted by radio listeners with a ***Code-O-Graph (left)***, saved us from spies and sabotage in the run-up to WWII. After Pearl Harbor, the ***Captain*** focused his activities on German and Japanese enemies.

Then the need for encryption & decryption got real...

and then the allies got hold of one of these German *Enigma Machines* that really helped us win the war.

11.1 Introduction

While evidence was accumulating that DNA was "the stuff of genes", many still believed that proteins were genes, at least until the experiments of Hershey and Chase. It was in this somewhat ambivalent context that investigators wondered about how genes (whether proteins or nucleic acids) might encode metabolically functional proteins such as enzymes… that of course are also proteins. In 1944, the physicist-philosopher Erwin Schrödinger published ***What Is Life? The Physical Aspect of the Living Cell***. *T*argeting the lay reader, Schrödinger took a stab at explaining life, heredity and development in terms of physical laws. Here in his own words is what he had to say about the chemistry of heredity that when deciphered, would dictate structures, or "patterns" of development (abridging and highlighting, courtesy of your author!):

> ***THE HEREDITARY CODE-SCRIPT*** (from https://criv.pw/v9v.pdf)
>
> *…Now, this pattern is known to be determined by the structure of that one cell, the fertilized egg. Moreover, we know that it is essentially determined by the structure of only a small part of that cell, its large nucleus. This* ***nucleus****… usually appears as a network of* ***chromatine****, distributed over the cell. But in the processes of cell division*

*(mitosis and meiosis) it is seen to consist of a **set of particles**, usually fibre-shaped or rod-like, called the **chromosomes**, which number in man, 48 (2 X 24)… I ought to have spoken of two sets, in order to use the expression in the customary strict meaning of the biologist. For the two sets are almost entirely alike. As we shall see in a moment, one set comes from the mother (egg cell), one from the father (fertilizing spermatozoon). **It is these chromosomes,** or probably only an axial skeleton fibre of what we actually see under the microscope as the chromosome, **that contain in some kind of code-script the entire pattern of the individual's future development and of its functioning in the mature state.** Every complete set of chromosomes contains the full code; so there are, as a rule, two copies of the latter in the fertilized egg cell, which forms the earliest stage of the future individual. In calling the structure of the chromosome fibres a code-script we mean that the all-penetrating mind, once conceived by Laplace, to which every causal connection lay immediately open, could tell from their structure whether the egg would develop, under suitable conditions, into a black cock or into a speckled hen, into a fly or a maize plant, a rhododendron, a beetle, a mouse or a woman. To which we may add, that the appearances of the egg cells are very often remarkably similar; and even when they are not, as in the case of the comparatively gigantic eggs of birds and reptiles, the difference is not been so much the relevant structures as in the nutritive material which in these cases is added for obvious reasons. **But the term code-script is, of course, too narrow. The chromosome structures**… are at the same time instrumental in bringing about the development they foreshadow. They **are law-code and executive power -or, to use another simile, they are architect's plan and builder's craft -in one.***

Once again, keep in mind that these speculations presage the identification of DNA as the genetic chemical. The terms *code, broken* and *deciphered* themselves came from the recent World War II history. Winning the war relied on *breaking* enemy codes (see the ***Enigma Machine*** at the top of this chapter), as well as hiding our own strategic battle information from the enemy (recall or look up the history of the Navajo *code talkers*).

In this chapter we look at just how the ***genetic code*** in DNA (and in fact in messenger RNA) was broken. We describe the elegant experiments that first deciphered the amino acid meaning of a few 3-base ***codons***, and then all 64 codons. Of these, 61 encode amino acids and three are ***stop codons***. Experiments similar to those that broke the genetic code also led to our under-standing of the mechanism of protein synthesis. Early studies indicated that genes and proteins are colinear, i.e., that the length of a gene was directly proportional to the polypeptide it encoded. It would follow then, that the lengths of mRNAs are also collinear with their translation products. ***Colinearity*** suggested the obvious hypotheses that translation proceeded in three steps (***initiation***, ***elongation*** and ***termination***), just like transcription itself.

We now know that initiation is a complex process involving the assembly of a translation machine near the 5' end of the mRNA. This machine consists of ribosomes, mRNA, several ***initiation factors*** and a source of chemical energy. Since *mature* mRNAs are actually longer than needed to specify a polypeptide (even after splicing!), one function of initiation *factors* is to position the ribosome and associated proteins near a ***start codon***. The start codon specifies the first amino acid in a new polypeptide. Once the ***initiation complex*** forms, elongation begins. Cycles of condensation reactions on the ribosome connect amino acids by peptide linkages, growing the chain from its amino-end to its carboxyl-end. Translation ends when the ribosome moving along the mRNA encounters a stop codon. We will look at how we came to understand the discrete steps of translation.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast the *mechanisms* and *energetics* of initiation, elongation and termination of translation and transcription.
2. speculate on why *the genetic code is universal* (or nearly so).
3. justify early thinking about a *4-base genetic code.*
4. justify early thinking about an *overlapping genetic code* (for example, one in which the last base of a codon could be the first base of the next codon in an mRNA.
5. explain why all *tRNA structures* share some, but not other features.
6. compare and contrast the roles of the ribosomal *A, E and P sites* in translation.
7. trace the formation of an *aminoacyl-tRNA* and the bacterial *Initiation Complex.*
8. describe the steps of translation that require chemical energy from NTPs.
9. formulate an hypothesis to explain why stop codons all begin with the base U.
10. create a set of rules for inferring an amino acid sequence from a DNA sequence.
11. speculate about why large eukaryotic genomes encode so few proteins.

11.2 Overview of the Genetic Code

The **genetic code** is the information for linking amino acids into polypeptides in an order based on the base sequence of 3-base code words (***codons***) in a gene and its messenger RNA (mRNA). With a few exceptions (some prokaryotes, mitochondria, chloroplasts), ***the genetic code is universal*** – it's the same in all organisms from viruses and bacteria to humans. Here we'll look at the genetic code itself and how information in the nucleotide sequences of transcribed genes is translated into polypeptides.

11.2.1 The (Nearly) Universal, Degenerate Genetic Code

The **genetic code** is the information to link amino acids into polypeptides in an order based on 3-base code words, or ***codons*** in a gene and its mRNA. Fig. 11.1 (below) is a representation of the genetic code in the 'language' of RNA.

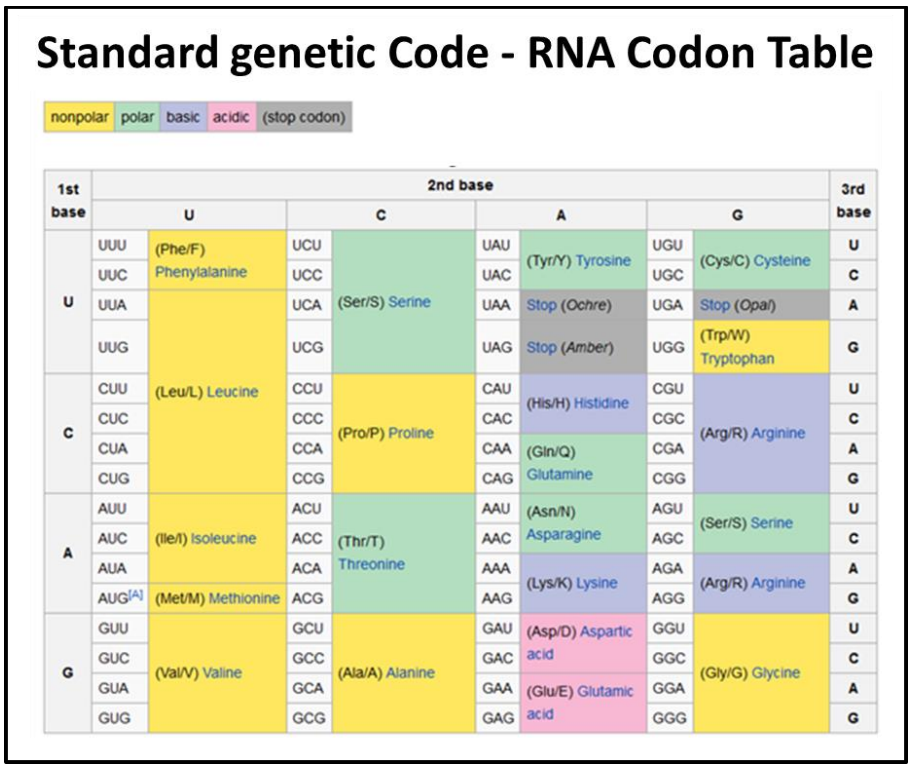


Fig. 11.1: The Universal *RNA Genetic Code Dictionary***.**

There is a single codon for two amino acids (methionine, tryptophan), but two or more for each of the other 18 amino acids. For the reason, we say that the genetic code is ***degenerate***. The three ***stop codons*** in the *Standard Genetic Code* 'tell' ribosomes the location of the last amino acid to add to a polypeptide. The last amino acid itself can be any amino acid consistent with the function of the polypeptide being made. In contrast, evolution has selected AUG as the ***start codon*** for all polypeptides (regardless of their function) *and* for the insertion of methionine within a polypeptide. Thus, all polypeptides begin life with a methionine at their amino-terminal end. We will see in detail that ribosomes are mRNA translation machines and that the biological equivalent of the *Enigma Machine* is the tRNA *decoding device*. Each amino acid attaches to a tRNA whose short sequence contains a 3-base *anticodon* that is complementary to an mRNA codon. Enzymatic reactions catalyze the ***dehydration***

synthesis reactions that link amino acids by *peptide bonds* in the order specified by mRNA codons.

 **201 The Genetic Code Dictionary** 

11.2.2 Comments on the Nature and Evolution of Genetic Information

The near-universality of the genetic code from bacteria to humans implies that the code originated early in evolution. It is probable that portions of the code were in place even before life began. Once in place however, the genetic code was highly constrained against evolutionary change. The degeneracy of the genetic code enabled (and contributed to) this constraint by permitting many base changes that do not affect the amino acid encoded in a codon.

The near universality of the genetic code and its resistance to change are features of our genomes that allow us to compare genes and other DNA sequences to establish evolutionary relationships between organisms (species), groups of organisms (genus, family, order, etc.) and even individuals within a species.

In addition to constraints imposed by a universal genetic code, some organisms show *codon bias*, a preference to use of some but not other codons in their genes. Codon bias is seen in organisms that favor A-T rich codons, or in organisms that prefer codons richer in G and C. Interestingly, codon bias in genes often accompanies a broader corresponding *genomic nucleotide bias*. An organism with an AT codon bias may also have an AT-rich genome (likewise GC-rich codons in GC-rich genomes). You can recognize genome nucleotide bias in Chargaff's base ratios!

Finally, we often think of genetic information as genes in terms of proteins. Obvious examples of *non-coding genetic information* include genes for rRNAs and tRNAs. The amount of these kinds of informational DNA (i.e., genes for polypeptides, tRNAs and rRNAs) as a proportion of total DNA can range across species, although it is higher in prokaryotes than eukaryotes. For example, ~88% of the *E. coli* circular chromosome encodes polypeptides, while that figure is less ~1.5% for humans. Some less obvious informative DNA sequences in higher organisms are transcribed (e.g., introns). Other informative DNA in the genome is never transcribed. The latter include regulatory DNA sequences, DNA sequences that support chromosome structure and other DNAs that contribute to development and phenotype. As for that amount of truly non-informative (useless) DNA in a eukaryotic genome, that amount is steadily shrinking as we sequence entire genomes, identify novel DNA sequences and discover novel RNAs (topics covered elsewhere in this text).

11.3 Gene and Protein Colinearity and Triplet Codons

Serious efforts to understand how proteins are encoded began after Watson and Crick used the experimental evidence of Maurice Wilkins and Rosalind Franklin (among others) to determine the structure of DNA. Most hypotheses about the genetic code assumed that DNA (i.e., genes) and polypeptides were ***colinear***.

11.3.1 Colinearity of Gene and Protein

For genes and proteins, colinearity just means that the length of a DNA sequence in a gene is proportional to the length of the polypeptide encoded by the gene. The gene mapping experiments in *E. coli* already discussed certainly supported this hypothesis. Fig. 11.2 illustrates the concept of colinearity of gene and protein.

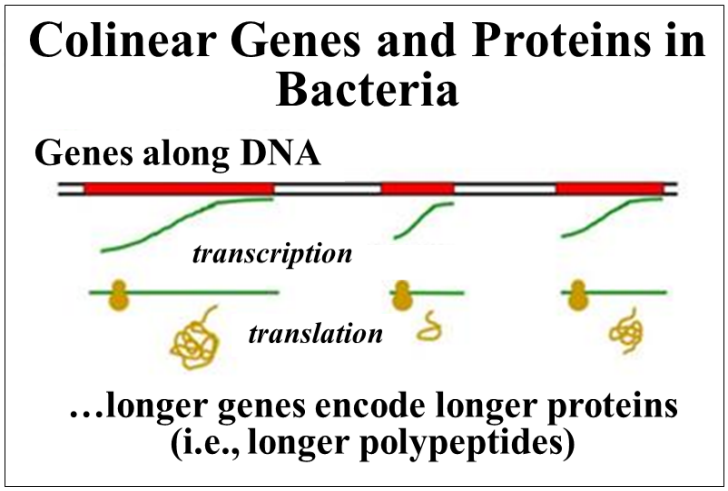


Fig. 11.2: Colinearity of genes and proteins (polypeptides) in bacteria.

If the genetic code is collinear with the polypeptides it encodes, then a one-base ***codon*** obviously does not work because such a code would only account for four amino acids. A two-base genetic code also doesn't work because it could only account for 16 (4^2) of the twenty amino acids found in proteins. However, three-nucleotide codons could code for a maximum of 4^3 or 64 amino acids, more than enough to encode the 20 amino acids. And of course, a 4-base code also works; it satisfies the expectation that genes and proteins are collinear, with the' advantage' that there would be 256 possible codons to choose from (i.e., 4^4 possibilities).

11.3.2 How is a Linear Genetic Code 'Read' to Account for All of An Organisms' Genes?

George Gamow (a Russian Physicist working at George Washington University) was the first to propose ***triplet codons*** to encode the twenty amino acids, the simplest hypothesis to account for the colinearity of gene and protein, and for encoding 20 amino acids. Once colinearity of gene and protein was accepted, a concern was whether there is enough DNA in an organism's genome to fit the all codons it needs to make all of its proteins? Assuming that genomes did not have a lot of extra DNA lying around, how might genetic information be compressed into short DNA sequences in a way that is consistent with the colinearity of gene and polypeptide. One idea assumed that there were 20 *meaningful* 3-base codons (one for each amino acid) and 44 *meaningless* codons, and that the *meaningful* codons in a gene (i.e., an mRNA) would be read and translated in an overlapping manner. A code where codons overlap by one base is shown in Fig. 11.3. You can figure out how compressed a gene could get with codons that overlapped by two bases.

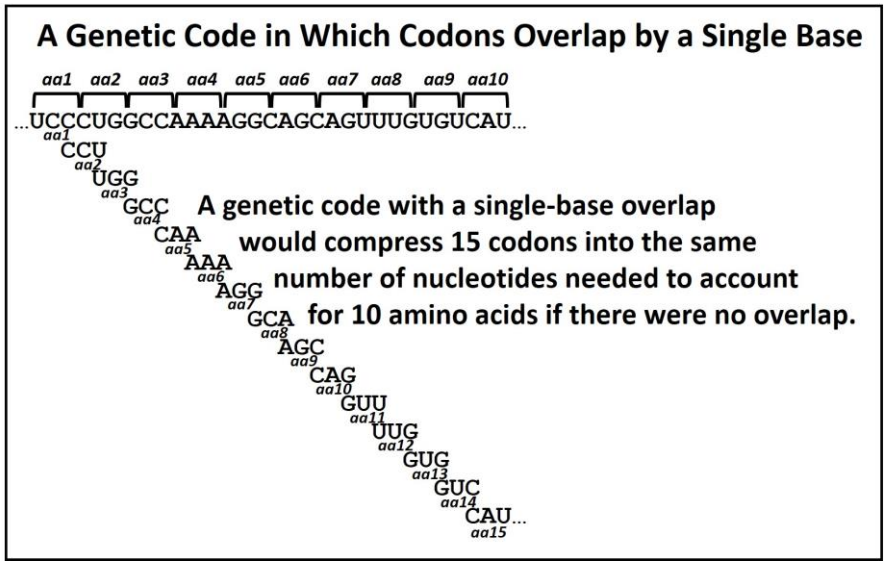


Fig. 11.3: A s*ingle-base overlapping genetic code* would get more genetic information in less DNA!

As attractive as an overlapping codon hypothesis was in achieving genomic economies, it sank of its own weight almost as soon as it was floated! If you look carefully at the example above, you can see that each succeeding amino acid would

have to start with a specific base. A look back at the table of 64 triplet codons quickly shows that only one of 16 amino acids, those that begin with a **C** can follow the first one in the illustration. Based on the amino acid sequences already accumulating in the literature, it was clear that virtually any amino acid could follow another in a polypeptide. Therefore, overlapping genetic codes are untenable. The genetic code must be non-overlapping!

Sidney Brenner and Frances Crick performed elegant experiments that directly demonstrated the non-overlapping genetic code. They showed that bacteria with a single base deletion in the coding region of a gene failed to make the expected protein. Likewise, deleting two bases from the gene. On the other hand, bacteria containing a mutant version of the gene in which three bases were deleted were able to make the protein. But the protein it made was slightly less active than bacteria with genes with no deletions.

The next issue was whether there were only 20 ***meaningful*** codons and 44 ***meaningless*** ones. If only 20 triplets actually encoded amino acids, how would the translation machinery recognize the correct 20 codons to translate? What would prevent the translational machinery from 'reading the wrong' triplets, i.e., reading an mRNA *out of phase*? If for example, the translation machinery began reading an mRNA from the second or third bases of a codon, wouldn't it likely encounter a meaningless 3-base sequence in short order?

One speculation was that the code was *punctuated.* That is, perhaps there were chemical equivalents of commas between the meaningful triplets. The commas would be of course, additional nucleotides. In such a punctuated code, the translation machinery would recognize the 'commas' and would not translate *any* meaningless 3-base triplet, avoiding out-of-phase translation attempts. Of course, a code with *nucleotide commas* would increase the amount of DNA needed to specify a polypeptide by a third!

So, Crick proposed the *Commaless Genetic Code*. He divided the 64 triplets into 20 *meaningful* codons that encoded the amino acids, and 44 *meaningless* ones that did not. The result was such that when the 20 *meaningful* codons are placed in any order, any of the triplets read in overlap would be among the 44 *meaningless* codons. In fact, he could arrange several different sets of 20 and 44 triplets with this property! Crick had cleverly demonstrated how to read the triplets in correct sequence without nucleotide commas.

202 Speculations About a Triplet Code

As we know now, the genetic code is indeed *commaless*… but not in the sense that Crick had envisioned. What's more, thanks to the experiments described next, we know that ribosomes read the correct codons in the right order because they know exactly where to start reading the mRNA!

11.4 Breaking the Genetic Code

Breaking the code began when Marshall Nirenberg and Heinrich J. Matthaei decoded the first triplet. They fractionated *E. coli* (Fig. 11.4) and then identified which fractions had to be added back together in order to get polypeptide synthesis in a test tube (***in vitro* translation**).

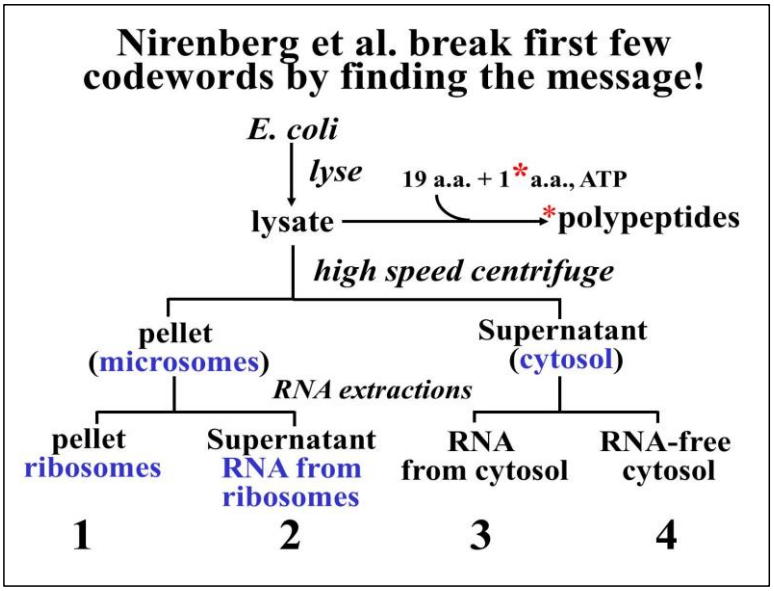


Fig. 11.4: Nirenberg et al.'s fractionation of bacterial cell RNAs.

Check out the original work in the classic paper by Nirenberg MW and Matthaei JH [(1961) *The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribo-nucleotides. Proc. Natl. Acad. Sci. USA 47:1588-1602*]. The various cell fractions isolated by this protocol were added back together along with amino acids (one of which was radioactive) and ATP as an energy source. After a short incubation, Nirenberg and his coworkers looked for the presence of high molecular weight radioactive proteins as evidence of cell-free protein synthesis.

They found that all four final sub-fractions (1-4 above) must be added together to make radioactive proteins in the test tube. One of the essential cell fractions consisted of RNA that had been gently extracted from ribosome (fraction 2 in the illustration). Reasoning that this RNA might be mRNA, they substituted a synthetic poly(U) preparation for this fraction in their cell-free protein synthesizing mix, expecting poly(U) to encode a simple repeating amino acid. Nirenberg and Matthaei set up 20 reaction tubes, with a different amino acid in each…, and made only poly-phenylalanine (Fig.11.5).

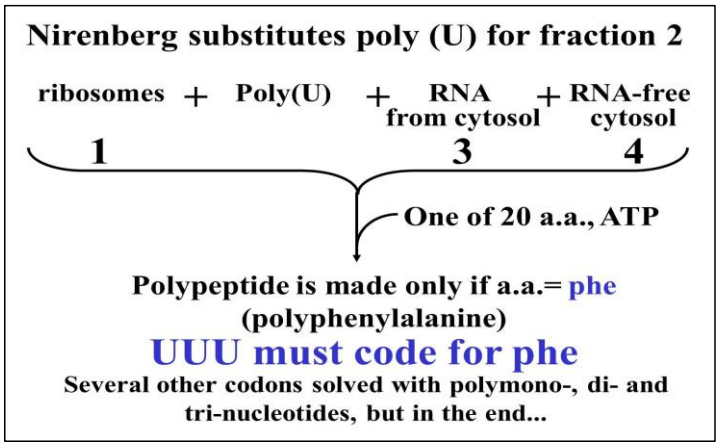


Fig. 11.5: Nirenberg et al.'s reconstitution of fractionated bacterial cell RNAs.

So, the triplet codon UUU means *phenylalanine*. When other polynucleotides were synthesized by G. Khorana, poly(A) and poly(C) were shown in quick succession to make poly-lysine and poly-proline in this experimental protocol. Thus, AAA and CCC must encode *lysine* and *proline* respectively. With a bit more difficulty and ingenuity, poly di- and tri-nucleotides used in the cell free system were able to decipher additional codons.

203 Deciphering the First Codon

M. W. Nirenberg, H. G. Khorana and R. W. Holley shared the 1968 Nobel Prize in Physiology or Medicine for their contributions to our understanding of protein synthesis. Deciphering the rest of the genetic code was based on Crick's realization that chemically, amino acids have no attraction for either DNA or RNA (or triplets thereof). Instead, he predicted the existence of an ***adaptor molecule*** that would contain nucleic acid and amino acid information *on the same molecule*. Today we recognize this molecule as **tRNA**, the genetic *decoding device*.

Nirenberg and Philip Leder designed the experiment that pretty much *broke* the rest of the genetic code. They did this by adding individual amino acids to separate test tubes containing tRNAs, in effect causing the synthesis of specific aminoacyl-tRNAs. They then mixed their amino acid-bound tRNAs with isolated ribosomes and synthetic triplets. Since they had already shown that synthetic 3-nucleotide fragments would bind to ribosomes, they hypothesized that triplet-bound ribosomes would in turn, bind appropriate amino acid-bound tRNAs. Fig. 11.6 summarizes their experiment.

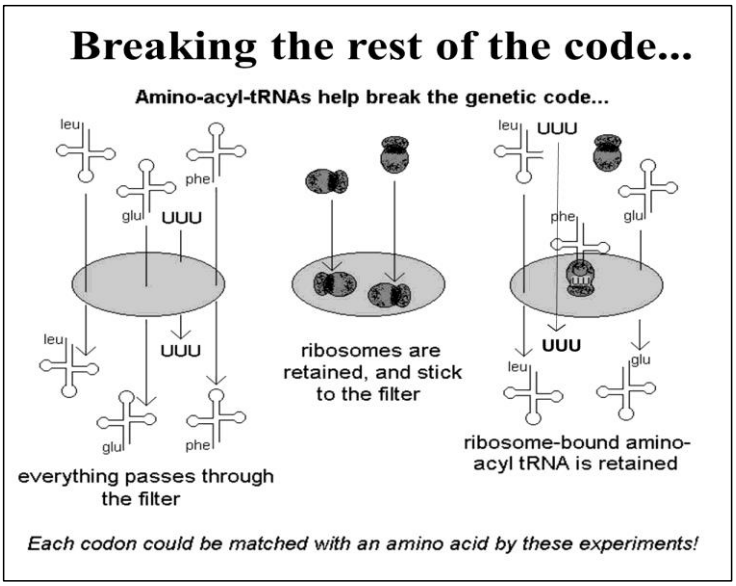


Fig. 11.6: Nirenberg and Leder's experiment that led to breaking the entire genetic code.

Various combinations of tRNA, ribosomes and aminoacyl-tRNAs were placed over a filter. Nirenberg and Leder knew that aminoacyl-tRNAs alone passed through the filter and that ribosomes did not. They predicted then, that triplets would associate with the ribosomes, and further, that this complex would bind the tRNA with the amino acid encoded by the bound triplet. This 3-part complex would also be retained by the filter, allowing the identification of the amino acid retained on the filter, and therefore the triplet code-word that had enabled binding the amino acid to the ribosome. Once deciphered, it was found that 61 of the codons specify amino acids, clearly making the code ***degenerate***.

204 Deciphering all 64 Triplet Codons

After the code was largely deciphered, Robert Holley actually sequenced a yeast tRNA. This first successful sequencing of a nucleic acid was possible because the RNA was short and contained several modified bases that facilitated the sequencing chemistry. Holley found the amino acid alanine at one end of his tRNA and he found one of the anticodons for an alanine codon roughly in the middle of the tRNA sequence. From regions of internal sequence complementarity, Holley predicted that this (and other) tRNAs would fold and assume a *stem-loop*, or *cloverleaf* structure with a central ***anticodon loop***.

Fig. 11.7 shows the stem-loop structure of a phenylalanine tRNA (left) and a subsequent computer-generated with a now familiar "L"-shaped molecular structure (right). Note the ***amino acid attachment site*** at the 3'-end at the top of the molecule, and the *anticodon loop* at the other, bottom 'end'.

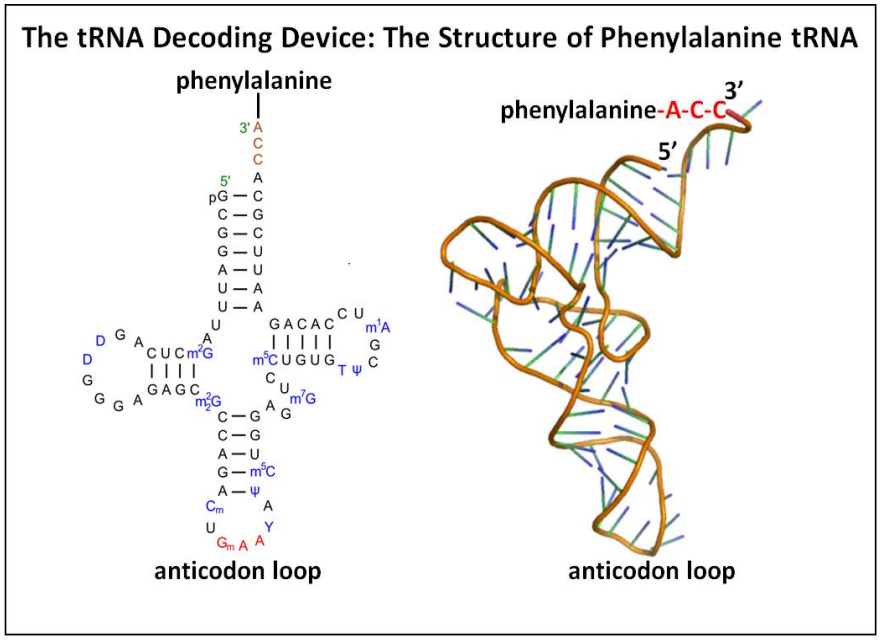


Fig. 11.7: Cloverleaf (LEFT) and computer-generated 3D (RIGHT) structures of a phenylalanyl- tRNA.

205-2 tRNA Structure and Base Modifications

After a brief overview of translation, we'll break this process down into its 3 steps and see how aminoacyl-tRNAs function in the initiation and elongation steps of translation, as well as the special role of an *initiator* tRNA.

11.5 Translation

Like any polymerization in a cell, translation occurs in three steps: ***initiation*** brings a ribosome, mRNA and an *initiator* tRNA together to form an initiation complex. ***Elongation*** is the successive addition of amino acids to a growing polypeptide. ***Termination*** is signaled by sequences (one of the stop codons) in the mRNA and protein ***termination factors*** that interrupt elongation and release a finished polypeptide. The events of translation occur at specific **A**, **P** and **E** sites on the ribosome (Fig. 11.8).

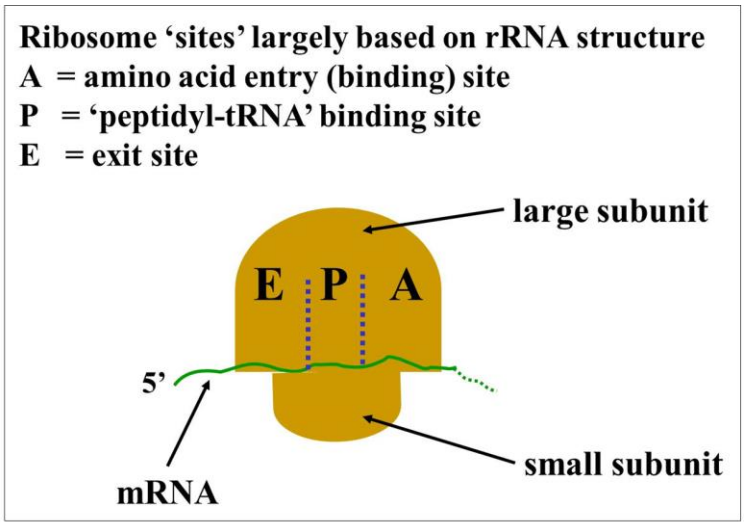


Fig. 11.8: A, P and E sites, mainly on the large ribosomal subunit, involved in mRNA translation.

11.5.1 Even Before Initiation - Making Aminoacyl-tRNAs

Translation is perhaps the most energy-intensive job a cell must do, beginning with the attachment of amino acids to their tRNAs. The basic amino-acylation reaction is the same for all amino acids. A specific ***aminoacyl-tRNA synthase*** attaches each tRNA to (i.e., ***charges***) an appropriate amino acid. Charging tRNAs itself proceeds in three steps and requires ATP (Fig. 11.9 below).

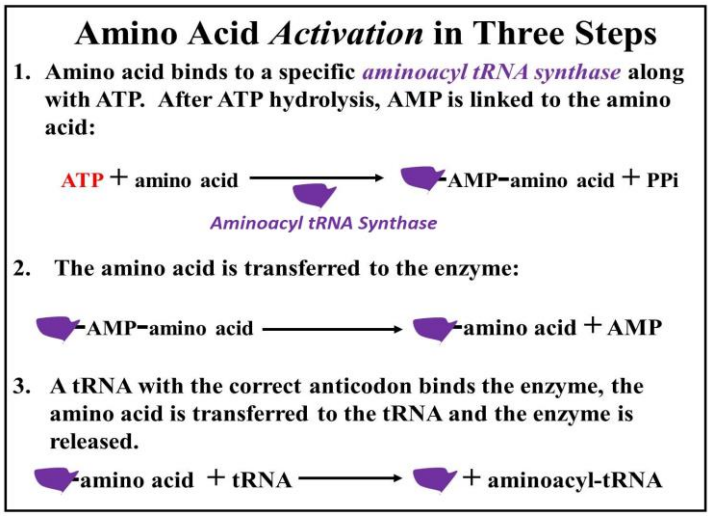


Fig. 11.9: Three steps of amino acid *activation* (tRNA acylation) require ATP hydrolysis.

In the first step, ATP and an appropriate amino acid bind to the aminoacyl-tRNA synthase. ATP is hydrolyzed, releasing a pyrophosphate (PPi) and leaving an enzyme-AMP-amino acid complex. Next, the amino acid is transferred to the enzyme, releasing the AMP. Finally, the tRNA binds to the enzyme, the amino acid is transferred to the tRNA and the intact enzyme is regenerated and released. The charged tRNA is now ready to use in translation.

Several studies had already established that polypeptides are synthesized from their amino (N-) terminal end to their carboxyl (C-) terminal end. When it became possible to determine the amino acid sequences of polypeptides, it turned out that around 40% of *E. coli* proteins had an N-terminal methionine. This suggested that *all* proteins begin with a methionine, but that the methionine was subsequently removed in a ***post-translation processing*** step. It also turned out that, even though there is only one codon for methionine, two different tRNAs for methionine could be isolated. One of the tRNAs was bound to a methionine modified by *formylation*, called **formyl-methionine-tRNA$_{fmet}$** (or ***fmet-tRNAf*** for short). The other was **methionine-tRNA$_{met}$** (***met-tRNA$_{met}$*** for short), charged with an unmodified methionine. ***tRNA$_{met}$*** and ***tRNAf*** each have an anticodon to AUG, the only codon for methionine. But they have different base sequences encoded by different tRNA genes. *tRNA$_{met}$* is used to insert methionine in the middle of a polypeptide. *tRNAf* is the *initiator* tRNA and is only used to start new polypeptides with formylmethionine. In prokaryotes, the amino group on

methionine on *met-tRNAf* is *formylated* by a ***formylating enzyme*** to make the *fmet-tRNAf*. This enzyme does not recognize methionine on *met-tRNA$_{met}$*. Fig. 11.10 (below) shows the structures of methionine (met) and formylated methionine (fmet).

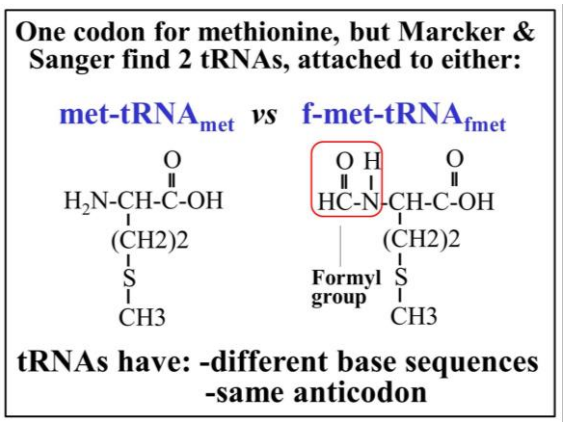


Fig. 11.10: Discovery of *met-tRNA* and *formyl-met-tRNA*, despite only one codon for methionine.

In *E. coli*, a ***formylase*** enzyme removes the formyl group from all N-terminal formyl methionines at some point after translation has begun. As we noted, the methionines themselves (and sometimes more N-terminal amino acids) are also removed from about 60% of *E. coli* polypeptides. Eukaryotes have inherited both the initiator *tRNAf* and the *tRNAmet*, using only *met-tRNAf* during initiation…, but the latter is never actually formylated in the first place! What's more, methionine is absent from the N-terminus of virtually all mature eukaryotic polypeptides.

Early in evolution, the need for an initiator tRNA must have ensured a correct starting point for translation on an mRNA and therefore growth of a polypeptide from one end to the other, that is, from its N- to its C-terminus. At one time, formylation of the N-terminal methionine may have served to block accidental addition of amino acids to the N-terminus of a polypeptide. Today, formylation seems to be a kind of *molecular appendix* in bacteria. In eukaryotes at least, evolution has selected other features to replace actual formylation as the protector of the N-terminus of polypeptides.

11.5.2 Translation *Initiation*

Now that we have charged the tRNAs, we can look more closely at the three steps of translation. Understanding translation initiation began with cell fractionation of *E. coli,* the purification of molecular components required for cell-free (*in vitro*) protein

synthesis and finally, *reconstitution* experiments. Cellular RNA was purified and the 30S ribosomal subunit was separated from a ribosomal extract. These were then added to initiation factor proteins purified from the bacterial cells. Reconstitution experiments revealed that when added to each other in the correct order, the separated fractions, along with the purified initiation factors, formed a stable 30S ribosomal subunit-mRNA complex. We now know that a ***Shine-Delgarno*** sequence in mRNAs forms H-bonds with its complementary sequence in the **16S rRNA** in 30S ribosomal subunit. The Shine-Delgarno sequence is a short nucleotide sequence in the ***5' untranslated region*** (***5'-UTR***) of the mRNA, just upstream of the initiator AUG codon. The stable binding of the small ribosomal subunit to the mRNA requires the participation of initiation factors **IF1** and **IF3**. In this event, IF1 and IF3 as well as the mRNA are bound to the 30S ribosomal subunit (shown in Fig. 11.11).

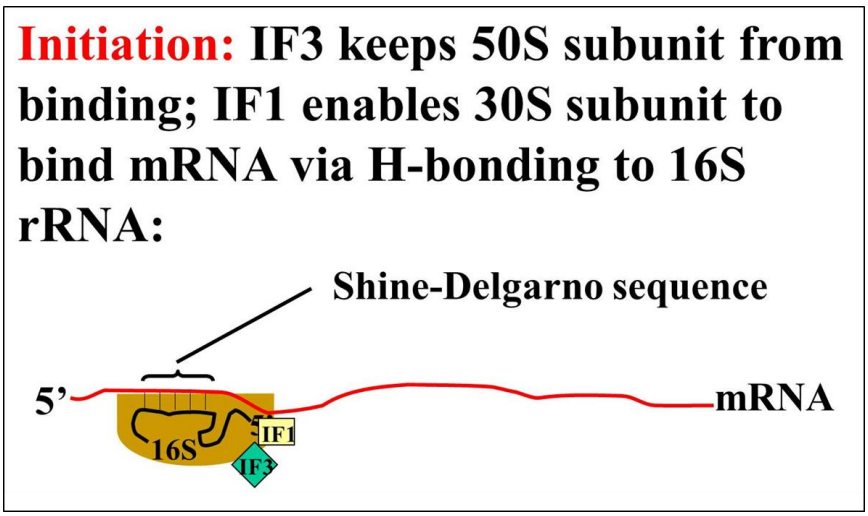


Fig. 11.11: Bacterial **T**ranslation Initiation: association of the small ribosomal subunit with mRNA requires two initiation factors (IF1 and IF3). The association is by complementary H-bonding between a region of the 16S rRNA and the Shine-Delgarno sequence near the 5' end of the mRNA.

206 Translation Initiation: mRNA Associates with 30S Ribosomal Subunit

Next, with the help of GTP and another initiation factor (**IF2**), the initiator *fmet-tRNAf* recognizes and binds to the AUG start codon found in all mRNAs. Some call the resulting structure (Fig.11.12 below) the ***Initiation Complex***, which includes the 30S ribosomal subunit, IFs 1, 2 and 3, and the *fmet-tRNAf*.

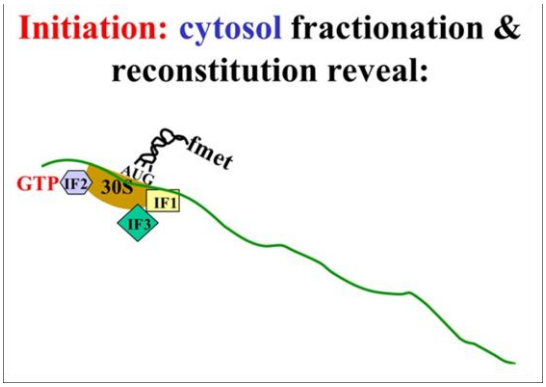


Fig. 11.12: Bacterial Translation Initiation: *GTP-bound IF2* enables binding of the first aminoacyl tRNA (the initiator *fmet-tRNA*$_{fmet}$) to the 30S ribosomal subunit/mRNA complex to create a bacterial *initiation complex*.

207 Initiation Complex Formation

In the last step of initiation, the large ribosomal subunit binds to this complex. IFs 1, 2 and 3 disassociate from the ribosome and the initiator *fmet-tRNA*$_{fmet}$ ends up in the *P* site of the ribosome. Some prefer to call the structure formed at this point the initiation complex (Fig. 11.13).

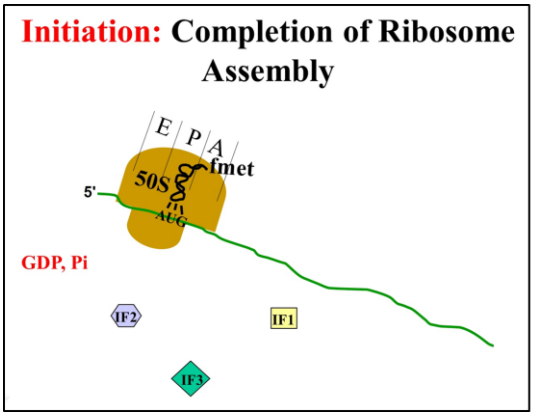


Fig. 11.13: Bacterial Translation Initiation: **a**ddition of the large ribosomal subunit to the *initiation complex, hydrolysis of GTP and* dissociation of initiation factors completes assembly of the ribosome on an mRNA.

Initiation can happen multiple times on a single mRNA, forming the polyribosome, or polysome (already described in Chapter 1). Each of the complexes formed above will engage in the elongation of a polypeptide described next.

208 Adding the Large Ribosomal Subunit

11.5.3 Translation Elongation

Elongation is a sequence of protein factor-mediated condensation reactions and ribosome movements along an mRNA. As you will see, polypeptide elongation proceeds in 3 discrete stages and requires a considerable input of free energy.

11.5.3.a Translation Elongation-1

As illustrated in Fig. 11.14, elongation starts with the entry of the second aminoacyl-tRNA (***aa2-tRNAaa2***) into the A site of the ribosome, a step that requires free energy. The energy is supplied by the hydrolysis of GTP bound *elongation factor* 2 (***EF2-GTP***). The *aa2-tRNAaa2* enters the ribosome A site based on codon-anticodon interaction, and the GDP dissociates from EF2.

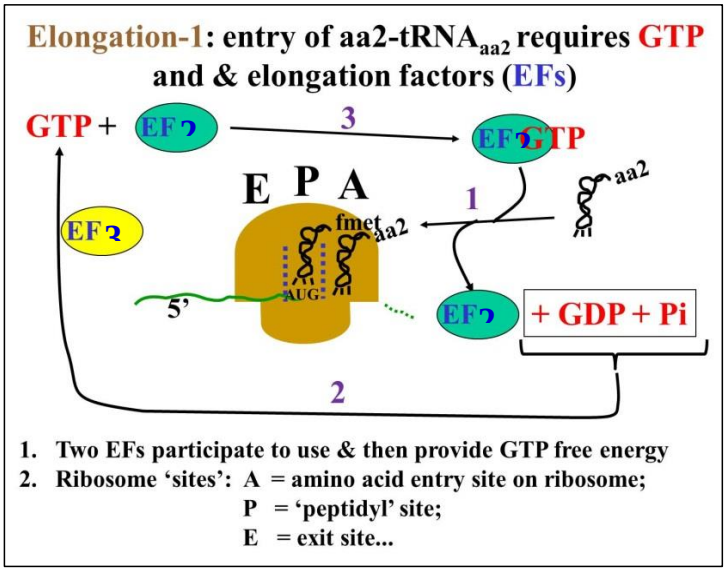


Fig. 11.14: Bacterial Translation Elongation: EF2-GTP and EF3 facilitate entry of the second aminoacyl tRNA into the A site of the ribosome to begin elongation; GTP is hydrolyzed.

To keep elongation moving along, *elongation factor* (EF3) re-phosphorylates the GDP to GTP, which can re-associate with free EF2.

209 Elongation: Elongation Factors and GTP

11.5.3.b Translation Elongation-2

Peptidyl transferase, a ***ribozyme*** component of the ribosome itself, links the incoming amino acid to a growing chain in a condensation reaction. Fig. 11.15 illustrates the result of peptidyl transferase action.

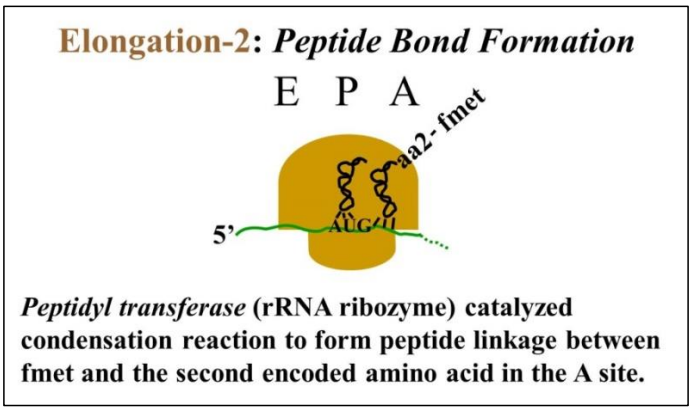


Fig. 11.15: Bacterial Translation Elongation: The *peptidyl transferase ribozyme* (part of the ribosome) catalyzes peptide bond formation between the fmet on its $tRNA_f$ and the second amino acid (aa2). The fmet is transferred from its $tRNA_f$ as it condenses with an aa2 still linked to its $tRNA_{aa2}$.

In this reaction, the fmet is transferred from the initiator *tRNAf* in the *P* site to *aa2-tRNAaa2* in the *A* site, forming a peptide linkage with aa2.

210 Elongation: A Ribozyme Catalyzes Peptide Linkage Formation

11.5.3.c Translation Elongation 3

Translocase catalyzes GTP hydrolysis as the ribosome moves (translocates) along the mRNA. After translocation, the next mRNA codon shows up in the *A* site of the ribosome and the first tRNA (in this example, *tRNAf*) ends up on the *E* site of the ribosome.

The movement of the ribosome along the mRNA is illustrated in Fig. 11.16 below.

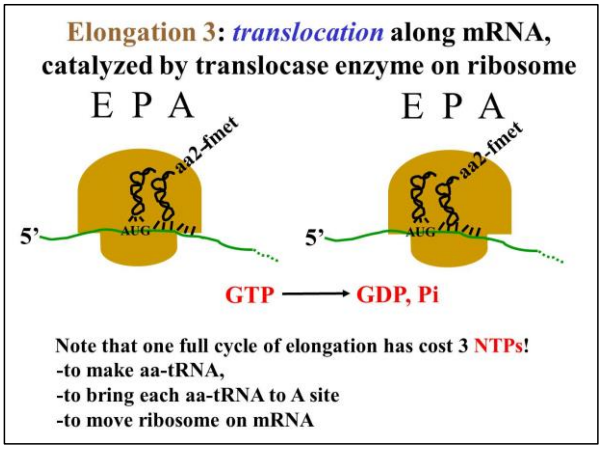


Fig. 11.16: Bacterial Translation Elongation: Translocase (on the ribosome) catalyzes movement of the ribosome along the mRNA, exposing the next codon in the A site with its attached peptidyl tRNA now in the peptidyl (P) site.

The *tRNAf*, no longer attached to an amino acid, will exit the E site as the next (3rd) aa-tRNA enters the empty A site based on a specific codon-anticodon interaction (assisted by elongation factors and powered by GTP hydrolysis) to begin another cycle of elongation. Note that in each cycle of elongation, an ATP is consumed to attach each amino acid to its tRNA, and two GTPs are hydrolyzed in the cycle itself. In other words, at the cost of three NTPs, protein synthesis is the most expensive polymer synthesis reaction in cells!

211 Elongation: Translocase Moves Ribosome Along mRNA

212 Adding the Third Amino Acid

213 Big Translation Energy Costs

As polypeptides elongate, they emerge from a groove in the large ribosomal subunit. As noted, a formylase enzyme in *E. coli* cytoplasm removes the formyl group from the exposed initiation fmet from all growing polypeptides. While about 40% of *E. coli* polypeptides still begin with methionine, specific proteases catalyze the hydrolytic removal of the amino-terminal methionine (and sometimes even more amino acids)

from the other 60% of polypeptides. The removal of the formyl group and one or more N-terminal amino acids from new polypeptides are examples of post-translational processing.

214 The Fates of fMet and Met; Cases of Post-Translational Processing

11.5.4 Translation Termination

Translation of an mRNA by a ribosome ends when translocation exposes one of the three stop codons in the A site of the ribosome. Stop codons are situated some distance from the 3' end of an mRNA. The region between a stop codon to the end of the mRNA is called the ***3' untranslated region*** of the messenger RNA (***3'UTR***).

Since there is no aminoacyl-tRNA with an anticodon to the stop codons (UAA, UAG or UGA), the ribosome actually stalls and the translation slow-down is just long enough for a protein ***termination factor*** to enter the A site. This interaction causes release of the new polypeptide and the disassembly of the ribosomal subunits from the mRNA. The process requires energy from yet another GTP hydrolysis. After dissociation, ribosomal subunits can be reassembled with an mRNA for another round of protein synthesis. Fig 11.17 illustrates translation termination.

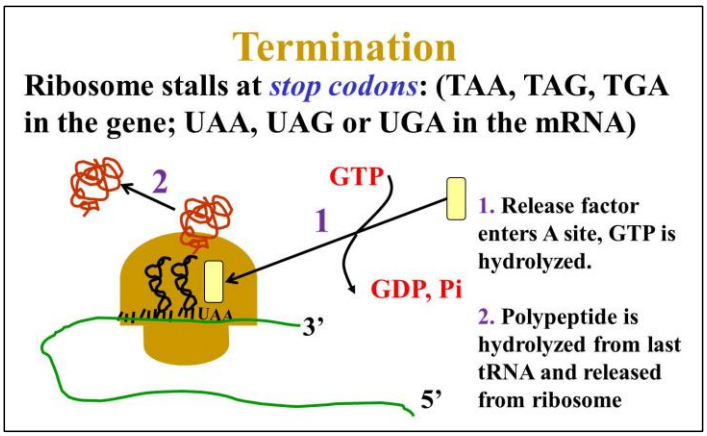


Fig. 11.17: : Bacterial Translation Termination: Translation stalls when the ribosome reaches a termination codon, GTP is hydrolyzed and a protein termination (*release*) *factor* enters the A site.

215-2 Translation Termination

We have seen some examples of post-translational processing (removal of formyl groups in *E. coli*, removal of the N-terminal methionine from most polypeptides, etc.) Most proteins, especially in eukaryotes, undergo one or more additional steps of post-translational processing before becoming biologically active. We will see examples in upcoming chapters.

11.6 How Can the Genetic Code be Degenerate and Accurate at the Same Time?

The A-T and G-C complementarity in DNA is known as standard Watson-Crick base pairing. Thinking about how a redundant, degenerate genetic code could work, Francis Crick realized that most often, the first and second bases base of codons for the same amino acid were fixed while the 3rd base was the most variable (with the obvious exceptions of codons for methionine and tryptophan!). In his *Wobble Hypothesis*, he proposed that the 3rd base in the tRNA anticodon could recognize more than one base in its codon in mRNA because it was able to shift (i.e., wobble) while the other two base pairings were fixed. According to Crick's hypothesis, the wobble would be due to a real freedom of movement, or flexibility of the 3rd base in the anticodon, allowing the formation thermodynamically stable non-Watson-Crick base pairs.

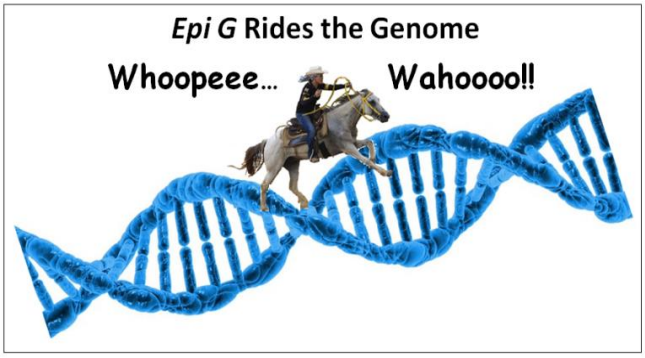
Some iText & VOP Key Words and Terms

64 codons	**genetic code**	**ribonucleoprotein**
adapter molecules	**initiation**	**ribosome**
amino terminus	**initiation complex**	**small ribosomal subunit**
aminoacyl tRNA	**initiation factors**	**start codon**
aminoacyl tRNA synthase	**initiator tRNA**	**stop codons**
amino acid attachment site	**large ribosomal subunit**	**termination**
anticodon	**meaningful codons**	**termination factor**
AUG	**mRNA, tRNA**	**translocation**
bacterial bound ribosomes	**nascent chains**	**triplets**
Carboxyl-terminus	**ochre, amber, opal**	**tRNA v. tRNAaa**
colinearity	**peptide linkage**	**UAG, UUA, UGA**
comma-less genetic code	**peptidyl transferase**	**universal genetic code**

degenerate genetic code	**polypeptide**	**UUU**
elongation	**polysome**	**Wobble Hypothesis**
free v. bound ribosomes	**reading phase**	

Chapter 12: Regulation of Transcription and Epigenetic Inheritance

Gene repression and induction (prokaryotes); Multiple transcription factors (eukaryotes); Regulatory elements in DNA; Memories of gene regulation (epigenetics)



12.1 Introduction

Cells regulate their metabolism in several ways. We have already seen that allosterically regulated enzymes monitor the cellular levels of metabolites. Recall that glycolytic intermediates rise and fall in cells based on cellular energy needs, binding to or dissociating from ***allosteric sites***. Allosteric enzymes respond to interaction with ***allosteric effectors*** with an increase or decrease in catalytic activity.

Cells can also control absolute levels of enzymes and other proteins by turning genes on and off, typically by controlling transcription. ***Transcription regulation*** usually starts with extracellular environmental signaling. The signals are chemicals in the air, in the water or in the case of multicellular organisms, in blood, lymph or other extracellular fluids. Bacterial and protist genes often respond to environmental ***toxins*** or fluctuating ***nutrient levels***. Familiar ***signal molecules*** in higher organisms include ***hormones*** released at the appropriate time in a sequential ***developmental program*** of gene expression, or in response to nutrient levels in body fluids.

Some signal molecules get into cells and binding to ***specific intracellular receptors*** to convey their instructions. Others bind to cell surface ***receptors*** that transduce their 'information' into intracellular molecular signals. When signaling leads to gene regulation,

responding cells ultimately produce ***transcription factors***. These in turn recognize and bind to specific *regulatory DNA sequences* associated with the genes that they control. DNA sequences that bind transcription factors are relatively short. They can be **proximal** (close) to the transcription start site of a gene, and/or in the case of eukaryotes, ***distal*** to (far from) it. We will see that binding some regulatory DNA sequences are ***enhancers***, turning on or increasing gene transcription. Others are ***silencers***, down-regulating, or suppressing transcription of a gene. Finally, DNA regulatory sequences are hidden behind a thicket of chromatin proteins in eukaryotes. When patterns of gene expression in cells change during development, chromatin is re-organized, cells differentiate, and new tissues and organs form. To this end, new patterns of gene expression and chromatin configuration in a cell must be remembered in its descendants.

In this chapter, we look at the path from cell recognition of a signal molecule to the interaction of regulatory proteins with DNA in both prokaryotic and eukaryotic cells. We also consider how eukaryotic cells *remember* instructions that alter chromatin configuration and patterns of gene expression, topics in the field of **epigenetics**.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast *transcription factors* and so-called *cis-acting elements.*
2. discuss the role of *DNA bending* in the regulation of gene expression.
3. explain the benefits of organizing bacterial genes into *operons, and why some* bacterial genes are not part of *operons.*
4. compare and contrast regulation of the *lac* and *trp* operons in *E. coli.*
5. define and describe *regulatory genes* and *structural genes* in *E. coli.*
6. discuss why a fourth gene was suspected in *lac operon* regulation.
7. distinguish between gene *repression* and *de-repression* and between *positive* and *negative* gene regulation, using real or hypothetical examples. For example, explain how it is possible to have repression by positive regulation.
8. draw and label all functional regions of prokaryotic and eukaryotic genes.
9. compare and contrast different mechanisms of gene regulation in eukaryotic cells.
10. describe the *transcription initiation complex* of a regulated gene in eukaryotes.
11. define and articulate differences between *gene expression* and *transcription regulation.*
12. define a gene.
13. distinguish between the roles of enhancers and other cis-acting elements in transcription regulation.
14. compare and contrast the *genome* and the *epigenome.*

12.2 Gene Regulation in Prokaryotes: the Lactose (*lac*) Operon

Many prokaryotic genes are organized in ***operons*** that are linked genes transcribed into a single mRNA encoding two or more proteins. Operons usually encode proteins with related functions. Regulating the activity of an operon (rather than multiple single genes encoding single proteins) allows better coordination of the synthesis of several proteins at once. In *E. coli*, the regulated ***lac operon*** encodes three enzymes involved in the metabolism of ***lactose*** (an alternative nutrient to glucose). Regulation of an operon (or of a single gene for that matter) can be by ***repression*** or by ***induction***. When a small metabolite in a cell binds to a regulatory ***repressor*** or ***inducer*** protein, the protein undergoes an allosteric change that allows it to bind to a regulatory DNA sequence… or to un-bind from the DNA.

We will see examples of such regulation in the ***lac*** and ***trp*** *operons.* Regulation of the *Lac* operon genes is an example of ***gene repression*** as well as ***induction***. We'll see that *Trp* (tryptophan) operon regulation is by ***gene repression***. In both operons, changes in levels of intracellular metabolites reflect the metabolic status of the cell, eliciting appropriate changes in gene transcription. Let's start with a look at *lac* operon regulation.

216 Overview of Prokaryotic Gene Regulation

The mRNA transcribed from the *lac operon* is simultaneously translated into three enzymes, as shown in Fig. 12.1.

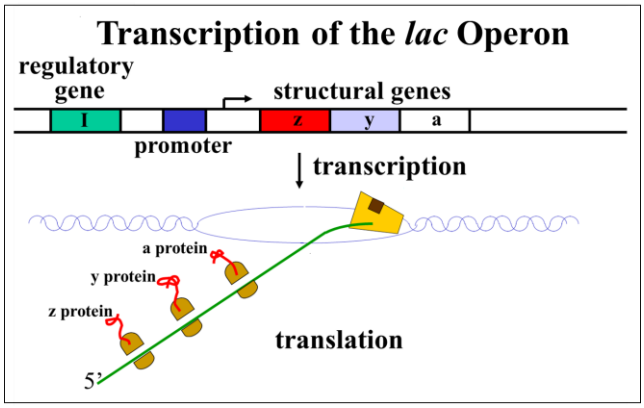


Fig. 12.1: Transcription of the *E. coli lac* operon produces a single mRNA encoding three polypeptides (nicknamed 'z', 'y' and 'a') which is translated into 3 enzymes involved in lactose metabolism.

12.2.1 Working Out Regulation of the Lactose (*lac*) Operon in *E. coli*

In the animal digestive tract (including ours), genes of the *E. coli* ***lac operon*** regulate the use of *lactose* as an alternative nutrient to glucose. Think cheese instead of chocolate! The operon consists of *lacZ*, *lacY*, and *lacA* genes that are called ***structural genes.*** By definition, structural genes encode proteins that participate in cell structure and metabolic function. As already noted, the *lac operon* is transcribed into an mRNA encoding the Z, Y and A proteins. Let's zoom in on a single *lac* operon in Fig. 12.2.

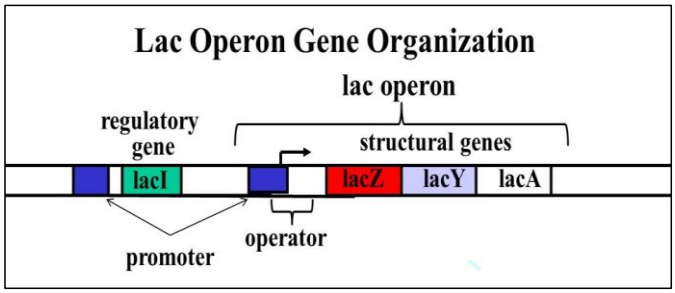


Fig. 12.2: The *lacZ*, *lacY* and *lacA* genes of the *lac* operon are all controlled by the single promoter to the left of the *lacZ* gene. A regulatory gene (lacI) with its own promoter lies further to the left of the *lac* operon.

The *lacZ* gene encodes ***β-galactosidase***, the enzyme that breaks lactose, a disaccharide, into *galactose* and glucose. The *lacY* gene encodes lactose ***permease***, a membrane protein that facilitates lactose entry into the cells. The role of the *lacA* gene product, ***transacetylase***, involved in lactose energy metabolism, is not well understood. The ***I gene*** to the left of the *lac Z* gene is a ***regulatory gene*** (to distinguish it from *structural genes*). Regulatory genes encode proteins that interact with regulatory DNA sequences associated with a gene to control transcription. As we describe next, the ***operator*** sequence separating the I and Z genes is a transcription regulatory DNA sequence.

The *E. coli lac* operon is usually silent (repressed) because these cells prefer glucose as an energy and carbon source. In the presence of sufficient glucose, a ***repressor protein*** (the I gene product) is bound to the ***operator***, blocking transcription of the *lac* operon. Even if lactose is available, cells will not be able to use it as an alternative energy and carbon source when glucose levels are adequate. However, when glucose levels drop, the *lac* operon is active and the three enzyme products are translated. We will see how *lac* operon transcription by both ***derepression*** and direct

induction can lead to maximal transcription of the *lac* genes only when necessary (i.e., in the presence of lactose *and* absence of glucose). Let's look at some of the classic experiments that led to our understanding of *E. coli* gene regulation in general, and of the *lac* operon in particular.

In the late 1950s and early 1960s, Francois Jacob and Jacques Monod were studying the use of different sugars as carbon sources by *E. coli*. They knew that *wild type E. coli* would ***not*** make the β-galactosidase, β-galactoside permease or β-galactoside transacetylase proteins when grown on glucose. Of course, they also knew that the cells would switch to lactose for growth and reproduction if they were deprived of glucose! They then searched for and isolated different *E. coli* mutants that could not grow on lactose, even when there was no glucose in the growth medium. Here are some of the mutants they studied:

1. One mutant failed to make active β-galactosidase but dis make permease.
2. One mutant failed to make active permease but made normal amounts of β-galactosidase.
3. Another mutant failed to make transacetylase…, but could still metabolize lactose in the absence of glucose. Hence the uncertainty of its role in lactose metabolism.
4. Curiously, one mutant strain failed to make any of the three enzymes!

Since double mutants are very rare and triple mutants even rarer, Jacob and Monod inferred that the activation of all three genes in the presence of lactose were controlled together in some way. In fact, it was this discovery that defined the operon as a set of genes transcribed as a single mRNA, whose expression could therefore be effectively coordinated. They later characterized the repressor protein produced by the *lacI* gene. Jacob, Monod and Andre Lwoff shared the Nobel Prize in Medicine in 1965 for their work on bacterial gene regulation. We now know that there are several layers of *lac* operon regulation. *Negative* or *positive* regulation of the *operon* depends on two regulatory proteins to control the rate of lactose metabolism.

12.2.2 Negative Regulation of the *lac* Operon by Lactose

Repression of *lac* operon activity involves a repressor protein that must be removed for gene expression to occur. The repressor protein product of the ***I gene*** is always made and present in *E. coli* cells. *I gene* expression is therefore defined as being ***constitutive***, meaning unregulated. In the absence of lactose in the growth medium, the repressor protein binds tightly to the operator DNA. While ***RNA polymerase*** is bound to the promoter and ready to transcribe the operon, the presence of the repressor protein bound to the operator sequence between the RNA polymerase and the Z gene transcription start site physically blocks forward movement of the polymerase. Under these conditions, little or no transcript is made.

Fig 12.3 shows the *lac* repressor protein in its repressed state.

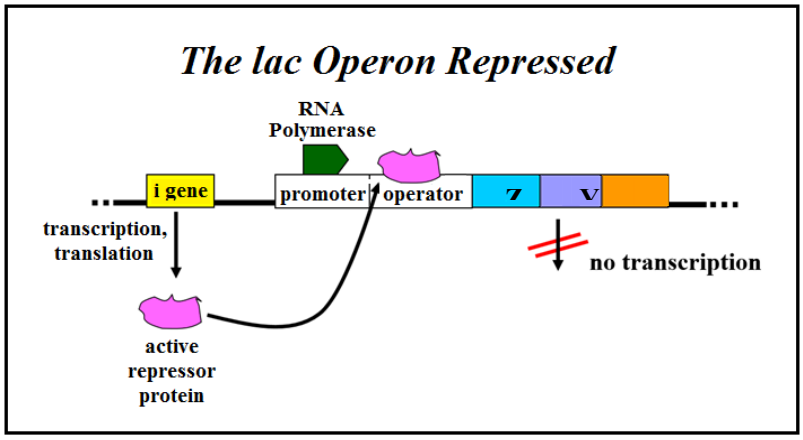


Fig. 12.3: *Negative regulation* of the *lac* operon: The *I gene* transcribes a *repressor* protein. When bound to the operator sequence (between the promoter and beginning of the Z gene), RNA polymerase is blocked and the operon cannot be transcribed. Transcription will require derepression (i.e., removal of the repressor protein).

If cells are grown in the presence of lactose, the lactose entering the cells is converted to ***allolactose***. Allolactose in turn, binds to the repressor sitting on the operator DNA to form a 2-part complex, as shown in Fig. 12.4.

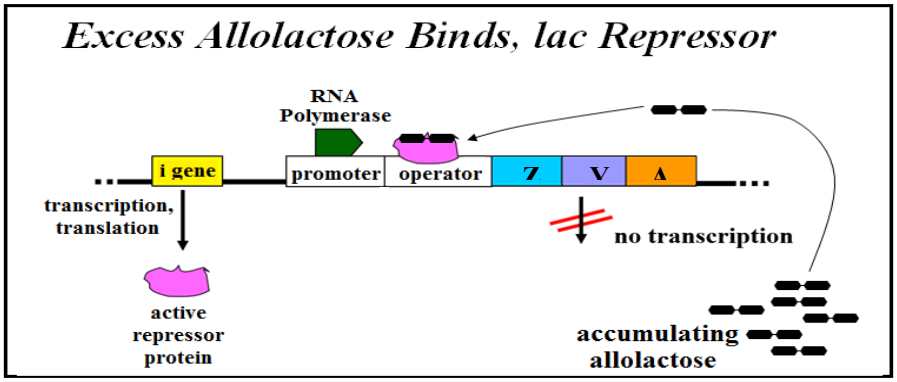


Fig. 12.4: *Negative regulation* of the *lac* operon: When present, lactose enters cells and is converted to *allolactose*. Accumulating allolactose binds to the *lac* repressor protein.

The allosterically altered repressor dissociates from the operator and RNA polymerase can transcribe the *lac* operon genes (Fig. 12.5).

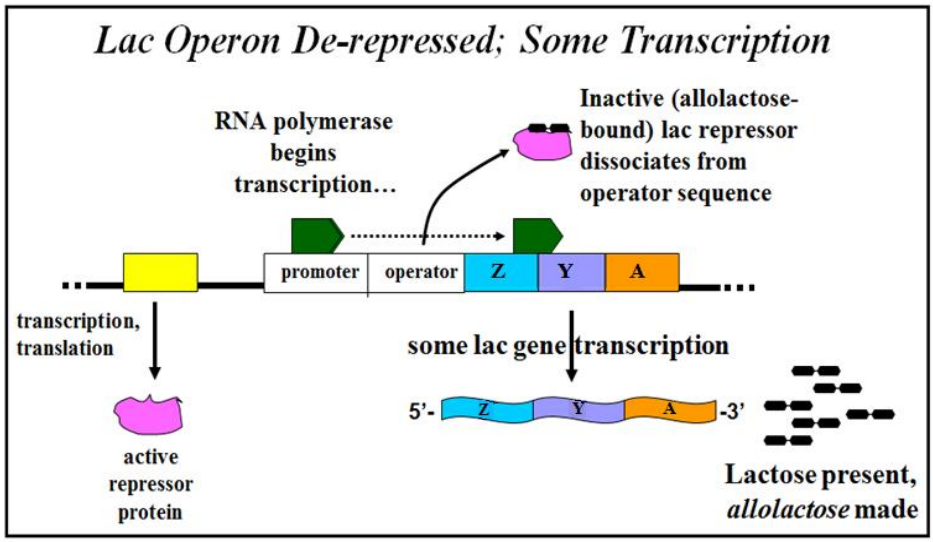


Fig. 12.5: *Negative regulation* of the *lac* operon: Allolactose binding causes allosteric change in the *lac* repressor that then dissociates from the operator sequence, allowing RNA polymerase to transcribe the operon.

12.2.3 Positive Regulation of the *Lac* Operon; Induction by Catabolite Activation

The second control mechanism regulating *lac* operon expression is mediated by CAP (cAMP-bound ***catabolite activator protein*** or cAMP receptor protein). When glucose is available, cellular levels of cAMP are low in the cells and CAP is in an inactive conformation. On the other hand, if glucose levels are low, cAMP levels rise and bind to the CAP, activating it. If lactose levels are also low, the cAMP-bound CAP will have no effect. If lactose is present and glucose levels are low, then allolactose binds the *lac* repressor causing it to dissociate from the operator region. Under these conditions, the cAMP-bound CAP can bind to the operator in lieu of the repressor protein. In this case, rather than blocking RNA polymerase, the activated cAMP-bound CAP induces even more efficient *lac* operon transcription. The result is synthesis of higher levels of *lac* enzymes that facilitate efficient cellular use of lactose as an alternative to glucose as an energy source. Fig. 12.6 shows maximal *lac* operon *activation* in high lactose and low glucose.

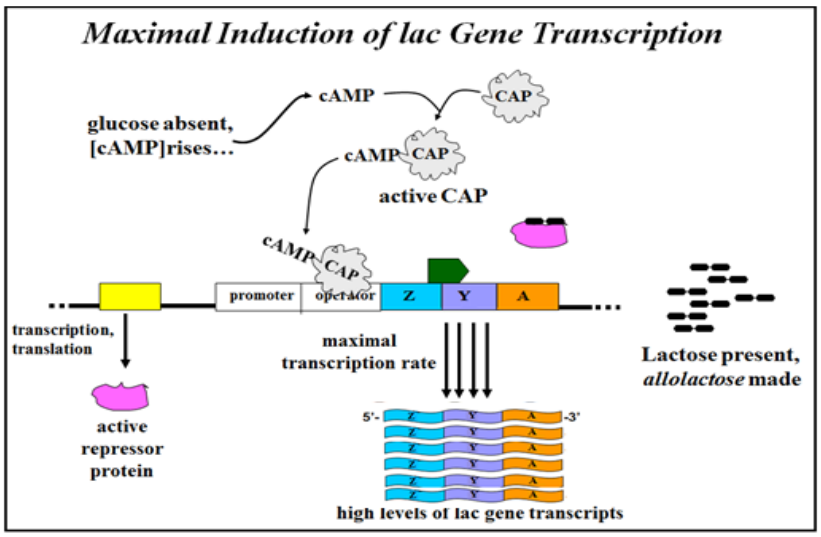


Fig. 12.6: *Positive regulation* of the *lac* operon: If lactose is present but glucose is unavailable, the operon is *derepressed* AND *induced*. Increased cAMP binds the *Catabolite Activator Protein* (*CAP*), which binds to the *lac* operator, inducing) *lac* gene transcription The *lac* operon is maximally transcribed under these conditions.

217 Regulation of the *lac* Operon

cAMP-bound CAP is a transcription ***inducer***. It forces DNA in the promoter-operator region to bend (Fig. 12.7).

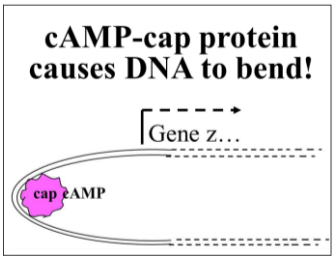


Fig. 12.7: *Positive regulation* of the *lac* operon: cAMP-CAP-bound operator bends the double helix. This loosens the H- bonds between the bases, making transcription factor access to template strands easier.

Binding of CAMP-CAP to the double helix loosens H-bonds between the strands making it easier for RNA polymerase to find and bind the promoter…, and for transcription to begin.

In recent years, additional layers of *lac* operon regulation have been uncovered. In one case, the ability of *lac permease* to transport lactose across the cell membrane is regulated. In another, additional operator sequences have been discovered to interact with a multimeric repressor to control *lac* gene expression. We'll consider *Inducer Exclusion* first.

12.2.4 *Lac* Operon Regulation by Inducer Exclusion

When glucose levels are high (even in the presence of lactose), phosphate is consumed in order to phosphorylate glycolytic intermediates. This keeps cytoplasmic phosphate levels low. Under these conditions, an unphosphorylated elongation factor ($EIIA^{Glc}$) binds to the *lactose permease* in the cell membrane, preventing the enzyme from bringing lactose into the cell. The role of phosphorylated and unphosphorylated $EIIA^{Glc}$ in regulating the *lac* operon is shown in Fig. 12.8.

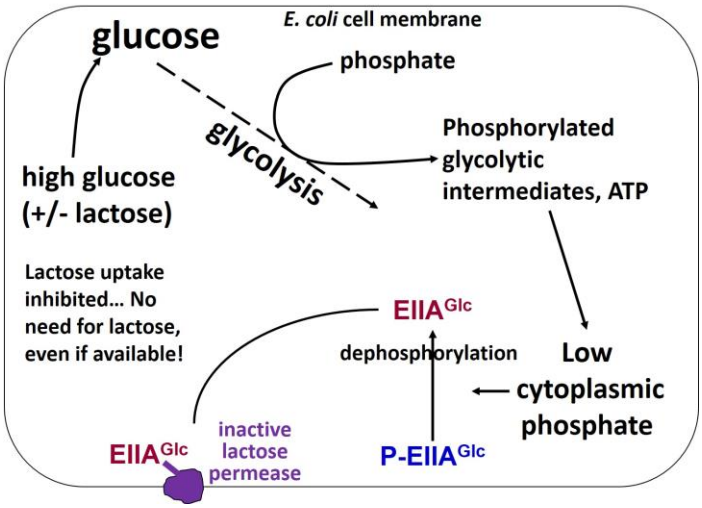


Fig. 12.8: *lac* operon regulation by *inducer exclusion*: High glucose levels accelerate glycolysis, depleting cellular phosphate. Low phosphate leads to the dephosphorylation of EIIAGlc, among other proteins. Dephosphorylated EIIAGlc is an inhibitor of *lactose permease* (i.e., the *lac*A protein). Without active permease, lactose can't get into cells, allolactose cannot be made, and the *lac* gene cannot be transcribed.

High glucose levels block lactose entry into the cells, effectively preventing allolactose formation and derepression of the *lac* operon. Inducer exclusion is thus a logical way for the cells to handle an abundance of glucose, whether or not lactose is present. On the other hand, if glucose levels are low in the growth medium, phosphate concentrations in the cells rise sufficiently for a specific kinase to phosphorylate the $EIIA^{Glc}$. Phosphorylated $EIIA^{Glc}$ then undergoes an allosteric change and dissociates from the lactose permease, making it active so that more lactose can enter the cell. In other words, the inducer is not "excluded" under these conditions!

The kinase that phosphorylates $EIIA^{Glc}$ is part of a phosphoenolpyruvate (*PEP)-dependent phosphotransferase* system (*PTS*) cascade. When extracellular glucose levels are low, the cell activates the PTS system in an effort to bring whatever glucose is around into the cell. The last enzyme in the PTS *phosphorylation cascade* is the kinase that phosphorylates $EIIA^{Glc}$. Now activated, phosphorylated $EIIA^{Glc}$ dissociates from the lactose permease. The now active permease can bring available lactose from the medium into the cell.

12.2.5 Structure of the *lac* Repressor Protein and Additional Operator Sequences

The *lac* repressor encoded by the I gene is a tetramer of identical subunits (Fig.12.9).

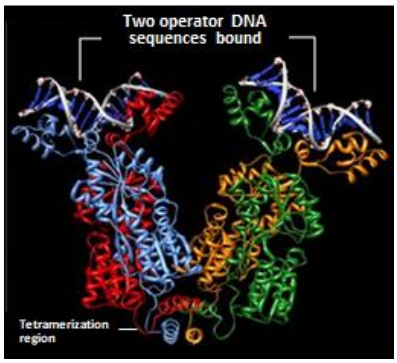


Fig. 12.9: Computer generated structure of the tetrameric lac repressor bound to DNA via *helix-turn-helix motifs.*

Each subunit contains a *helix-turn-helix motif* capable of binding to DNA. However, the operator DNA sequence downstream of the promoter in the operon consists of a pair of *inverted repeats* spaced apart in such a way that they can only interact with two

of the repressor subunits, leaving the function of the other two subunits unknown… that is, until recently! Two more operator regions were recently characterized in the *lac* operon. One, called ***O*$_2$**, is within the *lac z* gene itself and the other, called ***O*$_3$**, lies near the end of, but within the *lac I* gene. Apart from their unusual location within actual genes, these operators, which interact with the remaining two repressor subunits, went undetected at first because mutations in the O_2 or the O_3 region individually do not contribute substantially to the effect of lactose in derepressing the *lac* operon. Only mutating both regions at the same time results in a substantial reduction in binding of the repressor to the operon.

12.3 Gene Regulation in Prokaryotes: the Tryptophan (*trp*) Operon

If ample tryptophan (***trp***) is available, the tryptophan synthesis pathway can be inhibited in two ways. First, recall how feedback inhibition by excess trp can allosterically inhibit the trp synthesis pathway. A rapid response occurs when tryptophan is present in excess, resulting in rapid feedback inhibition by blocking the first of five enzymes in the trp synthesis pathway. The *trp operon* encodes polypeptides that make up two of these enzymes. ***Enzyme 1*** is a *multimeric* protein, made from polypeptides encoded by the *trp5* and *trp4* genes. The *trp1* and *trp2* gene products make up ***Enzyme 3***. If cellular tryptophan levels drop because the amino acid is rapidly consumed (e.g., due to demands for protein synthesis during rapid growth), *E. coli* cells will continue to synthesize the amino acid, as illustrated in Fig. 12.10.

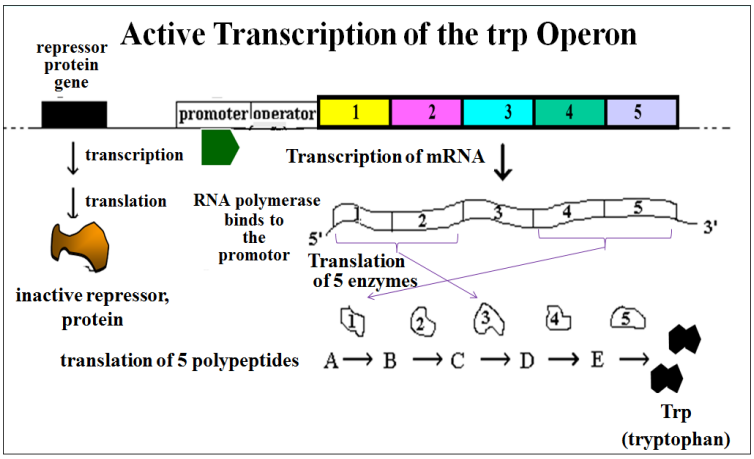


Fig. 12.10: Transcription of the 5-gene tryptophan (trp) operon: The 5 resulting enzymes are in the pathway for tryptophan synthesis. A tryptophan (trp) repressor gene to the left of the operator is always transcribed.

On the other hand, if tryptophan consumption slows down, tryptophan accumulates in the cytoplasm. Excess tryptophan will bind to the trp repressor. The trp-bound repressor then binds to the trp operator, blocking RNA polymerase from transcribing the operon. The repression of the trp operon by trp is shown in Fig. 12.11.

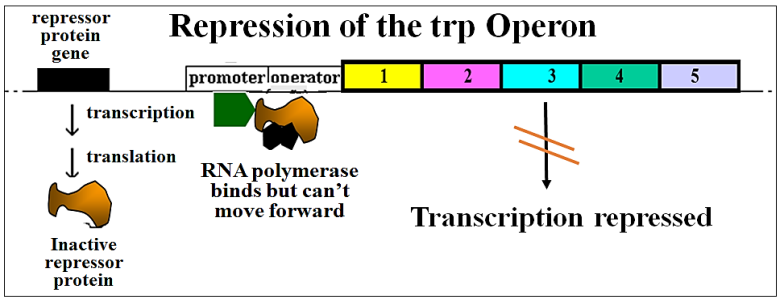


Fig. 12.11: When there is sufficient tryptophan in the cells, excess tryptophan binds to and changes the conformation of the trp repressor protein, which then recognizes and binds the trp operon operator, blocking RNA polymerase and repressing operon transcription.

In this scenario, tryptophan is a ***co-repressor***. The function of a co-repressor is to bind to a repressor protein and change its conformation so that it can bind to the operator.

219 Repression of the Tryptophan (TRP) Operon

12.4 The Problem with Unregulated (*Housekeeping*) Genes in All Cells

Before we turn our attention to the regulation of gene expression in eukaryotes, consider for a moment the expression of ***constitutive***, (unregulated, or ***housekeeping***) genes that are always active. The requirement that some genes are always "on" raises questions about cellular priorities of gene expression. Constitutive gene products are sets of many polypeptides that form large macromolecular complexes in cells, or enzyme sets that participate in vital biochemical pathways. How do cells maintain such polypeptides in stoichiometrically reasonable amounts? Or, can their levels rise or fall transiently without much effect? Recent studies suggest that transcription of *housekeeping* genes is in fact, not at all coordinated! We also saw that the efficiency of glycolysis relies in part on allosteric regulatory mechanisms that have evolved to control the activities of glycolytic enzymes rather than their transcription. While this takes care of some elements of metabolic control, a problem remains. Recall that protein synthesis is energy-intensive, each peptide linkage costing three NTPs (not to mention the waste of an additional NTP per phosphodiester linkage made in transcription of an mRNA!). The overproduction of

proteins would seem to be a waste of energy, and thus an evolutionary 'mistake'. While we may not know just how expensive it is to express housekeeping genes, whatever the expense, these energy expenses are the cost of evolving complex structures and biochemical pathways vital to their everyday function and survival. This is a good example of a major precept of evolutionary theory: evolution is an accumulation of improvements brought about by natural selection; it is not the result of perfect design in advance. Now back to our focus on regulated gene expression… in eukaryotes.

12.5 Gene Regulation in Eukaryotes

Let's recall an experiment described earlier and illustrated in Fig. 12.12.

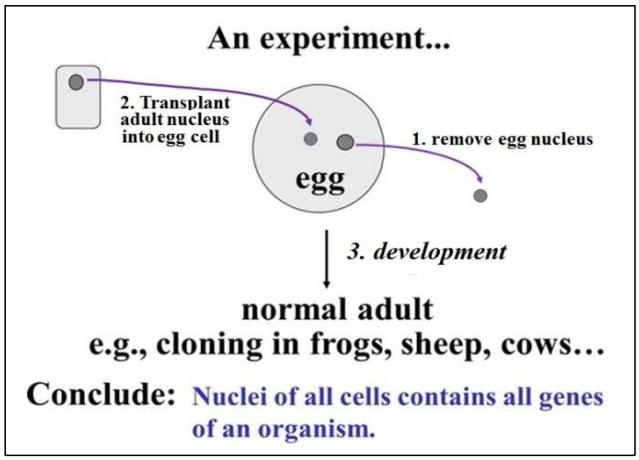


Fig. 12.12: Replacement of an egg nucleus with that from an adult cell enabled cloning of embryos and eventually complete organisms, proving that adult cells contain all of the genes required to program development of, and to make the organism.

Results of this experiment provided the evidence that even very different cells of an organism contain the same genes. In fact in any multicellular eukaryotic organism, every cell contains the same genes in their DNA. Therefore, the different cell types in an organism differ *not* in which genes they contain, but which sets of genes they express! Looked at another way, cells differentiate when they turn on new genes and turn off old ones. Thus, gene regulation produces different sets of gene products during differentiation, leading to cells that look and function differently in the organism.

220 An Experiment: All of an Organism's Cells Have the Same Genome

Compared to prokaryotes, many steps in eukaryotes lie between the transcription of an mRNA and the accumulation of a polypeptide end product. Eleven of these steps are shown below in the pathway from gene to protein Fig. 12.13.

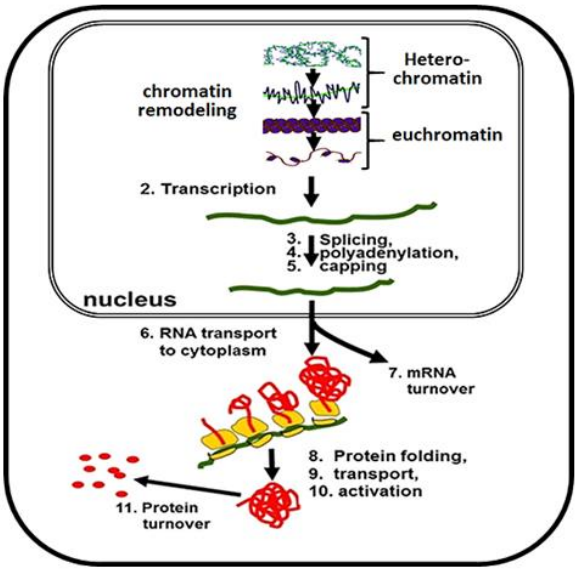


Fig. 12.13: Potentials for gene regulation in eukaryotes: Control of transcript and/or protein abundance, as well as protein activity can regulate cellular metabolism.

Theoretically, cells could turn on, turn off, speed up or slow down any of the steps in this pathway, changing the steady state concentration of a polypeptide in the cells. But the expression of a single gene is typically controlled at only one or a few steps. As is the case in bacteria, control of transcription initiation is perhaps the most common form of eukaryotic gene regulation, (in principle if not in detail).

221-2 Many Options for Regulating Eukaryotic Genes

12.5.1 Complexities of Eukaryotic Gene Regulation

Gene regulation in eukaryotes is more complex than in prokaryotes. This is in part because their genomes are larger and because they encode more genes. For example, the *E. coli* genome houses about 5,000 genes, compared to around 25,000

genes in humans. Furthermore, eukaryotes can produce even more than 25,000 proteins by *alternative splicing* of mRNAs and in at least a few cases, by initiating transcription from *alternative start sites* in the same gene. And of course, the activity of many more genes must be coordinated without the benefit of multigene operons! Finally, eukaryotic gene regulation is made more complicated because all nuclear DNA is wrapped in protein in the form of chromatin.

All organisms control gene activity with ***transcription factors*** that bind to specific DNA sequences (***cis regulatory elements***). In eukaryotes, these elements can be **proximal to** (near) the promoter of a gene, or ***distal to*** (quite far from) the gene they regulate. Fig. 12.14 is a eukaryotic gene, *mapped to* show its typical components (promoter, introns, exons…) and its associated *cis-acting* regulatory elements.

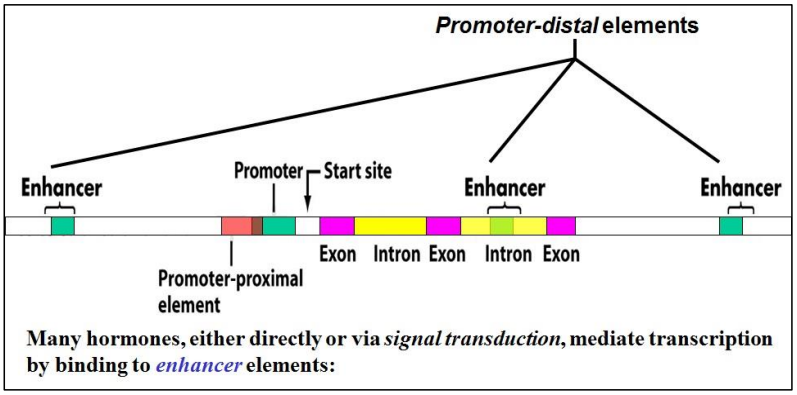


Fig. 12.14: Cis-acting sequence elements associated with a gene function as gene regulators when bound to regulatory proteins (trans-acting factors). Regulatory sequences may be near the gene promoter (proximal) or far from it (distal). Enhancers are examples of distal regulatory sequences.

Enhancers are typical *distal* cis elements that recognize and bind transcription factors to increase the rate of transcription of a gene. Oddly enough, these short DNA elements can be in the 5' or 3' non-translated region of the gene or even within introns, often thousands of base pairs away from the promoter and transcription start-site of the genes they control.

Upstream regulatory regions of eukaryotic genes (to the left of a gene promoter as shown above) often have distal binding sites for more than a few transcription factors, some with positive (*enhancing*) and others with negative (*silencing*) effects. Of course, which of these DNA regions are active in controlling a gene depends on which

transcription factor(s) are present in the nucleus. Sets of positive regulators can work together to coordinate and maximize gene expression when needed, and sets of negative regulators may bind negative regulatory elements to silence a gene.

222-2 Transcription Factors Bind DNA Near & Far

In eukaryotes we saw that the initiation of transcription involves many transcription factors (***TF***s) and RNA polymerase II acting at a gene promoter to form a ***transcription pre-initiation complex***. ***TFIID*** (or ***TATA binding protein***) is one of the first factors to bind, causing the DNA in the promoter region to bend, much like the CAP protein in bacteria. Once bound, *TFIID* recruits other transcription factors to the promoter. As in bacteria, bending the DNA loosens H-bonds between bases, facilitating unwinding the double helix near the gene. Bending eukaryotic DNA also brings regulatory proteins on distal enhancer sequences far from the promoter close to proteins bound to more proximal regulatory elements, as shown in Fig. 12.15.

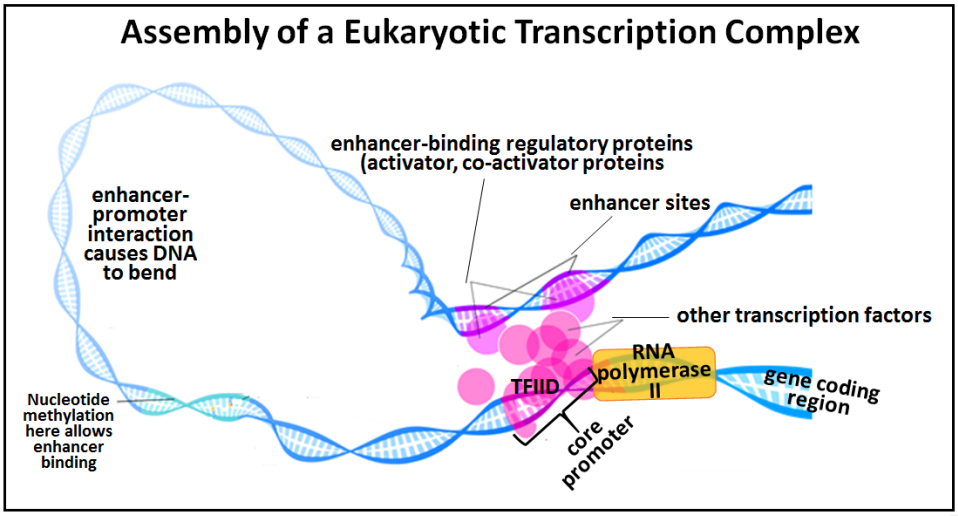


Fig. 12.15: Assembly of a eukaryotic transcription complex on a regulated gene. Distal regulatory protein/DNA interactions can cause DNA bending, recruiting transcription initiation factors and proximal regulatory factors to the transcription complex.

Nucleotide *methylation sites* may facilitate regulatory protein-enhancer binding. When such regulatory proteins, here called *activators* (i.e., of transcription), bind to their enhancers, they acquire an affinity for protein *cofactors* that enable recognition and

binding to other proteins in the transcription initiation complex. This attraction stabilizes the bend in the DNA that then makes it easier for RNA polymerase II to initiate transcription from the appropriate strand of DNA.

223-2 Assembling a Eukaryotic Transcription Initiation Complex

It is worth reminding ourselves that allosteric (shape) changes in proteins allow DNA-protein interactions (in fact, any interaction between macromolecules). The *lac* repressor we saw earlier is a transcription factor with *helix-turn-helix* DNA binding motifs. This motif and two others (*zinc finger,* and *leucine zipper)* characterize DNA binding proteins are illustrated in Fig. 12.16.

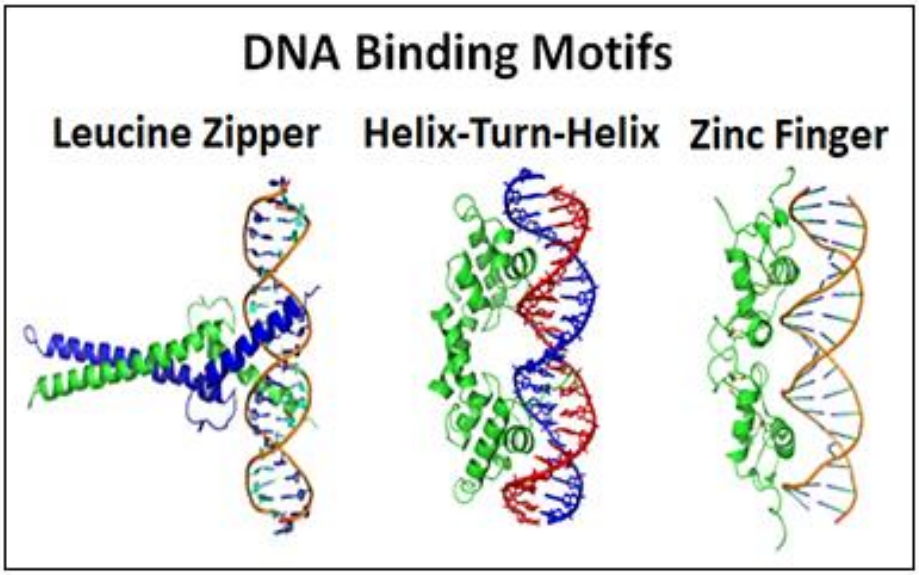


Fig. 12.16: Three DNA binding motifs commonly found in trans-acting (i.e. regulatory) protein factors.

DNA-binding motifs in each regulatory protein shown here bind one or more regulatory elements 'visible' to the transcription factor in the major groove of the double helix.

224-2 Transcription Factor Domains-Motifs Bind Specific DNA Sequences

We will look next at some common ways in which eukaryotic cells are signaled to turn genes on or off, or to increase or decrease their rates of transcription. As we describe these models, remember that eukaryotic cells regulate gene expression in response to changes in extracellular environments. These can be unscheduled, unpredictable changes in blood or extracellular fluid composition (ions, small metabolites), or dictated by changes in long-term genetic programs of differentiation and development. Some changes in gene expression even obey *circadian*, or daily rhythms, like the ticking of a clock. In eukaryotes, changes in gene expression, expected or not, are usually mediated by the timely release of chemical signals from specialized cells (e.g., hormones, cytokines, growth factors, etc.). We will focus on some better-understood models of gene regulation by these chemical signals.

12.5.2 Regulation of Gene Expression by Hormones that Enter Cells and Those That Don't

Gene-regulatory (cis) elements in DNA and the transcription factors that bind to them have co-evolved. But not only that! Eukaryotic organisms have evolved complete pathways that respond to environmental or programmed developmental cues and lead to an appropriate cellular response. In eukaryotes, chemicals released by some cells signal other cells to respond, thus coordinating the activity of the whole organism. Hormones released by cells in endocrine glands are well-understood signal molecules; hormones affect *target cells* elsewhere in the body.

225 Chemicals That Control Gene Expression

12.5.2.a How Steroid Hormones Regulate Transcription

Steroid hormones cross the cell membranes to have their effects. Common steroid hormones include testosterone, estrogens, progesterone, glucocorticoids and mineral corticoids.

Once in target cell, such hormones bind to a ***steroid hormone receptor*** protein to form a ***steroid hormone-receptor complex***. The receptor may be in the cytoplasm or in the nucleus but in the end, the hormone-receptor complex must bind to DNA regulatory elements of a gene, either to enhance or silence transcription. Therefore, a steroid hormone must cross the plasma membrane, and may also need to cross the nuclear envelope. Follow the binding of a steroid hormone to a cytoplasmic receptor in Fig. 12.17 (below). Here the hormone (the triangle) enters the cell. An allosteric change in the receptor releases a protein subunit called Hsp90 (the black rectangle in the illustration). The remaining, now-active hormone-bound receptor enters the nucleus.

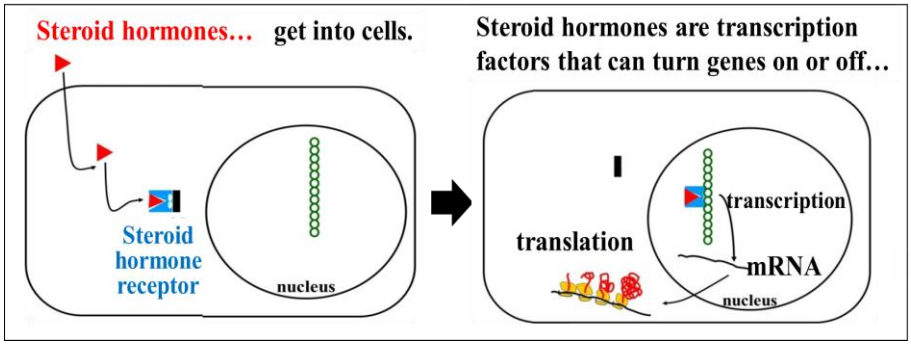


Fig. 12.17: Steroid hormones regulate genes. Once in a target cell, the hormone binds a receptor in the nucleus or in the cytoplasm. As shown here, a cytoplasmic hormone-receptor complex moves into the nucleus. Now acting as transcription factor, the hormone-receptor complex binds to regulatory DNA sequences to modulate gene transcription or turn genes on or off.

The fascinating thing about Hsp90 is that it was first discovered in cells subjected to heat stress. When the temperature gets high enough, cells shut down most transcription and instead transcribe Hsp90 and/or other special *heat shock* genes. The resulting ***heat shock proteins*** seem to protect the cells against metabolic damage until temperatures return to normal. Since most cells never experience such high temperatures, the evolutionary significance of this protective mechanism is unclear. But as we now know, heat shock proteins have critical cellular functions, in this case blocking the DNA-binding site of a hormone receptor until a specific steroid hormone binds to it.

Back to hormone action! No longer associated with the Hsp90 protein, the receptor bound to its hormone *cofactor* binds to a cis-acting transcription control element in the DNA, turning transcription of a gene on or off. The hormone receptors for some steroid hormones are already in the nucleus of the cell, so the hormone must cross not only the plasma membrane, but also the nuclear envelope in order to access the receptor. As for steroid hormone functions, we already saw that glucocorticoids turn on the genes of gluconeogenesis. Steroid hormones also control sexual development and reproductive cycling in females, sexual development and sperm maturation in males, salt and mineral homeostasis in the blood, metamorphosis in arthropods, etc., all by regulating gene expression.

226 Steroid Hormones Regulate Gene Transcription

12.5.2.b How Protein Hormones Regulate Transcription

Compared to steroids, soluble proteins are soluble and of course large, with highly charged surfaces. Even protein-derived hormones like adrenalin, though small, are charged and hydrophilic. Large and/or hydrophilic signal molecules cannot get across the phospholipid barrier of the plasma membrane. To have any effect at all, they must bind to receptors on the surface of cells. These receptors are typically membrane glycoproteins. The information carried by protein hormones must be conveyed into the cell indirectly by a process called ***signal transduction***. One of two well-known pathways of signal transduction is illustrated below in Fig. 12.18.

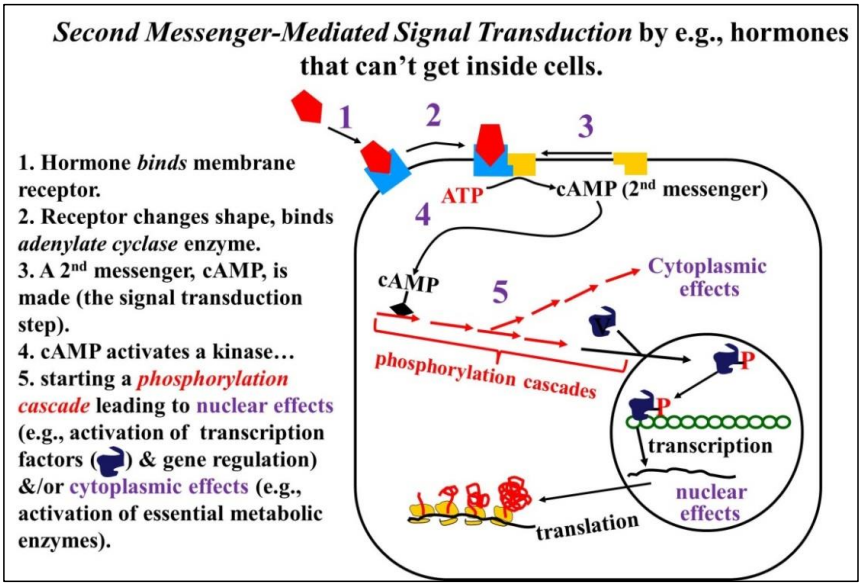


Fig. 12.18:Large or polar signal molecules bind to membrane receptors, resulting in *signal transduction,* Here, a *second messenger* molecule (cAMP) forms in the cytoplasm to deliver the hormonal message. A *phosphorylation cascade* leads to responses including gene regulation and/or control of existing protein activity.

In this pathway, hormone binding to a cell-surface receptor results in formation of ***cAMP***, a ***second messenger***. cAMP forms when the hormone-receptor in the membrane binds to and activates a membrane-bound ***adenylate cyclase*** enzyme. Once formed, cAMP binds to and activates a protein kinase to initiate a protein ***phosphorylation cascade*** in the cytoplasm. The *cascade* is a series of protein

phosphorylations. The last in the series phosphorylated proteins can often be (as suggested in the illustration) one of many activating or inactivating enzymes that regulate cellular metabolism, or it could be an activated transcription factor that binds to a cis-regulatory DNA sequence to regulate gene expression. cAMP was the first *second messenger* metabolite to be discovered.

Some hormones bind to membrane receptors that are themselves protein kinases. In this case, binding of the signal protein (e.g. hormone) to its receptor causes an allosteric change that activates the linked receptor kinase. This starts a phosphorylation cascade without the aid of cAMP. The cascade ends with the activation of an active transcription factor as shown in Fig. 12.19.

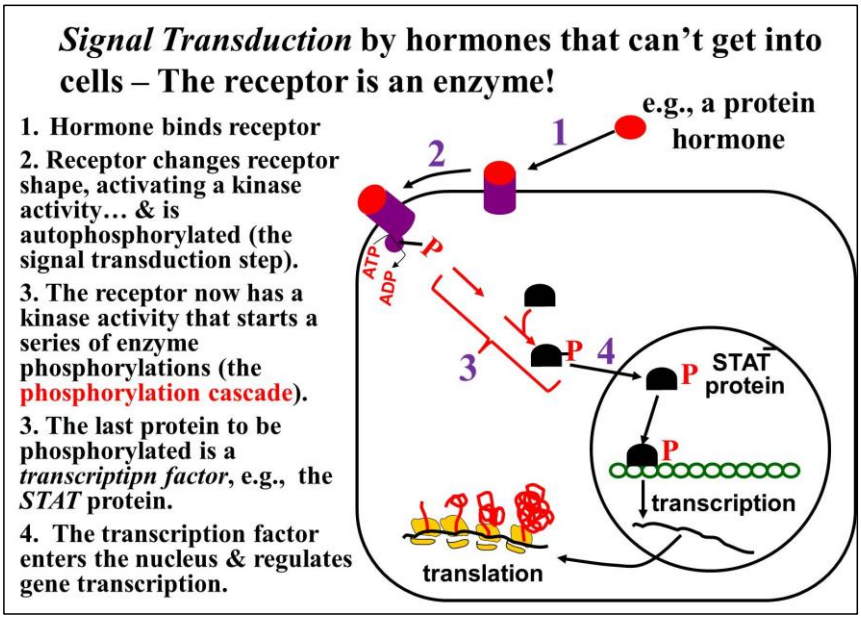


Fig. 12.19: *Enzyme-linked receptors* transduce hormonal signals directly, activating receptor kinases on the cytoplasmic surface of the cell, initiating phosphorylation cascades leading to any of several responses.

We'll take a closer look at signal transduction in another chapter.

227 Signal Transduction Can Lead to Gene Regulation

12.6 Regulating Eukaryotic Genes Means Contending with Chromatin

Consider again the different levels of chromatin structure (Fig. 12.20).

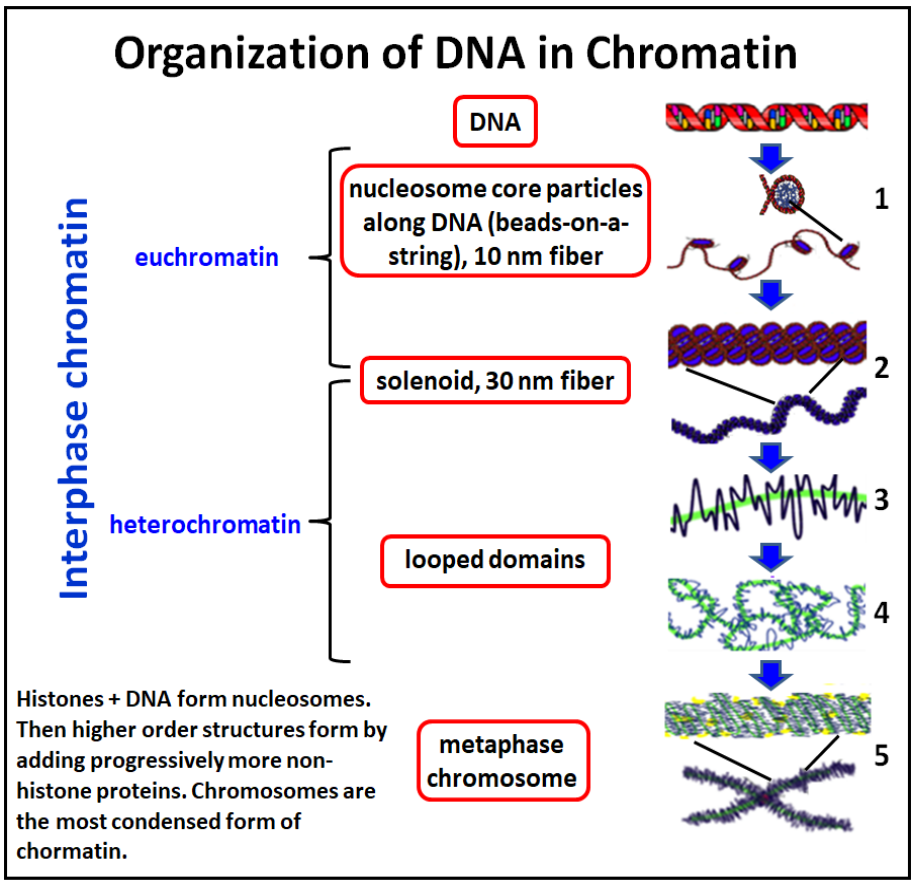


Fig. 12.20: Chromatin Organization: different levels of chromatin structure result from differential association of DNA with chromosomal proteins.

Transcription factors bind specific DNA sequences by detecting them through the grooves (mainly the major groove) in the double helix. The drawing above reminds us however, that unlike the nearly naked DNA of bacteria, eukaryotic (nuclear) DNA is coated with

proteins that in aggregate, are greater in mass than the DNA that they cover. The protein-DNA complex of the genome is of course, chromatin. Again as a reminder, DNA coated with histone proteins forms the 9 nm diameter *beads-on-a-string* structure in which the beads are the ***nucleosomes***. The association of specific non-histone proteins with the nucleosome 'necklace' causes it to fold over on themselves to form the ***30 nm solenoid***. As we saw earlier, it is possible to selectively extract chromatin. Take a second look at the results of typical extractions of chromatin from isolated nuclei (Fig. 12.21).

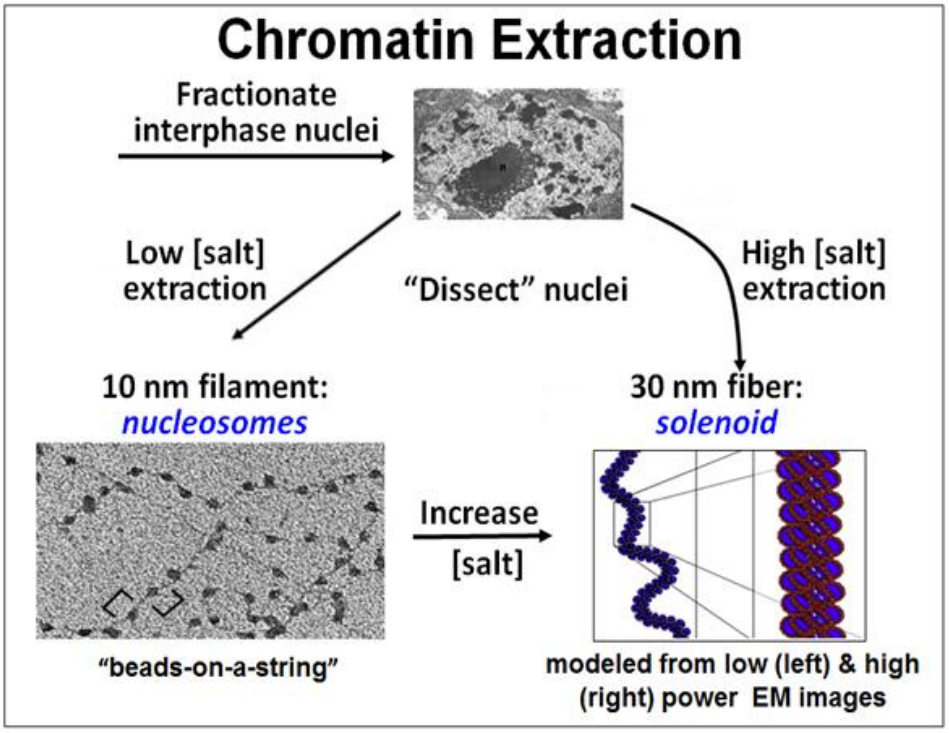


Fig. 12.21: High salt chromatin extraction from nuclei (or high salt treatment of 10 nm filaments) yields 30 nm *solenoid* structures, essentially coils of 10 nm filaments.

Further accretion of non-histone proteins leads to more folding and the formation of ***euchromatin*** and ***heterochromatin*** characteristic of non-dividing cells. In dividing cells, the chromatin further *condenses* to form the ***chromosomes*** that separate during either *mitosis* or *meiosis*.

Recall that biochemical analysis of the 10 nm filament extract revealed that the DNA wraps around histone protein octamers, the nucleosomes or beads in this beads-on-a-string structure. Histone proteins are highly conserved in the eukaryotic evolution; they are not found in prokaryotes. They are also very basic (many *lysine* and *arginine* residues) and therefore very positively charged. This explains why they can arrange themselves uniformly along DNA, binding to the negatively charged *phosphodiester backbone* of DNA in the double helix.

Since the DNA in euchromatin is less tightly packed than it is in heterochromatin, perhaps active genes are to be found in euchromatin and *not* in heterochromatin. Experiments in which total nuclear chromatin extracts were isolated and treated with the enzyme deoxyribonuclease (DNAse) revealed that the DNA in active genes was degraded more rapidly than non-transcribed DNA. More detail on these experiments can be found in the two links below.

228 Question: Is Euchromatic DNA Transcribed?

229 Experiment and Answer: Euchromatin *is* Transcribed

The results of such experiments are consistent with the suggestion that active genes are more accessible to DNAse because they are in less coiled, or less condensed chromatin. DNA in more condensed chromatin is surrounded by more proteins, and thus is less accessible to, and protected from DNAse attack. When packed up in chromosomes during mitosis or meiosis, all genes are largely inactive.

Regulating gene transcription must occur in non-dividing cells or during the interphase of cells, times when it is easier to silence or activate genes by ***chromatin remodeling*** (i.e., changing the shape of chromatin). Changing chromatin conformation involves chemical modification of chromatin proteins and DNA. For example, chromatin can be modified by histone acetylation, de-acetylation, methylation and phosphorylation reactions, catalyzed by *histone acetyltransferases* (*HAT* enzymes), de-acetylases, methyl transferases and kinases, respectively. For example, acetylation of lysines near the amino end of histones H2B and H4 tends to unwind nucleosomes and open the underlying DNA for transcription. De-acetylation promotes condensation of the chromatin in the affected regions of DNA. Likewise, methylation of lysines or arginines (basic amino acids that characterize histones!) in H3 and H4 typically opens up DNA for transcription, while demethylation has the opposite effect. These chemical modifications affect recruitment of other proteins that alter chromatin conformation and ultimately activate or block transcription. This reversible acetylation and its effect on chromatin are illustrated in Fig. 12.22 (below).

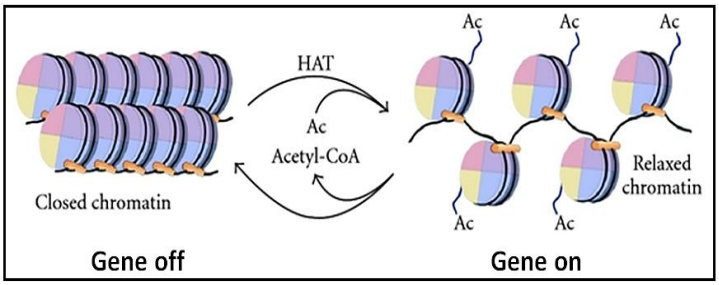


Fig. 12.22: Chemical modification of histones can *open* or *close* chromatin to transcription.

Nucleosomes themselves can move, slide and otherwise be repositioned by complexes that hydrolyze ATP for energy to accomplish the physical shifts. Some cancers are associated with mutations in genes for proteins involved in chromatin remodeling. Failures of normal remodeling could adversely affect normal cell cycling and normal replication. In fact, a single, specific pattern of methylation may *mark* DNA in multiple cancer types (check out Five Cancers with the Same Genomic Signature - Implications).

12.7 Regulating all Genes on a Chromosome at Once

Recall that male cells have only one X chromosome and that one of the X chromosomes in human female adult somatic cells is inactivated, visible in the nucleus as a *Barr body*. Inactivation of one of the X chromosomes in *Drosophila* females. But both males and females require X chromosomes gene expression during embryogenesis. At that time, given the difference in X chromosome ***gene dosage*** between males and females, how do male embryos get by with only on X chromosome?

Experiments looking at the expression of X chromosome genes in male *and* female flies revealed similar levels of gene products. It turns out that A protein, called ***CLAMP*** (**C**hromatin-**L**inked **A**daptor for **M**ale-specific lethal **P**rotein), was shown to bind to ***GAGA*** nucleotide repeats lying between the genes for histones 3 and 4. There are about 100 repeats of the five-gene histone locus on X chromosomes, and so, about 100 *GAGA* repeats. All those *CLAMP* proteins bound to those *GAGA* repeats can interact with a *nuclear body* called ***HLB*** (**H**istone **L**ocus **B**ody). The *HLB* was previously implicated in higher levels of X chromosome gene expression. A recent study suggests that once bound to the *GAGA* sequences, *CLAMP* recruits ***MSL*** (Male-Specific-Lethal) proteins to form an ***MSL complex***. This complex (the associated *HLB*/*CLAMP*/ *MSL*) has been shown to boost X chromosome gene transcription in male fruit flies. The resulting MSL protein complexes that form then globally increase X chromosome gene expression,

compensating for the lower X gene dosage in males. Read the original research at Increasing the Gene Expression of an Entire X Chromosome (L.E. Reider et al. (2018) Genes & Development 31:1-15). And finally, there is emerging evidence that the HLB action may also be involved in inactivation of an entire female X chromosome later in embryogenesis in females!

12.8 Mechanoreceptors: Capturing Non-Chemical Signals

A discussion of hormone and other signal molecule-based signal transduction would be incomplete without acknowledging that cells sometimes need to respond to mechanical stimuli, such as *pressure* or *stretching*. There are receptors called ***piezoreceptors*** for that (piezo, meaning squeeze, or press)!

Piezoreceptors in cells lining the urinary bladder signal when the bladder is full, with the expected response. Piezoreceptors in the endothelial cells of blood vessels alert the cells to changes in blood pressure and mediate appropriate responses. In sensory cells in the skin, piezoreceptors enable nerve ending responses to touch or pain. Another example of *piezoreception* is found in cellular *proprioreceptors*, as in proprio, meaning one's own self, or in other words, a kind of self-awareness receptor. Proprioreceptors in joints respond to changes in posture to let us know where our arms and legs are at any given moment! The domain structure of one *piezoreceptor* protein was shown to include *springy* domains that could respond to mechanical forces. For more details on piezoreceptor structure, see https://www.nature.com/articles/s41586-019-1499-2. Fig. 12.23 illustrates how a piezoreceptor may respond to pressure or stretch to open an Ca^{++} channel.

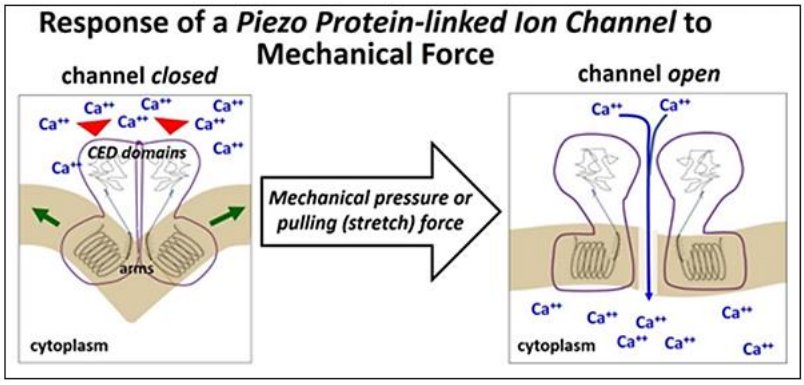


Fig. 12.23: Extracellular pressure (arrowheads) on a CED domain or activating spring-like arms by stretching the cell membrane (e.g., from inside the cell (arrow) opens a piezo-linked ion channel.

12.9 Epigenetics

Aristotle thought that an embryo emerged from an amorphous mass, a “less fully concocted seed with a nutritive soul and all bodily parts”. The much later development of the microscope led to more detailed (if inaccurate) descriptions of embryonic development. In 1677, no less a luminary than Anton von Leeuwenhoek, looking at a human sperm with his microscope, thought he saw a miniature human inside! The tiny human, or ***homunculus***, became the epitome of ***preformation*** theory.

William Harvey, also in the 17th century, described changes in morphology in the developing embryos of chickens (and other animals). Harvey coined the term ***epigenesis*** to counter the notion that any tiny adult structures in eggs or sperm simply grew bigger during embryonic gestation. Meanwhile, other experiments were leading embryologists to the conclusion that the physical and chemical environment of an embryo strongly affected development. Thus temperature, pH, and in the case of chicken eggs, the position of Incubation can affect embryonic development. In a series of very elegant experiments reported in 1924, Hans Speeman reported that cells associated with differentiation of one region of an embryo could be transplanted to a different part of the same embryo, or to another embryo entirely, where it would induce new tissue development. He won the 1935 Nobel Prize in Physiology and Medicine for his discovery of embryonic ***organizers*** that induced morphogenesis.

Other embryologists demonstrated that cells killed by freezing or boiling still induced morphogenesis after being placed on an embryo. This clearly implicated a role for actual chemicals in embryogenesis. The fact that differences in physical or chemical environment could affect embryonic development led many to conclude that environment played the dominant role and that genes played only a minor one in an organism’s ultimate phenotype. Unlike most of his fellow embryologists, Conrad Waddington believed in a more equitable role of genes and environment in determining phenotype. Adapting the term epigenesis, he coined the term *epigenetics* to describe the impact of environment on embryonic development (1942, *The Epigenotype. Endeavour. **1**: 18–20*).

At the time, the concept of epigenetics led to the *nature vs. nurture* controversy. According to the nature side, inheritance was mainly genetic, while the nurture side gave the dominant role to the environmental chemistry. We now know that differences in environmental influence can and do cause individuals with the same genes (genotype) to vary in appearance (phenotype). A modern version of the *nature vs. nurture* argument has more to do with complex traits, for example how much do genetics vs. environment influence intelligence, psychology and behavior. There is much to-do and little evidence to resolve these questions, and likely too many factors affecting these traits to separate them experimentally.

These days, epigenetic (*epi* meaning 'over' or above) studies look closely at protein interactions in eukaryotes affecting gene expression, in other words at interactions superimposed on genes. These interactions change the structure NOT of genes or DNA, but of the proteins (and other molecules) that affect how DNA and genes are used. As we have seen, the control of transcription involves transcription factors that recognize and bind to regulatory sequences in DNA such as enhancers or silencers. These protein-DNA interactions often require selective structural changes in the conformation of the chromatin surrounding genes. These changes can be profound and stable, and they are not easily undone.

12.9.1 Epigenetic Inheritance in Somatic Cells

Examples of somatic cell epigenetics include the inheritance of chromatin protein alterations that accompany changes in gene expression that occur in development. Given an appropriate signal, say a hormone at the right time, a few cells respond with chromatin rearrangements and the expression of a new set of genes. The new pattern of gene expression defines a cell that has differentiated. Hundreds, even thousands of such changes to chromatin and gene expression accompany progress from fertilized egg to fully mature eukaryotic organism. Every one of these changes in a cell is passed on to future generations of cells by mitosis, accounting for different tissues and organs in the organism. In this way, the many different *epigenomes* representing our differentiated cells are *heritable*.

Thus, somatic cell epigenetics is the study of when and how undifferentiated cells (embryonic and later, adult stem cells) acquire their differentiated characteristics and how they then pass on the information for differentiated state to progeny cells. As we'll see shortly, epigenetic inheritance is not limited to somatic cells, but can span generations! First, let's look at this brief history of our changing understanding of evolution…

Let's start with Jean-Baptiste Lamarck who proposed (for instance) that the ancestors of giraffes had short necks, but evolved longer and longer necks because longer necks enabled them to reach food higher up in the trees. That character would be inherited by the next giraffe generation. According to Lamarck, evolution was ***purposeful***, with the goal of improvement. Later, Darwin published his ideas about evolution by *natural selection*, where nature selects from pre-existing traits in individuals (the raw material of evolution). The individual that just randomly happens to have a useful trait then has a survival (reproductive) edge in an altered environment. Evolution is thus ***not purposeful***. Later still, with the rediscovery of Mendel's genetic experiments, it became increasingly clear that it is an organism's genes that are inherited, are passed down the generations, and are the basis of an

organism's traits. By the start of the 20th century, Lamarck's notion of purposefully acquired characters was discarded. With this brief summary, let's look at epigenetic inheritance across generations of sexually reproductive species.

12.9.2 Epigenetic Inheritance in the Germ-Line

Epigenetic inheritance implies in addition to our DNA blueprint, there is also an epigenetic blueprint. This means that in addition to passing on the genes of a male and female parent, epigenomic characteristics (which genes are expressed and when) are also passed to the next generation. Waddington suspected as much early on, calling the phenomenon *genetic assimilation*, and once again created controversy! Does genetic assimilation make Lamarck right after all? Prominent developmental biologists accused Waddington of promoting purposeful evolution. Waddington and others denied the accusation, trying to explain how epigenetic information might be heritable, without leading to *purposeful* evolution. So, is there in fact, an *epigenetic code*?

Data from the small Swedish town of Överkalix led to renewed interest in epigenetic phenomena. Consider the meticulous harvest, birth, illness, death and other demographic and health records collected and analyzed by L. O. Bygren and colleagues in Sweden. A sample of Bygren's data is illustrated in Fig. 12.24.

Grand-parent	Food supply	Grandson relative risk of death from cardio disease and diabetes	Granddaughter relative risk of death from cardio disease and diabetes
Grandfather	poor	-35%	No change
Grandfather	abundant	+67%	No change
Grandmother	poor	No change	-49%
Grandmother	abundant	No change	+113%

Fig. 12.24: Summary of health, birth, death and demographic records by L/. O. Bygren for the town of Överkalix

It looked to the good doctor as if environment was influencing germline inheritance! It is as if the environment was indeed causing an *acquired change* in the grandparent that is passed not to one, but through two generations… and in a sex-specific way!

230 Epigenetic Inheritance: First Inkling

This phenomenon was subsequently demonstrated experimentally. In one experiment, pregnant rats were exposed to a toxin. Rat pups born to exposed mothers suffered a variety of illnesses. This might be expected if the toxic effects on the mother were visited on the developing pups, for example through the placenta. However, when the diseased male rat pups matured and mated with females, the pups in the new litter grew up suffering the same maladies as the male parent. This *even though the pregnant females in this case were NOT exposed to the toxins.* Because the original female was already pregnant when she was exposed, the germ line cells (eggs, sperm) of her litter had not suffered mutations *in utero*. This could only mean that the epigenetic patterns of gene expression caused by the toxin in pup germ line cells (those destined to become sperm & eggs) *in utero* were retained during growth to sexual maturity, to be passed on to their progeny even while gestating in a normal unexposed female.

231-2 Experimental Demonstration of Germ-Line Epigenetic Inheritance

For some interesting experimental findings on how diet influences epigenetic change in Drosophila click Dietary Change Causes Heritable Change in Drosophila Epigenome. For recent evidence for a role of male DNA methylation in trans-generational epigenetic inheritance, check out More on Epigenetic Inheritance across Generations. For an amusing (but accurate) explanation of epigenetics, check out the YouTube at *https://www.youtube.com/watch?v=kp1bZEUgqVI*. So, given the reality of epigenetic inheritance, let's consider a most intriguing question: Is epigenetic inheritance (like Mendelian genetic inheritance) the result of natural selection?

A number of studies have correlated adverse conditions during pregnancy in animals (including humans) with high incidences of health, behavioral and other anomalies in the adults. This was the case in Holland for children and grandchildren of mothers pregnant during the famine of the last winter of WWII, a season that became known as the ***Dutch Hunger Winter of 1944-1945***. As adults, these descendants had higher incidences heart disease, obesity and diabetes. Such correlations were attributed to epigenetic changes in embryonic DNA *in utero* (read more at Moms-environment-during-pregnancy-can-affect-her-grandchildren.

Here is the question that should occur to us: Why should so many survivors of life in an undernourished womb have suffered the same epigenetic changes at the same time? A study of methylation patterns in specific DNA regions in survivors of *Dutch Hunger Winter* pregnancies and a control group that missed the famine suggests an intriguing answer. The study found unique DNA methylation patterns in those surviving adults.
To explain the survival of so many newborns that then suffered adult health anomalies, the investigators suggested first that epigenetic modifications occur at random in

embryos…, and then suggested that some of these (i.e., the ones that *marked* the suffering adults) were selected during the pregnancy because they actually conferred a survival advantage to embryos (see details and a more complete discussion at Natural Selection *in utero*). Could the many known cases of epigenetic inheritance by large cohorts of individuals be explained by the survival advantages of ***epigenetic natural selection***? In other words, could epigenetic inheritance be subject to natural selection in the same way as genetic inheritance…, perhaps with more complex results?

These days, the term epigenetics describes heritable changes in chromatin modifications and gene expression. We now know that epigenetic configurations of chromatin that are most stable include patterns of histone modification (*acetylation, phosphorylation, methylation…*) or DNA (*methylation, phosphorylation…*). Such changes can convert the 30nm fiber to the 10nm 'beads-on-a-string' nucleosome necklace… and *vice versa*. Such changes in chromatin (chromatin remodeling) lead to altered patterns of gene expression, whether during normal development or when deranged by environmental factors (abundance or limits on nutrition, toxins/poisons or other life-style choices). The active study of DNA methylation patterns even has its own name, *methylomics*! Check out Epigenetics Definitions and Nomenclature for more epigenetic nomenclature.

Let's close this chapter with some socially, culturally and personally relevant questions and some observations. For example, consider that the epigenetic consequences of smog that Los Angelinos endured for decades are not known. And apart from the obvious effects of lead toxicity to folks drinking lead-laden water in Flint Michigan (especially in children), any epigenetic effects of the exposure remain to be seen.

Epigenetics continues to be a hot topic, so there's more to think about! If environmental causes of epigenetic change persist in a unique or isolated location or demographic, could the natural selection of epigenetic traits result in geographic or demographic differences in epigenetic characters just as it has done for genetic characters? Can we trace an epigenetic ancestry in the same way as our genetic one? That seems to be the case in a recent comparison of patterns of methylation of DNA between Mexican and Puerto Ricans of Hispanic origin. The study revealed that DNA regions known to be sensitive to diesel emissions or intra-uterine exposure to tobacco (and even social stressors) were differentially methylated in the two groups. A summary of this study (elifesciences-articles/20532.pdf) is at biotechniques.com/epigenetics-culture-&-ethnicity.

Finally, can we think of epigenetic selection of what are clearly harmful traits, as instead analogous to genetic selection of β-globin gene mutations (e.g., sickle cell anemia) that confer a survival advantage in one context (a prevalence of malarial parasites in mosquitoes) but are harmful in another? Here's a bottom line you cannot avoid! Can you be sure that your smoking habit *won't* affect the health of your children or grandchildren?

What about your eating habits? Drinking? It is not *just a little* scary to know that I have a gullible germline epigenome that can be influenced by *my* behavior, good and bad. And that my children (and maybe grandchildren) will inherit my epigenetic legacy long before they get my house and my money. And even that may not be the limit; epigenetic memory in *C. elegans* can stretch to 14 generations (see Epigenetic Memory in Caenorhabditis elegans)! Also, read about epigenetic inheritance resulting from Dad's cocaine use at Sins of the Father.

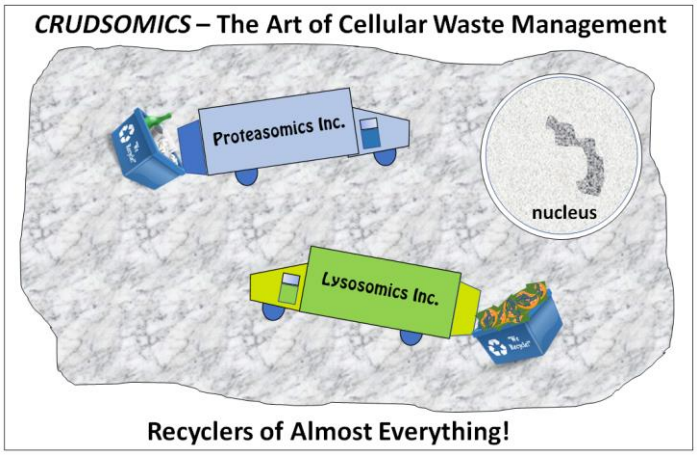
Some iText & VOP Key Words and Terms

10 nm fiber	**galactose**	**pseudogene**
3' non-transcribed DNA	**galactoside**	**PTS**
30 nm solenoid fiber	**gene activation**	**regulatory genes**
5' non-transcribed DNA	**gene derepression**	**second messenger**
adaptive immune system	**gene expression**	**nucleosomes**
adult stem cells	**gene induction**	**O_1 and O_2 lac operators**
Allolactose	**gene regulation**	**operator**
antisense RNA	**gene repression**	**operon regulation**
basic v. non-basic proteins	**HAT enzymes**	**PEP-dependent P-transferase system**
beads-on-a-string	**helix-turn-helix motif**	**phage DNA**
β-galactosidase	**heterochromatin**	**phosphodiester backbone**
cAMP	**histone acetylation**	**phosphorylation**
cAMP receptor protein	**histone kinases**	**pluripotent cells**
CAP protein	**histone methyl transferases**	**polycistronic mRNA**

CAT box	histone methylation	positive regulation
catabolite activator protein	histone phosphorylation	promoter
chromatin remodeling	housekeeping genes	proximal regulatory element
cis-acting elements	inducer exclusion	signal transduction
condensed chromatin	interphase	steroid hormone
developmental program	introns	steroid hormones
differential gene	lac operon	structural genes
distal regulatory element	lacI gene	TATA box
DNA bending	lactose	tetrameric lac repressor
DNAse	lactose permease	totipotent cells
embryonic stem cells	lactose repressor	transcription factors
enhancers	lacZ, lacY and lacA genes	transcription regulation
environmental signals	leucine zipper motif	transcription start site
epigenome	levels of chromatin structure	translation regulation
euchromatin	major groove	trp operon
exons	minor groove	trp repressor
extended chromatin	miRNA (micro RNA)	zinc finger motif
fully differentiated cells	negative regulation	

Chapter 13: Post Transcriptional Regulation of Gene Expression

Regulating gene expression with short and long non-coding RNAs, CRISPR-Cas and protein phosphorylation; Control gene expression by regulating translation



13.1 Introduction

The metabolic potential of cells is flexible, depending on various mechanisms that ultimately determine the levels and activities of proteins that dictate a cell's metabolic state. We have seen some of these regulatory mechanisms:

- the *regulation of transcription* by extracellular chemical signals or developmental chemical prompts, and
- the control of enzyme or other protein activity by *allosteric regulation* or chemical modification (e.g., *phosphorylation* or *dephosphorylation*).

In this chapter, we look at different kinds of ***post-transcriptional regulation***, events somewhere between mRNA transcription and controls on the activity of finished proteins. These control mechanisms are most diverse in eukaryotes. Like other pathways for regulating gene expression, post-transcriptional regulation begins with extracellular chemical signaling. Responses include changes in the rate of polypeptide translation, and changes in macromolecular ***turnover rate*** (e.g., changes in the ***half-life*** of specific RNAs and proteins in cells). Regardless of mechanism, each up- or down-regulation of gene expression contributes to changes in the ***steady state*** of a particular RNA or protein

required for proper cell function. In considering *post-transcriptional regulation*, we will see how cells use specific proteins and different non-coding RNA transcripts to target unwanted proteins or RNAs for degradation.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain what it is about *C. elegans* makes it a model organism for studying development and the regulation of gene expression.
2. compare and contrast the origins and functions of *miRNA* and *siRNA.*
3. search for examples of *miRNAs, siRNAs,* lncRNAs and circRNAs that regulate the expression of specific genes and explain their mechanisms.
4. explain how a *riboswitch* functions to control bacterial gene expression.
5. explain the origins and roles of bacterial *CRISPR-Cas immune* system components.
6. explain how *eif2* activity is modulated to coordinate red cell heme and globin levels.
7. describe how eukaryotic cells degrade unwanted proteins and speculate on how bacteria might do so.
8. answer the questions "How did *junk DNA* arise?" and "Does junk DNA have value?"

13.2 Post-transcriptional Control of Gene Expression

Not too long ago we thought that very little of the eukaryotic genome was ever transcribed. We also thought that the only non-coding RNAs were tRNAs and rRNAs. Now we know that other RNAs play roles in gene regulation and the degradation of *spent* cellular DNA or unwanted foreign DNA. These are discussed in detail below.

13.2.1 Riboswitches

The *riboswitch* is a bacterial transcription mechanism for regulating gene expression. While this mechanism is not specifically post-transcriptional, it is included here because the action occurs after transcription initiation and aborts completion of an mRNA. When the mRNA for an enzyme in the guanine synthesis pathway is transcribed, it folds into stem-&-loop structures. Enzyme synthesis will continue for as long as the cell needs to make guanine. But if guanine accumulates in the cell, excess guanine will bind stem-loop elements near the 5' end of the mRNA, causing the RNA polymerase and the partially completed mRNA dissociate from the DNA, prematurely ending transcription. The ability to form folded, stem-loop structures at the 5' ends of bacterial mRNAs seems to have allowed the evolution of translation regulation strategies. Whereas guanine interaction with the stem-loop structure of an emerging 5' mRNA can abort its own transcription, similar small metabolite/mRNA and even protein/mRNA interactions can also regulate (in this case prevent) translation. The basis of guanine riboswitch regulation of expression of a guanine synthesis pathway enzyme is shown below in Fig. 13.1.

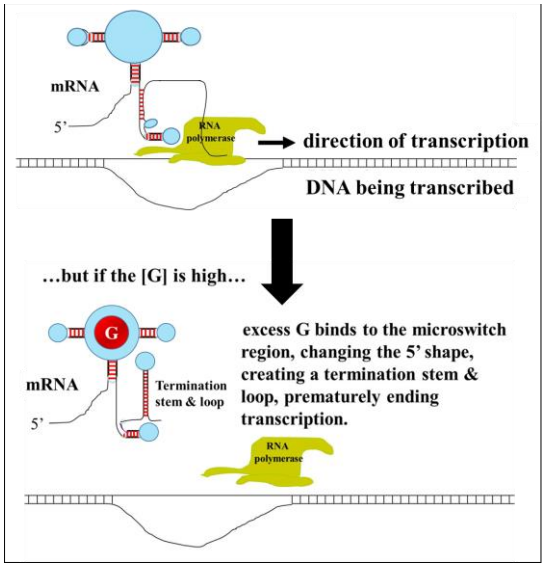


Fig. 13.1: Bacterial guanine riboswitch: The 'switch' is an mRNA encoding an enzyme in the guanine synthesis pathway. Excess guanine binds and distorts the stem-loop structure of the mRNA, causing termination of further gene transcription.

As we will see shortly, 5' mRNA folded structures also play a role in eukaryotic translation regulation.

232 Riboswitches Interrupt Bacterial Transcription

233 Small Metabolites Also Regulate Bacterial mRNA translation

13.2.2 CRISPR/Cas: a Prokaryotic Adaptive Immune System

In higher organisms, the *immune system* is *adaptive*. It remembers prior exposure to a pathogen and can thus mount a response to a second exposure to the same pathogen. The discovery of an '*adaptive immune system'* in many prokaryotes (bacteria, archaebacteria) was therefore something of a surprise. The CRISPR story begins in 1987 in Japan with the discovery by Y. Ishino et al. of cluster of regularly-interspaced short palindromic DNA sequences in *E. coli*. Clustered DNA repeats were known of course, but they were always consecutive and uninterrupted by other

sequences (think rRNA genes). Ishiino's cluster was at the time of unknown origin and function. In 1993 similar clustered repeats were found in several strains of *Mycobacterium tuberculosis* (the cause of tuberculosis), but the interspaced sequences interrupting the palindromic repeats differed between the strains. By 2001, such clustered, interrupted repeats, by then found in many prokarya and archaea, were named **CRISPR** (***C***lustered ***R***egularly ***I***nterspaced ***S***hort ***P***alindromic ***R***epeats).

The discovery of an array of ***Cas*** gene repeats linked to some of the CRISPR repeats in 2002. This was followed in 2005 by the revelation that some of the interspaced sequences were derived from non-host origins, specifically phage DNA and plasmids, and in the same year, the protein encoded by the Cas gene was shown to have helicase and nuclease activity. This led to a suggestion that CRISPR and Cas were part of a bacterial *adaptive immune system*, a bacterial response to phage infection or other invasive DNA and for the lucky bacterial survivor, protection against a second infection! In 2012 Jennifer Doudna and Emmanuelle Charpentier showed how the CRISPR/Cas9 system in streptococcal bacteria could rapidly and accurately edit any DNA sequence, including genomic DNA. Let's look more closely at CRISPR and Cas DNAs, how they work as an adaptive immune system and how they can be used to edit DNA.

CRISPR (***C***lustered ***R***egularly ***I***nterspaced ***S***hort ***P***alindromic ***R***epeat) RNAs are derived from phage transcripts that have interacted with CRISPR-Associated (*Cas*) proteins. They make up the ***CRISPR/Cas*** system that seems to have evolved to fight of viral infection by targeting phage DNA for destruction. When viral DNA gets into a cell during a phage infection, it can generate a CRISPR/Cas gene array in the bacterial genome, with ***spacer*** DNA sequences separating repeats of the CRISPR genes. These remnants of a phage infection are the *memory* of this *prokaryotic immune system*. When a phage attempts to re-infect a previously exposed cell, *spacer RNAs* and Cas genes are transcribed. After Cas mRNA translation, the Cas protein and spacer RNAs will engage and target the incoming phage DNA for destruction to prevent infection. Thus, the CRISPR/Cas systems (there is more than one!) *remember* prior phage attacks and transmit that memory to progeny cells. The CRISPR/Cas9 system in *Streptococcus pyogenes* is one of the simplest of the CRISPR/Cas immune defense systems (Fig. 13.2, below).

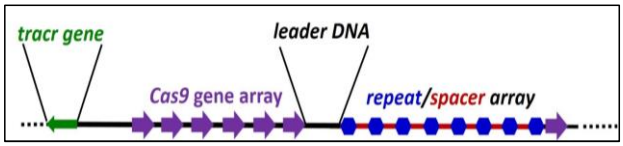


Fig. 13.2: The array of CRISPR/Cas9 genes, spacers and other components in the genome of *Streptococcus pyogenes*.

The CRISPR/Cas gene array consists of the following components:

- ***Cas***: Genes native to host cells
- ***CRISPR***: 24-48 bp repeats native to host cells
- ***Spacer DNA***: DNA between CRISPR repeats: typically, phage DNA from prior phage infection or plasmid transformation
- **leader DNA**: Contains promoter for CRISPR/spacer RNA transcription
- **tracr gene**: Encodes transcription activator (tracr) RNA (not all systems)

Let's look at CRISPR/Cas in action.

13.2.2.a The CRISPR/Cas 'Immune' Response

Consider the mechanism of action of this prokaryotic immune system. The action begins when infectious phage DNA gets into the cell (Fig. 13.3, below).

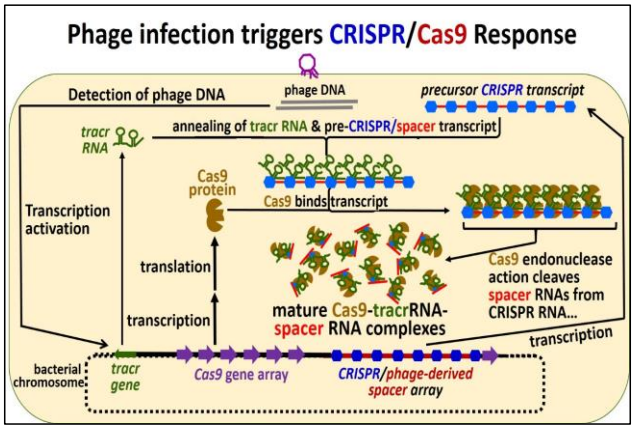


Fig. 13.3: Phage infection triggers formation of *CRISR/Cas9 array* (see text for details).

Let's summarize what has happened here:

a) *Incoming phage DNA* was detected after phage infection.
b) Then the *tracr* and *Cas* genes are transcribed along with the C*RISPR/spacer* region. *Cas* mRNAs are translated to make the *Cas* protein. Remember, the *spacer* DNAs in the CRISPR region are the legacy of a prior phage infection.
c) *CRISPR/spacer* RNA forms hydrogen bonds with a complementary region of the *tracr* RNA as the two RNAs associate with *Cas* proteins.
d) *Cas* protein endonucelases hydrolyze *spacer* RNA from *CRISPR* RNA sequences. The spacer RNAs remain associated with the complex while the actual, imperfectly *palindromic CRISPR* sequences (shown in blue in the illustration above) fall off.

In the next steps, *phage-derived spacer RNAs*, now called ***guide RNAs*** (or ***gRNAs***) 'guide' mature *Cas9/tracrRNA/spacer* RNA complexes to new incoming phage DNA resulting from a phage attack. Fig. 13.4 (below) illustrates the association of the complex with the incoming phage DNA and subsequent events.

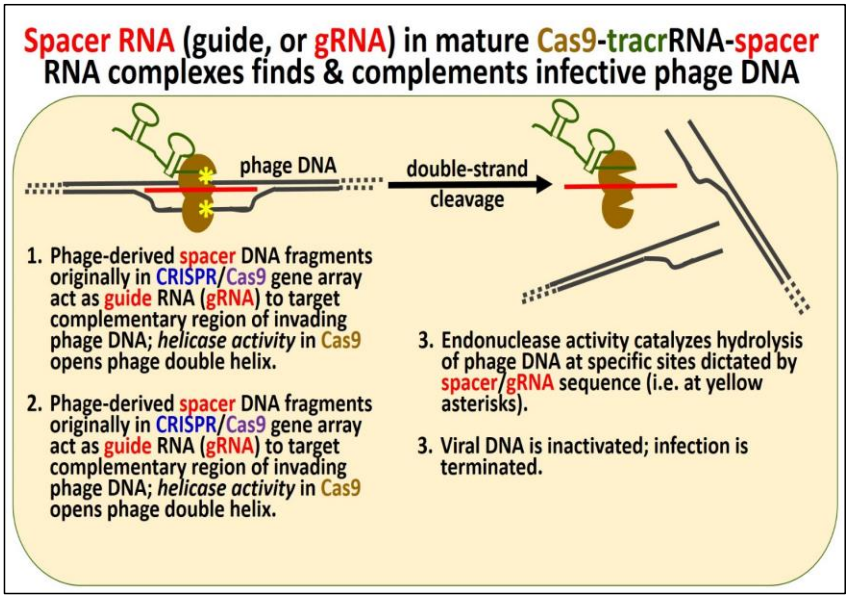


Fig. 13.4: Phage spacers in a CRISPR/Cas9 array (derived from an earlier phage infection) protects against phage re-infection (see text for details) .

Once again, let's summarize:

a) *Spacer* (i.e., *gRNA*) in the complex targets *incoming phage* DNA.
b) *Cas helicase* unwinds *incoming phage DNA* at complementary regions.
c) *gRNA* H-bonds to *incoming phage DNA*.
d) *Cas endonucleases* create a double-stranded break (hydrolytic cleavage) *at specific sites* in *incoming phage DNA*. Because precise site DNA strand cleavage is guided by RNA molecules, CRISPR/Cas endonucleases are classified as *type V restriction enzymes*.
e) The *incoming phage DNA* is destroyed and a new phage infection is aborted.

Check out More about CRISPR in Wikipedia to learn more about how bacteria acquire spacer DNAs, and therefore how this primitive adaptive immune system 'remembers') in the first place.

13.2.2.b Using CRISPR/Cas to Edit/Engineer Genes

Early studies demonstrated the reproducible cleavage of incoming phage DNA at specific nucleotides. Several labs quickly realized that it might be possible to adapt the system to cut DNA at virtually any specific nucleotide in a target DNA! It has turned out that the system works both *in vivo* and *in vitro, allowing* virtually unlimited potential for editing genes and RNAs in a test tube… or in ***any*** cell. Here is the basic process:

a) Engineer ***gDNA*** with a Cas-specific DNA sequence that targets a desired target in genomic DNA.
b) Fuse the ***gDNA*** to ***tracr DNA*** to make a *single guide DNA* (***sgDNA***) so that it can be made as a single guide transcript (***sgRNA***).
c) Engineer a ***CRISPR/Cas9*** gene array that substitutes this *sgDNA* for its original *spacer* DNAs.
d) Place engineered array in a plasmid next to *regulated promoters*.
e) *Transform* cells by '*electroporation*' (works for almost any cell type!)
f) *Activate the promoter* to transcribe the CRISPR/Cas9 genes…

The applications are powerful… and controversial!

13.2.2.c CRISPR…, the Power and the Controversy

The application of gene editing with CRISPR/Cas systems has already facilitated studies of gene function *in vitro*, in cells and in whole organisms. Click CRISPR Applications from NEB for a description of CRISPR/Cas applications already on the market! The efficiency of specific gene editing using CRISPR/Cas systems holds great promise for understanding basic gene structure and function, for determining the genetic basis of disease, and for accelerating the search for gene therapies. Here are a few examples of how CRISPR/Cas editing is being applied.

- One can engineer an sgRNA with desired mutations targeting specific sites in chromosomal DNA. Then clone sgRNA into the CRISPR/Cas9 array on a plasmid. After transformation of appropriate cells, the engineered CRISPR/Cas9 forms a complex with target DNA sequences. Following nicking of both strands of the target DNA, DNA repair can insert the mutated guide sequences into the target DNA. The result is loss or acquisition of DNA sequences at *specific, exact sites*, or ***Precision Gene Editing***. The ability to do this in living cells that has excited the basic and clinical research communities.

- Before transforming cells, engineer the ***CRISPR/Cas9*** gene array on the plasmid to eliminate both ***endonuclease*** activities from the Cas protein. Upon transcription of the array in transformed cells, the *CRISPR/Cas9-sgRNA* still finds an *sgRNA*-targeted gene. However, lacking CAS protein endonuclease

activities, the complex that forms just sits there ***blocking transcription***. This technique is sometimes referred to as ***CRISPRi*** (*CRISPER interference*), by analogy to *RNA interference* or *RNAi* (see below). Applied to organisms (and not just in vitro or to cells), it mimics the much more difficult ***knockout*** mutation experiments that have been used in studies of behavior of cells or organisms rendered unable to express a specific protein.

- There are now several working CRISPR/Cas systems capable of *Precision Gene Editing*. They are exciting for their speed, precision, their prospects for rapid, targeted gene therapies to fight disease, and their possibilities to alter entire populations (called ***Gene Drive***). By inserting modified genes into the germline cells of target organisms, gene drive can render harmless entire malarial mosquito populations, to eliminate pesticide resistance in e.g. insects, eliminate herbicide resistance in undesirable plants, or genetically eliminate invasive species. For more information, click Gene drive; for an easy read about this process and the controversies surrounding applications of CRISPR technologies to mosquitoes in particular, check out J. Adler, (2016) *A World Without Mosquitoes*. Smithsonian, 47(3) 36-42, 84.

- It is possible to delete an entire chromosome from cells. This bit of global genetic engineering relies on identifying multiple unique sequences on a single chromosome and then targeting these sites for CRISPR/Cas. When the system is activated, the chromosome is cut at those sites, fragmenting it beyond the capacity of DNA repair mechanisms to fix the situation. Imagine the possibilities for therapeutically removing **aneuploidies**, e.g., extra chromosomes such as found in ***trisomy 21*** (the cause of *Down's Syndrome),* or those correlated with some cancers. Click Using CRISPR/Cas9 to delete a chromosome to learn more.

- For yet another twist on CRISPR power, check CRISPR-Based ID of genes in situ in Chromatin in Fixed Cells to read about a technique to identify and locate specific DNA sequences in cells without disturbing chromatin structure.

But a 'dark side' of the CRISPR/Cas (and now, related gene editing technologies) was quickly recognized by its practitioners, notably Jennifer Doudna herself. If for no other reason than its efficiency and simplicity, applications of CRISPR to precision gene editing has raised ethical issues. Clearly, the potential exists for abuse or even for use with no beneficial purpose at all. It is significant that, as in all discussions of biological ethics, scientists are very much engaged in the conversation. Listen to Dr. Doudna talk about CRISPR and its potential to do good in the world, and its potential to be abused by the foolish and unscrupulous (https://www.youtube.com/watch?v=TdBAHexVYzc).

We will no doubt continue to edit genes with CRISPR/Cas, and we can look for a near future Nobel Prize for its discovery and application! If you still have qualms, maybe RNA editing will be the answer. Check out the link at Why edit RNA? for an overview of the possibilities! Finally, "*mice and men*" (and women and babies too) have antibodies to Cas9 proteins, suggesting prior exposure to microbial CRISPR/Cas9 antigens. This observation may limit clinical applications of the technology! See Uncertain Future of CRISPR-Cas9 Technology.

13.2.3 The Small RNAs: miRNA and siRNA in Eukaryotes

Micro RNAs (miRNAs) and small interfering RNAs (siRNAs) are found in *C. elegans*, a small nematode (roundworm) that quickly became a model for studies of cell and molecular biology and development shown in a fluorescence micrograph in Fig. 13.5 below.

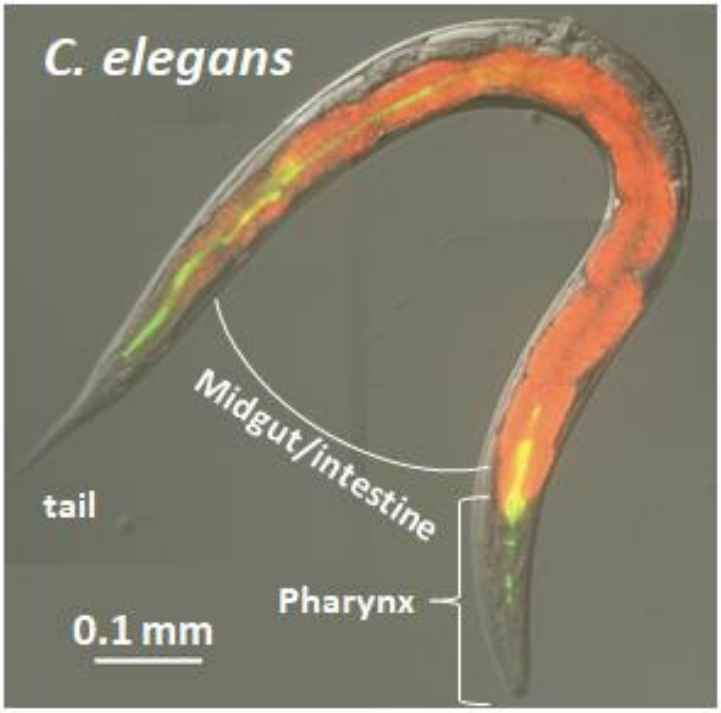


Fig. 13.5: Anatomy of the roundworm *Caenorhabditis elegans.*

The particular attractions of *C. elegans* are (a) its genome has ~21,700 genes, comparable to the ~25,000 genes in a human genome!; (b) it uses the products of these genes to produce an adult worm consisting of just 1031 cells organized into all of the major organs found in higher organisms; (c) it is possible to trace the embryonic origins of every single cell in its body!

13.2.3.a Small Interfering RNA (siRNA)

siRNA was first found in plants as well as in *C. elegans*. However, siRNAs (and miRNAs) are common in many higher organisms. siRNAs were so-named because they *interfere* with the function of other RNAs foreign to the cell or organism. Their action was dubbed *RNA interference* (***RNAi***). For their discovery of siRNAs, A. Z. Fire and C. C. Mello shared the 2006 Nobel Prize in Physiology or Medicine. Fig 13.6 below illustrates the action of siRNA targeting foreign DNA.

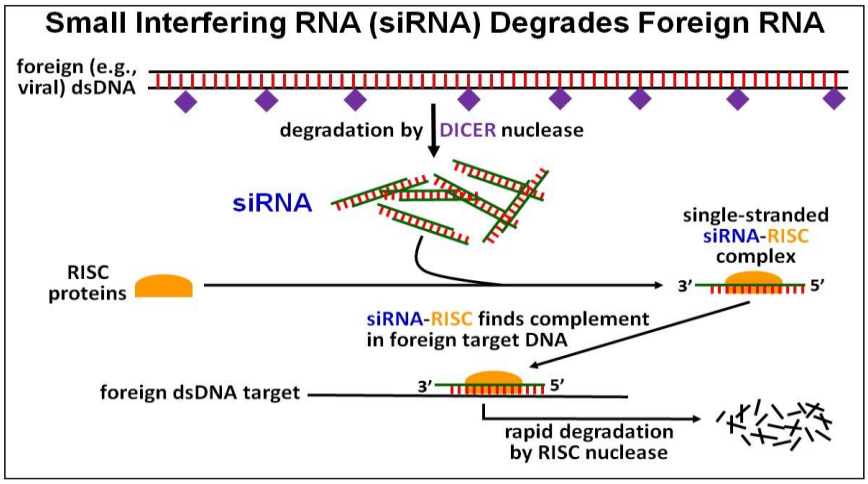


Fig. 13.6: Mechanism of action of siRNA (small interfering RNA) defense against foreign DNA (see text for details).

When cells recognize foreign double-stranded RNAs (e.g., some viral RNA genomes) as alien, the ***DICER*** a *nuclease* called hydrolyzes them. The resulting short double-stranded hydrolysis products (the ***siRNAs***) combine with ***R****NAi* ***I****nduced* ***S****ilencing* ***C****omplex* (***RISC***) proteins. The *antisense* siRNA strand in the resulting ***siRNA-RISC*** complex binds to complementary regions of foreign RNAs, targeting them for degradation. Cellular use of RISC to control gene expression in

this way may have derived from the use of RISC proteins by miRNAs as part of a cellular defense mechanism, to be discussed next. Custom-designed siRNAs have been used to disable expression of specific genes in order to study their function *in vivo* and *in vitro*. Both siRNAs and miRNAs are being investigated as possible therapeutic tools to interfere with RNAs whose expression leads to cancer or other diseases.

234 siRNA Post Transcriptional Regulation

235 Did siRNA Coopt RISC strategy to Trash Corrupt or Worn out RNA?

For an example check out a YouTube video of unexpected results of an RNAi experiment at https://www.youtube.com/watch?v=-3XboKthxM8. In the experiment described, RNAi was used to block embryonic expression of the ***orthodenticle*** (***odt***) gene that is normally required for the growth of horns in a dung beetle. The effect of this *knock-out mutation* was, as expected, to prevent horn growth. What was unexpected however, was the development of an eye in the middle of the beetle's head ('third eye' in the micrograph). The 3rd eye not only looks like an eye, but is a functional one. This was demonstrated by preventing normal eye development in *odt*-knockout mutants. The 3rd eye appeared and was responsive to light! Keep in mind that this was a beetle with a 3rd eye, not *Drosophila*! To quote Justin Kumar from Indiana University, who though not involved in the research, stated that "…lessons learned from *Drosophila* may not be as generally applicable as I or other Drosophilists, would like to believe … The ability to use RNAi in non-traditional model systems is a huge advance that will probably lead to a more balanced view of development."

13.2.3.b Micro RNAs (miRNA)

miRNAs target unwanted *endogenous* cellular RNAs for degradation. miRNAs are transcribed from genes now known to be widely distributed in eukaryotes. As they are transcribed, pre-miRNAs fold into a stem-loop structure that is lost during cytoplasmic processing. Like SiRNAs, mature miRNAs combine with ***RISC*** proteins. The RISC protein-miRNA complex targets old or no-longer needed mRNAs or mRNAs damaged during transcription. An estimated 250 miRNAs in humans may be sufficient to H-bond to diverse target RNAs; only targets with strong complementarity to a RISC protein-miRNA complex will be degraded. The pathway from pre-miRNA transcription through processing ultimately to target mRNA degradation is shown in Fig. 13.7, below.

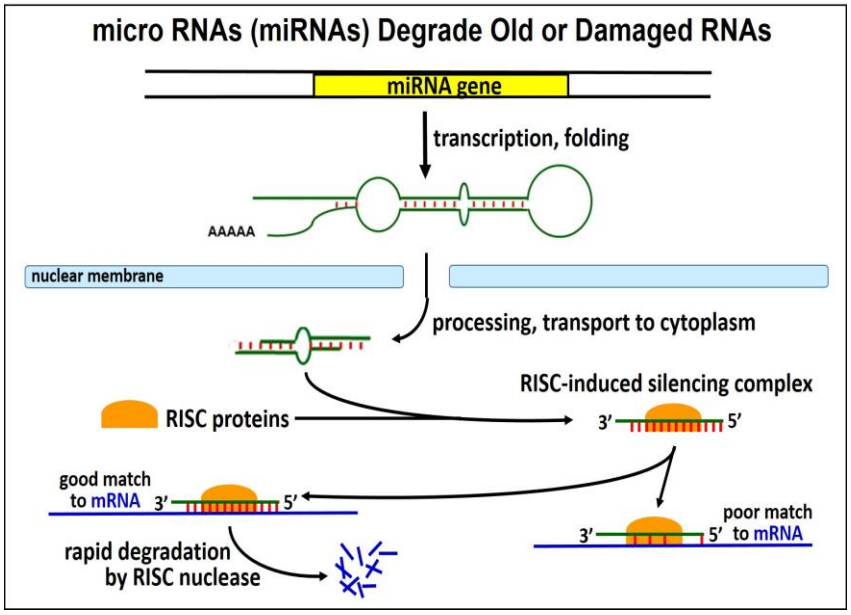


Fig. 13.7: Mechanism of action of miRNA (micro RNA) in degrading unwanted (e.g., old or damaged) RNA (see text for details).

236 miRNA Post-Transcriptional Regulation

13.2.4 Long Non-Coding RNAs

Long non-coding RNAs (***lncRNAs***) are a yet another class of eukaryotic RNAs. The latter include transcripts of antisense, intronic, intergenic, pseudogene and retroposon DNA. Retroposons are one kind of transposon, or mobile DNA element; pseudogenes are recognizable genes with mutations that make them non-functional. While some *lncRNAs* might turn out to be incidental transcripts that the cell simply destroys, others have a role in regulating gene expression.

A recently discovered lncRNA is XistAR that, along with the Xist gene product, is required to form *Barr bodies*. Barr bodies form in human females when one of the X chromosomes in somatic cells is inactivated. For a review of lncRNAs, see Lee, J.T. (2012. *Epigenetic Regulation by Long Noncoding RNAs*; Science 338, 1435-1439).

An even more recent article (at ***l***ncRNAs and smORFs) summarizes the discovery that some long non-coding RNAs contain short open reading frames (***smORFs***) that are actually translated into short peptides of 30+ amino acids! Who knows? The human genome may indeed contain more than 21,000-25,000 protein-coding genes!

13.2.5 Circular RNAs (circRNA)

Though discovered more than 20 years ago, circular RNAs (circRNAs) are made in different eukaryotic cell types. Click Circular RNAs (circRNA) to learn more about this peculiar result of *alternative* splicing. At first circRNAs were hard to isolate. When they were isolated, circRNAs contained "scrambled" exonic sequences and were therefore thought to be nonfunctional errors of mRNA splicing. In fact, circRNAs are fairly stable. Their levels can rise and fall in patterns suggesting that they are functional molecules. Levels of one circRNA, called ***circRims1***, rise specifically during neural development. In mice, other circRNAs accumulate during synapse formation, likely influencing how these neurons will ultimately develop and function. Thus, circRNAs do not seem to be 'molecular mistakes'. In fact, *errors in their own synthesis* may be correlated with disease! Speculation on the functions of circRNAs also includes roles in gene regulation, particularly the genes or mRNAs from which they themselves are derived.

13.3 "Junk DNA" in Perspective

Not long ago we thought that less than 5% of a eukaryotic genome was transcribed (i.e., as mRNA, rRNA and tRNA), and that much of the non-transcribed genome served a structural function… or no function at all! The latter, labeled *junk DNA*, included non-descript intergenic sequences, pseudogenes, 'dead' transposons, long stretches of intronic DNA, etc. Thus, junk DNA was DNA we could do without. Junk DNAs were thought to be accidental riders in our genomes, hitchhikers picked up on the evolutionary road. But, while miRNA genes are a small proportion of a eukaryotic genome, their discovery, and that of more abundant *lnc RNAs* suggest a far greater amount of functional DNA in the genome. Might there be in fact, no such thing as "junk DNA"?

The debate about how much of our genomic DNA is a relic of past evolutionary experiments and without genetic purpose continues. Read all about it at Junk DNA - not so useless after all and Only 8.2% of human DNA is functional. Perhaps we need to re-think what it means for DNA to be "junk" or to be without "genetic purpose". Maintenance of more than 90% of our own DNA with no known genetic purpose surely comes at an energy cost. At the same time, all of that DNA is grist for future selection, a source of the diversity required for long-term survival. The same natural selection that picks up 'hitchhiker' DNA sequences, as we have seen, can at some point, put them to work!

13.4 The RNA Methylome

Call this an *RNA epi-transcriptome* if you like! Recall that methyl groups direct cleavage of ribosomal RNAs from eukaryotic 45S pre-RNA transcripts. tRNAs among other transcripts are also post-transcriptionally modified. Known since the 1970s, such modifications were thought to be non-functional. But are they?

13.5 Eukaryotic Translation Regulation

mRNAs are made to be translated, and so they are! Nonetheless, translation is regulated, largely by controlling translation initiation. Here's a reminder of the basics of eukaryotic *initiation*… In many respects, the overall process is similar to prokaryotic translation initiation described elsewhere. The 40S ribosomal subunit itself can bind to and scan an mRNA, seeking the start site of an ORF (open reading frame) encoding a polypeptide. When GTP-bound *eukaryotic initiation factor 2* (**GTP-*eIF2***) binds met-tRNAf, it forms a *ternary complex* (**TC**). The TC can associate with the scanning 40S subunit. When a TC-associated scanning subunit encounters the start site of the ORF, scanning stalls. Additional eIFs help form the *initiation complex*, positioning the initiator tRNA anticodon over the start site AUG in the mRNA. The initiation complex then recruits the large (60S) ribosomal subunit.

Consider translation initiation in eukaryotes (Fig. 13.8, below). Note that the binding of the 60S ribosomal subunit to the initiation complex (lower left) causes the release of all the eIFs and hydrolysis of the GTP on eIF2; GDP remains bound to eIF2 upper right).

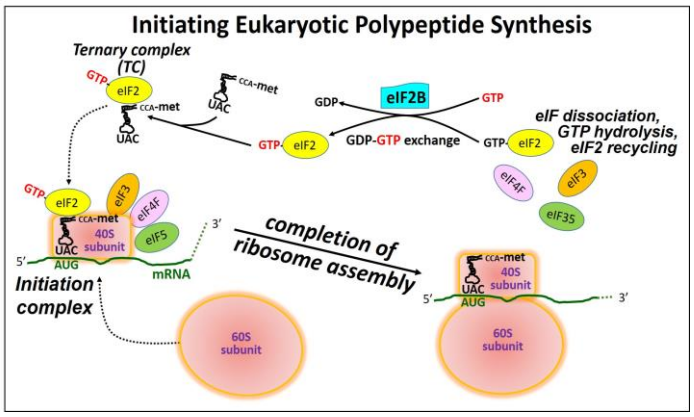


Fig. 13.8: Review of the steps in translation initiation (see text for details).

For protein syntheses to continue, new GTP must replace GDP on eIF2. Another initiation factor, ***eIF2B***, facilitates this GTP/GDP swap, recycling GTP-eIF2 for use in initiation. The regulation of translation is superimposed on these basic processes. But where? What steps in initiation are controlled?

We know that CAP and poly(A) tails on mRNAs are required for efficient translation because mRNAs engineered to lack one and/or the other are poorly translated. But there is little evidence that cells control capping or polyadenylation, or the structures themselves. Instead, translation is regulated, largely by targeting interactions with structural features and sequences in the 5' region of mRNAs. Fig. 13.9 (below) shows key structural motifs in a gene that can contribute to translational control.

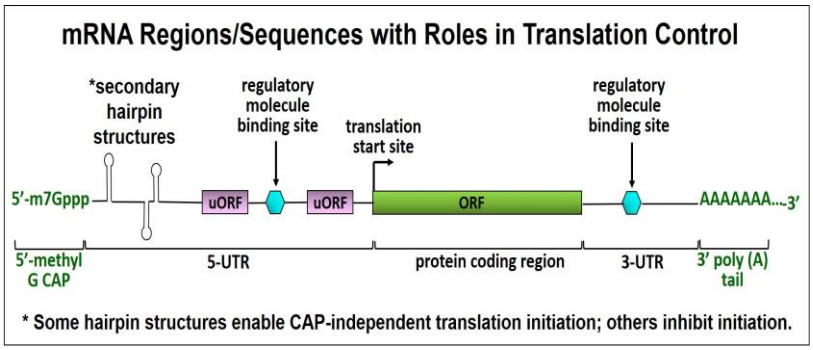


Fig. 13.9: Regions and specific sequences known to be involved in regulating translation.

Regulation of translation may be global, affecting the synthesis of many polypeptides. Or it may be specific, affecting a single polypeptide. Global regulation involves changes in the activity of eukaryotic initiation factors (eIFs) that would typically affect all cellular protein synthesis. Specific regulation involves binding sequences or regions on one or a few mRNAs that recognize and bind specific regulatory proteins and/or other molecules. In the latter case, such binding controls translation of only those mRNAs, without affecting general protein biosynthesis. We will consider three examples of translational control of gene expression.

13.5.1 Specific Translation Control by mRNA Binding Proteins

Translation initiation complexes typically scan the 5'-UTR of an mRNA. When it finds the translation start site, it can bind the large subunit and begin translating the polypeptide. Here we consider the control of synthesis of ***ferritin***, a cytplasmic iron-binding protein. Iron is of course insoluble and must be bound to proteins, both in cells

and in the blood (the latter is a story for another time and place!). Once in the cell, iron binds to the ***ferritin***. As you may guess, cellular iron metabolism depends of levels of ferritin in the cell, which is regulated at the level of translation.

Ferritin is actually a two-subunit protein, and the 5'-UTR of the mRNAs for both polypeptide chains contain iron-responsive elements (IREs). These IREs are stem-loop structures that specifically recognize and bind ***iron regulatory proteins*** (***IRP1***, ***IRP2***). In iron-deficient cells, the ***IRPs*** normally bind to the IRE to block ferritin translation as illustrated in Fig. 13.10 (below).

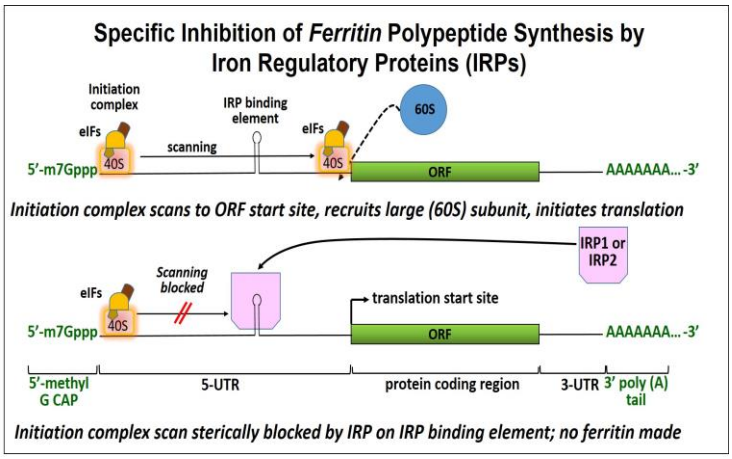


Fig. 13.10: Specific inhibition of ferritin polypeptide synthesis by iron regulatory proteins.

Normally, the initiation complex scans the 5'-UTR of an mRNA. When it finds the normal translation start site, it can bind the large subunit and begin translating the polypeptide. In iron-deficient cells, scanning by the initiation complex is thought to be physically blocked by steric hindrance.

13.5.2 Coordinating Heme and Globin Synthesis

Consider that *reticulocytes* (the precursors to *erythrocytes*, the red blood cells in mammals) synthesize ***globin*** proteins. They also synthesize ***heme***, an iron-bound porphyrin-ring molecule. Each globin must bind to a single heme to make a hemoglobin protein subunit. Clearly, it would not do for a reticulocyte to make too much globin protein and not enough heme, or *vice versa*. It turns out that ***hemin*** (a precursor to heme) regulates the initiation of translation of both α– and β–globin mRNAs. Recall that, to sustain globin mRNA translation, the ***GDP-eIF2*** generated

after each cycle of translation elongation must be exchanged for fresh GTP. This is facilitated by the ***eIF2B*** initiation factor. ***eIF2B*** can exist in phosphorylated (inactive) or un-phosphorylated (active) states. Making sure that globin is not under- or overproduced relative to heme biosynthesis involves controlling levels of active *vs*. inactive *eIF2B* by ***hemin***. Hemin accumulates when there is not enough globin polypeptide to combine with heme in the cell.

Excess hemin binds and inactivates an ***HCR kinase***, preventing phosphorylation of *eIF2B*. Since unphosphorylated *eiF2B* is active, it facilitates the GTP/GDP swap needed to allow continued translation. Thus, ongoing initiation ensures that globin mRNA translation can keep up with heme levels. In other words, if hemin production gets ahead of globin, it will promote more globin translation. When globin and heme levels become approximately equimolar, hemin is no longer in excess. It then dissociates from the active HCR kinase. The now- active kinase catalyzes *eIF2B* phosphorylation. *Phospho-eIF2B* is inactive and cannot facilitate the GTP/GDP swap on eIF2. Globin mRNA translation initiation, thus blocked, allows a lower rate of globin polypeptide translation to keep pace with heme synthesis. Fig. 13.11 (below) illustrates the regulation of globin mRNA translation initiation by hemin.

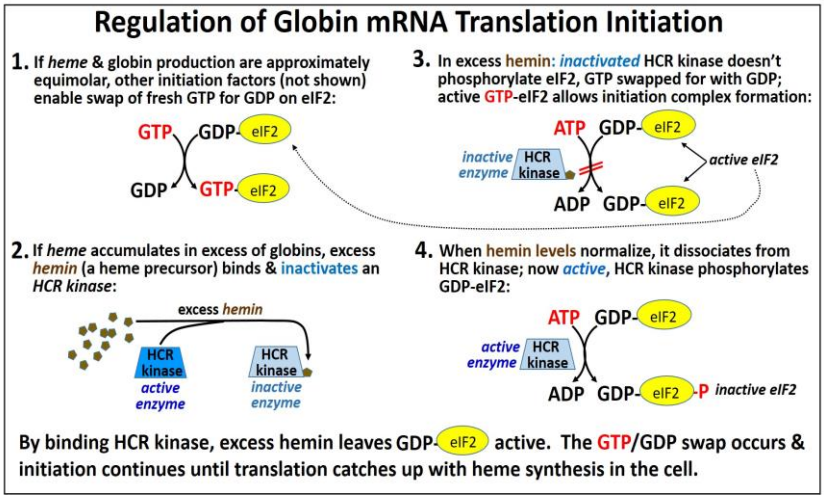


Fig. 13.11: Regulation of globin mRNA translation initiation by hemin, a heme precursor (see text for details)

237 Translation Regulation of Globin Polypeptide Synthesis

13.5.3 Translational Regulation of Yeast GCN4

Like the coordination of heme and globin production, the regulation of the GCN4 protein is based on controlling the ability of the cells to swap GTP for GDP on eIF2. However, this regulation is quite a bit more complex, despite the fact that yeast is a more primitive eukaryote! GCN4 is a ***global transcription factor*** that controls the transcription of as many as 30 genes in pathways for the synthesis of 19 out of the 20 amino acids! The discovery that amino acid starvation caused yeast cells to increase their production of amino acids in the cells led to the discovery of the *General Amino Acid Control* (***GAAC***) mechanism involving GCN4. GCN is short for *General Control Nondepressible*, referring to its global, positive regulatory effects. It turns out that the GCN4 protein is also involved in stress gene expression, glycogen homeostasis, purine biosynthesis…, in fact in the action of up to 10% of all yeast genes! Here we focus on the GAAC mechanism.

Yeast cells provided with ample amino acids do not need to synthesize them. Under these conditions, GCN4 is present at basal (i.e., low) levels. On the other hand, when the cells are starved of amino acids, GCN4 levels increase as much as ten-fold within two hours, resulting in an increase in general amino acid synthesis. Amino acid starvation signals an increase in the activity of ***GCN2***, a *protein kinase*. The GCN2 kinase catalyzes phosphorylation of GDP-eIF2. As we have seen, phosphorylated eIF2B cannot exchange GTP for GDP on the eIF2, with results shown in Fig.13.12.

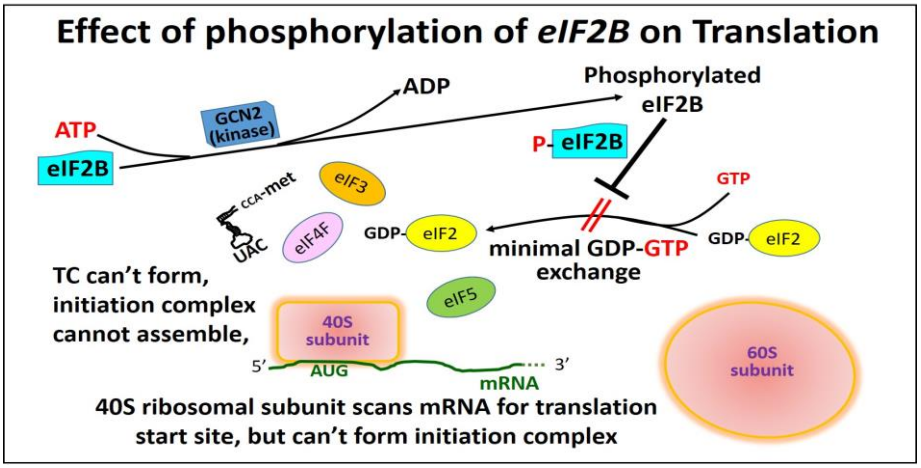


Fig. 13.12: Role of GCN2 kinase in regulating translation by phosphorylating initiation factor eIF2B, preventing formation of an initiation complex (see text for details).

There is a paradox here. You would expect a slowdown in GTP-eIF2 regeneration to inhibit overall protein synthesis, and it does. However, the reduced levels of GTP-eIF2 somehow also stimulate translation of the GCN4 mRNA, leading to increased transcription of the amino acid synthesis genes. In other words, amino acid starvation leads yeast cells to use available substrates to make their own amino acids in order that protein synthesis can continue… at the same time as initiation complex formation is disabled! Let's accept that paradox for now and look at how amino acid starvation leads to increased translation of the GCN4 protein and the up-regulation of amino acid biosynthesis pathways. To begin with, we are going to need to understand the structure of GCN4 mRNA. In Fig. 13.13 below, note the 4 short ***uORF***s in the 5'UTR of the RNA; these play a key role in GCN4 translation regulation.

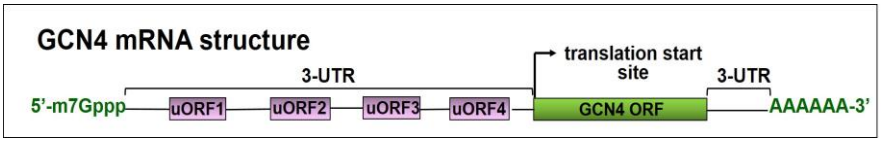


Fig. 13.13: Multiple short *open reading frames* (u*ORF*s) in the 5' untranslated region (*5'UTR*) of *GCN4* mRNA play a role in regulating its translation.

Recall that eIF2 binds with GTP and the initiator met-tRNA to form a ternary complex (TC). .We also noted earlier TC-associated 40S ribosomal subunits scan mRNAs to find open reading frame translation start sites, allowing initiation complexes form. When a 60S ribosomal subunit binds, polypeptide translation starts. But while uORFs in the GCN4 mRNA encode only a few amino acids before encountering a stop codon, they can also be recognized during scanning. When TCs and 40S subunits are plentiful, they engage uORFs in preference to the GCN4 coding region ORF (Fig. 13.14).

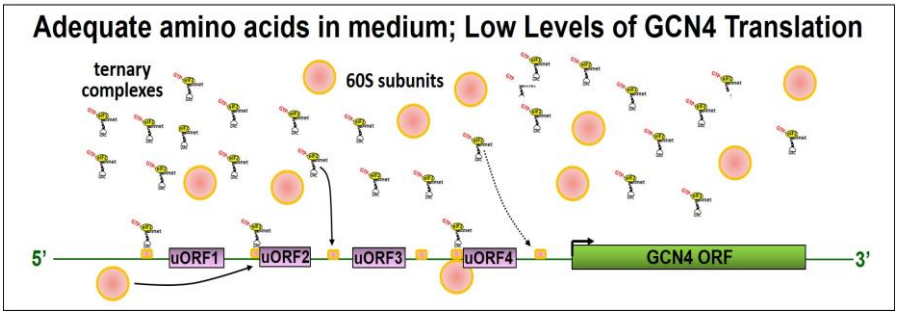


Fig. 13.14: GCN4 translation is reduced when cellular amino acid levels are normal (see text for details).

Under these conditions, active eIF2B allows the GTP/GDP swap on GDP-eIF2, leading to efficient GTP-eIF2 recycling and high TC levels. TCs bound to small (40S) subunits during scanning and/or at the start sites of uORFs, form initiation complexes that then bind 60S ribosomal subunits and begin uORF translation. The effect is to slow down scanning past the uORFs, thereby inhibiting initiation complex formation at the actual GCN4 ORF.

What happens in amino acid-starved cultures of yeast cells, when GTP-eIF2 cannot be efficiently regenerated and TCs are in short supply? To review, amino acid starvation signals an increase in GCN2 kinase activity resulting in phosphorylation and inactivation of eIF2B. Inactive *phospho*-eIF2 will not facilitate the GTP/GDP swap at GDP-eIF2, inhibiting overall protein synthesis. The resulting reduction in GTP-eIF2 also lowers the levels of TC and TC-associated 40S subunits. In Fig. 13.15, see how this phenomenon up-regulates GCN4 translation, even as the translation of other mRNAs has declined.

Under these conditions, initiation complex formation at uORFs is less likely, and scanning by remaining TC-40S complexes is more likely to reach the GCN4 ORF and increase GCN4 translation! The increased production of the gene-regulatory GCN4 protein then turns on virtually all of the genes for enzymes of amino acid biosynthesis. The cells thus make their own amino acids when the medium has few to contribute! For a good review of the translation-level regulation in general, and of GCN4, click Regulation of GCN4 Translation.

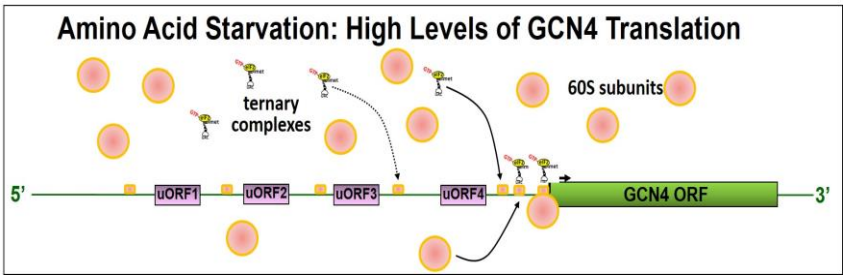


Fig. 13.15: GCN4 translation is highest when cellular amino acid levels are reduced (amino acid starvation). GCN4 then regulates the expression of many genes, including those encoding enzymes needed for amino acid synthesis (see text for details).

13.6 Protein Turnover in Eukaryotic Cells: Regulating Protein Half-Life

We have already seen that organelles have a finite life span, or half-life. Recall that lysosomes participate in destroying worn out mitochondria, including their molecular components. We also saw the role of small RNAs (especially *miRNA*) in destroying old,

damaged or otherwise unwanted RNAs from cells. All cell structures and molecules have a finite ***half-life***, defined as the time it takes for half of them to disappear in the absence of new synthesis of the structure or molecule. As we already know, the steady-state level of any cellular structure or molecule exists when the rate of its manufacture or synthesis is balanced by the rate of its turnover. Of course, steady state levels of things can change. For example, the level of gene expression (the amount of a final RNA or protein gene product in a cell) can change if rates of transcription, processing or turnover change. We should also expect the same for the steady-state levels of cellular proteins. Here we consider the factors that govern the half-life of cellular proteins.

The half-life of different proteins seems to be inherent in their structure. Thus, some amino acid side chains are more exposed at the surface of the protein and are thus more susceptible to change or damage over time than others. Proteins with fewer 'vulnerable' amino acids should have a longer half-life than those with more of them. Proteins damaged by errors of translation, folding, processing gone awry or just worn out from use or 'old age' will be targeted for destruction. All molecules have a half-life! The mechanism for detecting and destroying unwanted old, damaged or misbegotten proteins involves a 76-amino acid polypeptide called ***ubiquitin*** that targets the protein for destruction, delivering it to a large complex of polypeptides called the ***proteasome***. Here is what happens:

1. The first step is to activate an ubiquitin. This starts when ATP hydrolysis fuels the binding of ubiquitin to an ***ubiquitin-activating enzyme***.

2. An ***ubiquitin-conjugating enzyme*** then replaces the ubiquitin-activation enzyme.

3. The protein destined for destruction replaces the ubiquitin-conjugating enzyme.

4. Several more ubiquitins then bind to this complex.

5. The ***poly-ubiquinated protein*** delivers its protein to one of the 19S 'CAP' structures of a proteasome.

6. After binding to one of the CAP structures of a proteasome, the poly-ubiquinated target proteins dissociate and the ubiquitins are released and recycled as the target protein unfolds (powered by ATP hydrolysis). The unfolded protein then enters a 20S core proteasome.

The target protein is digested to short peptide fragments by proteolytic enzymes in the interior of the proteasome core. The fragments are release from the CAP complex at the other end of the proteasome and digested down to free amino acids in the cytoplasm.

There is a mind-boggling variety of proteins in a cell…, and there are as many as 600 different ubiquitin proteins, encoded by as many genes! Presumably, each ubiquitin handles a subclass of proteins based on common features of their structure. The structurally complex 26S proteasome is smaller than a eukaryotic small ribosomal subunit (40S), but it is nevertheless one of the largest cytoplasmic particles, even without the benefit of any RNA in its structure!

Fig 13.16 details the role of ubiquitin in the degradation of a worn-out protein by a proteasome. Click on Proteasomes in Action to see an animated version of this illustration.

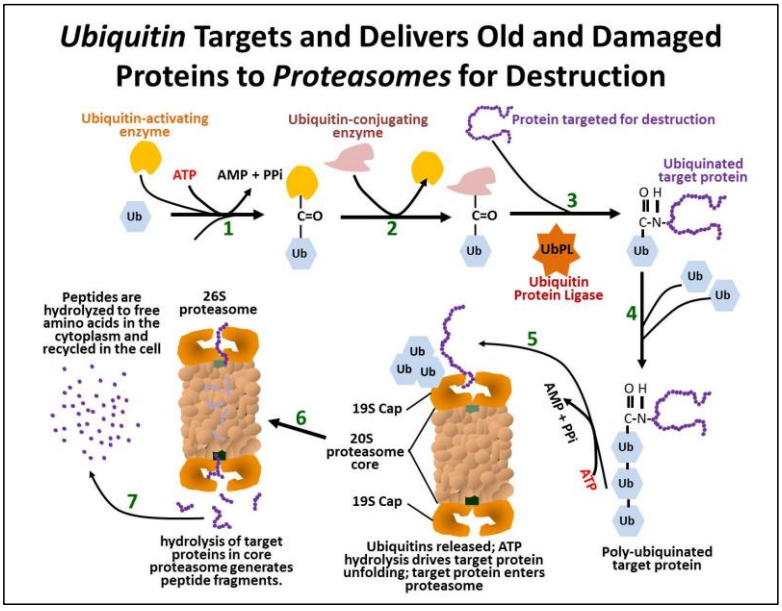


Fig. 13.15: Mechanism of ubiquitin and proteasome action in the removal/destruction of old and damaged proteins in cells, and the recycling of their amino acids (see text for details).

Some iText & VOP Key Words and Terms

19S proteasome cap complex	**gene editing**	**RISC endonuclease**
20S proteasome complex	**global transcription factor**	**RISC proteins**
amino acid starvation	**globin**	**RNA interference**

Barr Bodies	**gRNA**	**RNA turnover rates**
C. elegans	**GTP/GDP swap**	**RNAi**
Cas	**GTP-eiF2 recycling**	**RNA-induced silencing complex**
Cas helicase activity	**half life**	**sgRNA**
Cas9 endonuclease	**HCR kinase**	**siRNA (small interfering RNA)**
circRNA	**heme**	**small RNAs**
circular RNA	**hemin**	**smORF**
CRISPR	**HRC kinase**	**spacer RNA**
CRISPR interference	**Initiation complex scanning**	**steady state**
CRISPR/Cas	**iron regulatory protein**	***Streptococcus pyogenes***
CRISPR/Cas9 gene array	**IRP**	**tracr**
CRISPRi	**Junk DNA**	**tracr gene**
dicer	**lncRNA**	**tracr RNA**
eiF2 phosphorylation	**long non-coding RNA**	**translation elongation**
eiF2B	**micro RNA**	**ubiquitin**
EIIA^{Glc}	**miRNA**	**ubiquitination**
Ferritin	**mRNA scanning**	**uORF**
GAAC	**proteasome**	**XistAR**
GCN2	**protein turnover rates**	**Yeast GCN4**
GDP-eIF2	**riboswitch**	
Gene Drive	**RISC**	

Chapter 14: Repetitive DNA, A Eukaryotic Genomic Phenomenon

Mini-Satellite DNA, Microsatellite DNA, Telomeres, Ribosomal RNA genes, Transposons (Selfish, Junk DNA or Architects of the Genome?)

14.1 Introduction

Because of their small size, bacterial genomes have few ***repetitive DNA*** sequences. In contrast, repetitive DNA sequences make up a large part of a eukaryotic genome. Much of this repeated DNA consists of identical or nearly identical sequences of varying length repeated many times in a genome. Examples include ***satellite DNA*** (*minisatellite* and *microsatellite* DNA) and ***transposons***, or ***transposable elements***. Here we look at experiments that first revealed the existence and proportion of repeated DNA in genomes. Next we describe Barbara McClintock's even earlier (and pretty amazing!) discovery of transposable elements. After we describe the different classes of transposons and different mechanisms of ***transposition***, we tackle the question of why they and other repetitive DNAs even exist. Elsewhere we introduced the notion of ***junk DNA*** as DNA sequences that serve no known purpose. Is repeated DNA actually *junk DNA*? In particular, are transposable elements *junk*? We are now learning that transposons and other repetitive DNAs can have specific functions, from regulating gene expression to reshaping genomes to increasing genetic diversity in evolution. So, far from being 'junk', much redundant DNA exists in genomes because of evolutionary selection.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast *renaturation kinetic data.*
2. explain *CoT curves* and *DNA complexity.*
3. list physical and chemical properties of *main band* and *satellite DNA*s.

4. outline an experiment to determine if a given sequence of DNA is repetitive or not.
5. summarize how Barbara McClintock revealed the genetics of maize *mosaicism.*
6. outline the experiments suggesting that the *Ds* gene moves from one locus to another in the maize genome.
7. compare and contrast *cut-&-paste* and *replicative transposition.*
8. compare the behaviors of *autonomous* and *non-autonomous* transposons.
9. list the differences between *Mu phage* infection and transposition.
10. describe the common structural features of transposons.
11. compare the mechanisms of *LINE* and *SINE* transposition.
12. speculate on how species avoid potentially lethal consequences of transposition.
13. speculate on which came first in evolution: DNA transposons, RNA transposons or retroviruses, and explain your reasoning.

14.2 The Complexity of Genomic DNA

By the 1960s, when Roy Britten and Eric Davidson were studying eukaryotic gene regulation, they knew that there was more than enough DNA to account for the genes needed to encode an organism. It was also likely that DNA was more structurally complex than originally thought. They knew that ***cesium chloride (CsCl) density gradient centrifugation*** separated molecules based on differences in density and that fragmented DNA would separate into main and minor bands of different density in the centrifuge tube. The minor band was dubbed ***satellite DNA***, recalling the *Sputnik* satellite recently launched by Russia (or moons as satellites of planets!). DNA bands of different density could not exist if the proportions of A, G, T and C in DNA (already known to be species-specific) were the same throughout a genome. Instead, there must be regions of DNA that are richer in A-T than G-C pairs and vice versa. Analysis of satellite bands that moved further on the gradient (i.e., that were more dense) than the main band were indeed richer in GC content. Those that lay above the main band were more AT-rich.

Consider early estimates of how many genes it might take to make a human, mouse, chicken or petunia: about 100,000! We know now that it takes fewer! Nevertheless, even with inflated estimates of the number of genes it takes to make a typical eukaryote, their genomes contain 100-1000 times more DNA than necessary to account for 100,000 genes. How then to explain this *extra* DNA? Britten and Davidson's elegant experiments that measured DNA ***renaturation kinetics*** revealed some physical characteristics of genes and so-called 'extra' DNA. Let's look at these experiments in some detail.

14.2.1 The Renaturation Kinetic Protocol

The first step in a renaturation kinetic experiment is to shear DNA isolates to an average size of 10 Kbp by pushing high molecular weight DNA through a hypodermic needle at constant pressure. The resulting double-stranded fragments (***dsDNA***

fragments) are heated to 100^{0}C to ***denature*** (separate) the two strands. The solutions are then cooled to 60^{0}C to allow the single stranded DNA (***ssDNA***) fragments to slowly re-form complementary double strands. At different times after incubation at 60^{o}C, the partially renatured DNA was sampled and ssDNA and dsDNA were separated and quantified. The experiment is summarized in Fig. 14.1.

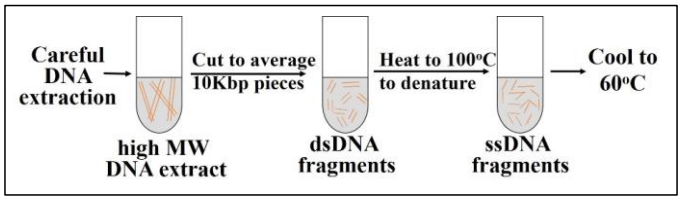


Fig. 14.1: Renaturation kinetics protocol: Double-stranded (ds)DNA is mechanically sheared to ~10Kb fragments, heated to denature the DNA, then cooled to let single strands find their complements and renature.

14.2.2 Renaturation Kinetic Data

Britten and Davidson then plotted the percent of DNA that had renatured over time. Fig. 14.2 is the plot of data from a renaturation kinetics experiment using *rat* DNA, showing the rate of dsDNA formed at different times (out to many days!).

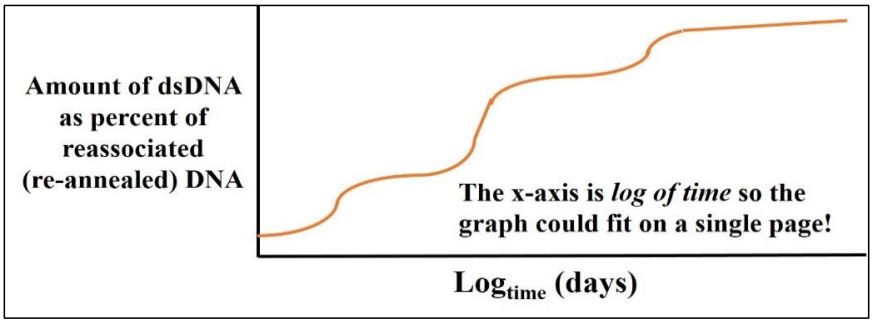


Fig. 14.2: Plot of rat dsDNA formed over time during renaturation of denatured DNA.

In this example, the DNA fragments could be placed in three main groups with different overall rates of renaturation. Britten and Davidson reasoned that the dsDNA that had formed most rapidly was composed of sequences that must be more highly repetitive than the rest of the DNA. The rat genome also had a lesser amount of more

moderately repeated dsDNA fragments that took longer to anneal than the highly repetitive fraction, and even less of a very slowly re-annealing DNA fraction. The latter sequences were so rare in the extract that it could take days for them to re-form double strands, and were classified as non-repetitive, unique (or nearly unique) sequence DNA, as illustrated in Fig. 14.3.

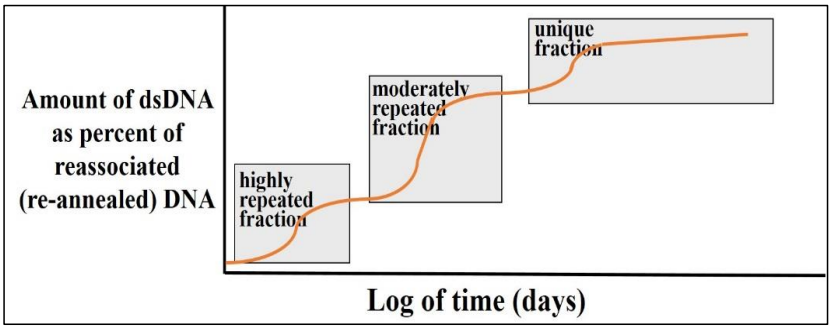


Fig. 14.3: The 3 'phases' of the curve in Fig. 14.2 highlighted to identify the 3 fractions of repeated and almost unique DNA sequences in the rat genome.

It became clear that the rat genome, and in fact most eukaryotic genomes, consists of different classes of DNA that differ in their redundancy. From the graph, a surprisingly a large fraction of the genome was repetitive to a greater or lesser extent.

238 Discovery of Repetitive DNA

When renaturation kinetics were determined for *E. coli* DNA, only one 'redundancy class' of DNA was seen (Fig. 14.4).

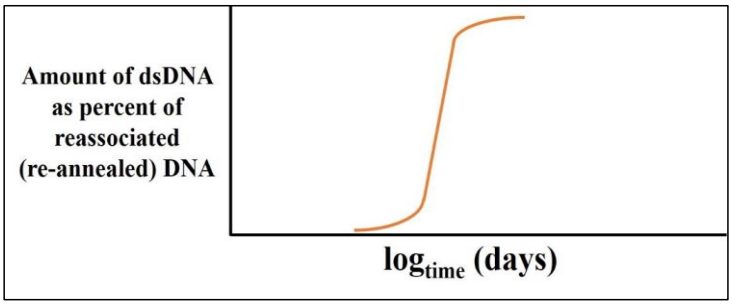


Fig. 14.4: Plot of *E. coli* dsDNA formed over time during renaturation of denatured DNA.

Based on *E. coli* gene mapping studies and the small size of the *E. coli* 'chromosome', the reasonable assumption was that there is little room for 'extra' DNA in a bacterial genome, and that the single class of DNA on this plot must be unique-sequence DNA.

14.2.3 Genomic Complexity

Britten and Davidson defined the relative amounts of repeated and unique (or single-copy) DNA sequences in an organism's genome as its ***genomic complexity***. Thus, prokaryotic genomes have a lower genomic complexity than eukaryotes. Using the same data as is in the previous two graphs, Britten and Davidson demonstrated the difference between eukaryotic and prokaryotic *genome complexity* by a simple expedient. Instead of plotting the fraction of dsDNA formed *vs.* time of renaturation, they plotted the percent of re-associated DNA against the **concentration of the re-natured DNA multiplied by the time that DNA took to reanneal** (the ***CoT*** value). When ***CoT*** values from rat and *E. coli* renaturation data are plotted on the same graph, you get the ***CoT curves*** in the graph in Fig. 14.5.

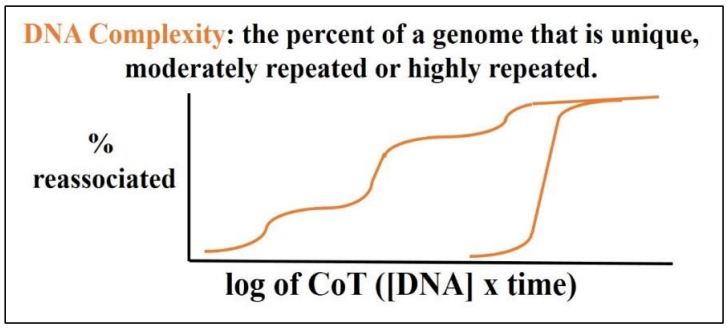


Fig. 14.5: DNA complexity revealed by plotting rat and *E. coli* DNA renaturation kinetics as the percent of re-associated dsDNA over CoT (Concentration of re-associated dsDNA X Time).

This deceptively simple extra calculation (from the same data!) allows a direct comparison of the *complexities* of different genomes. These *CoT curves* tell us that ~100% of the bacterial genome consists of unique sequences (curve at the far right), compared to the rat genome with two DNA redundancy classes…, and at the right of the curve, a small fraction of unique sequence DNA. Prokaryotic genomes are indeed largely composed of unique (non-repetitive) sequence DNA that must include single-copy genes (or operons) that encode proteins, ribosomal RNAs and transfer RNAs.

239 CoT Curves and DNA Complexity Explained!

14.2.4 Functional Differences between Cot Classes of DNA

The next question of course was what kinds of sequences are repeated and which are 'unique' in eukaryotic DNA? Eukaryotic satellite DNAs, transposons and ribosomal RNA genes were early suspects. To start answering these questions, satellite DNA was isolated from the CsCl gradients, made radioactive and then heated to separate the DNA strands. In a separate experiment, renaturing rat DNA was sampled at different times of renaturation. The isolated ***Cot fractions*** were once again denatured and mixed with heat-denatured radioactive satellite DNA. The mixture was then cooled a second time to allow renaturation. The experimental protocol is illustrated in Fig. 14.6.

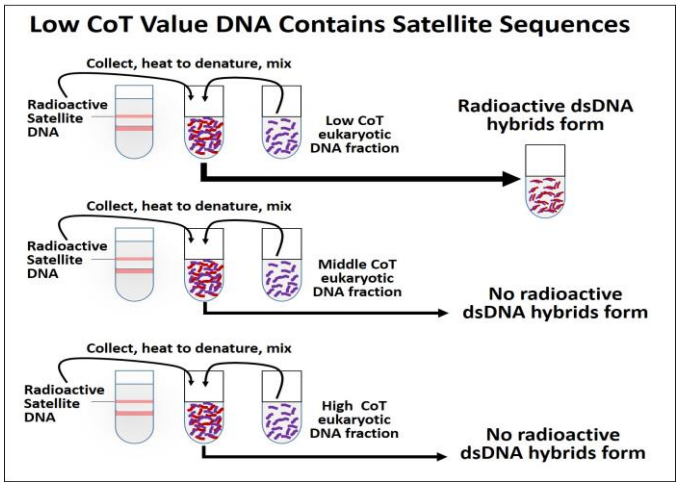


Fig. 14.6: Experimental demonstration of *satellite DNA* in *low CoT* renaturation kinetic fractions (see text for details).

The results of this experiment showed that radioactive satellite DNA only annealed to DNA from the *low Cot fraction* of DNA. Satellite DNA is thus *highly repeated* in the eukaryotic genome.

In similar experiments, isolated radioactive rRNAs made radioactive formed RNA-DNA hybrids when mixed and cooled with the denatured *middle CoT* of eukaryotic DNA. Thus, rRNA genes were moderately repetitive. With the advent of recombinant DNA technologies, the redundancy of other kinds of DNA were explored using cloned genes (encoding rRNAs, mRNAs, transposons and other sequences) to probe DNA fractions obtained from renaturation kinetics experiments. Results of such experiments are summarized in Table 14.1(below).

Repetitive DNAs in Eukaryotes

Class	Type	Copy number; % of Genome e.g. Mammals	Sub-type	Organization */or Properties	Unit Length (bp)	Location	Function (if known), Examples
Highly Repetitive	*Satellite DNAs*	Up to 10^6; 10-15%	*Microsatellite*	Tandem repeats (*VNTRs)	2-8 bp X 5-50	Centromeres, Heterochromatin, Dispersed	Spindle fiber attachment, gene regulation
			Minisatellite	Tandem repeats (*VNTRs)	10-60 bp X ?	Dispersed	Gene Regulation
			Telomeres (sub-category of minisattelite DNAs)	Tandem Repeats	4-6 bp	Chromosome ends	Prevent chromosome shortening during replication
Moderately Repetitive	*Transposable Elements*	10-10^5; 20-45% (>80% in Maize!)	*DNA Transposons*	Move via DNA intermediates ("cut-&-Paste")	Up to 7,000 bp	Dispersed	P-element, Mariner, Ac, Ds
			Retrotransposons: LTR elements, LINES & SINES	Move via RNA intermediates	80-400 bp (SINEs),	Dispersed	Alu
					Up to 7000 bp (LTR, LINEs)	Dispersed	L1
	rRNA genes	*<1%*	*45S rRNA genes*	Tandem 45S rDNA repeats	13.7 Kbp	Nucleolus	Translation
			5S rRNA genes	Tandem 5SrDNA repeats	120 bp	Dispersed	Translation
Unique Sequence	*Transcribed genes, introns, intergenic DNA*	Single copy sequences; ~50% in aggregate	*Protein-coding genes (introns + exons)*	*~25000 in humans*	variable	Dispersed	Just about everything else!

** VNTR: Variable Number of Tandem Repeats*

The table compares properties (lengths, copy number, functions, percent of the genome, location in the genome, etc.) of different kinds of repetitive sequence DNA. The observation that most of a eukaryotic genome is made up of repeated DNA, and that transposons can be as much as 80% of a genome was a surprise! We'll focus next on the different kinds of *transposable elements*.

240 Identifying Different Kinds of DNA Each CoT Fraction

241 Some Repetitive DNA Functions

14.3 The 'Jumping Genes' of Maize

Barbara McClintock's report that bits of DNA could jump around and integrate themselves into new loci in DNA was so dramatic and arcane that many thought the phenomenon was either a one-off, or not real! Only with the subsequent discovery of transposons in bacteria (and in other eukaryotes) were McClintock's jumping genes finally recognized for what they were. To begin the tale, let's look at the maize reproduction in Fig. 14.7 below.

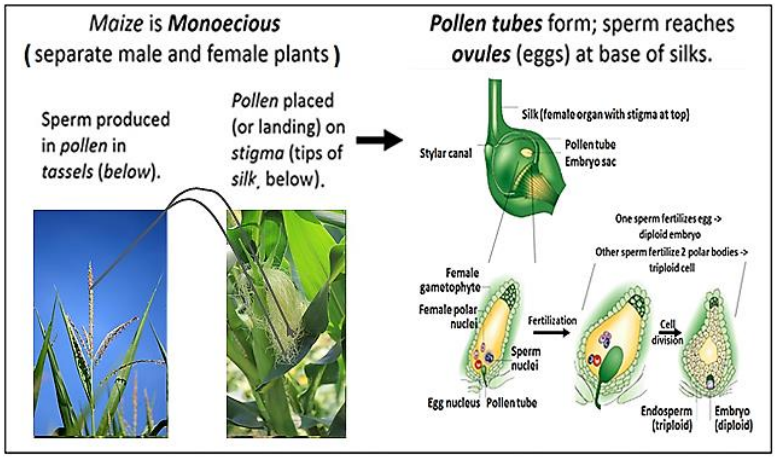


Fig. 14.7: Life-style of maize. Sperm from male pollen lands on femaler stigmata atop corn silk, tunneling through a pollen tube of its own making to reach and fertilize eggs (ovules) at the base of the silks. While one sperm will fertilize the egg; it begins embryogenesis. Another sperm fertilizes polar bodies left over from oogenesis, creating triploid cells that become the endosperm.

The different colors of corn seeds (kernels) result from ***anthocyanin*** pigments that are expressed differentially by cells of the *aleurone* tissue. Mclintock was studying the inheritance of color variation, which ranged from colorless (white or yellow due to an absence of *anthocyanins*) to brown, purple, spotted or streaked. The ***mosaic*** of kernel colors is vivid in the corncobs in the photo in Fig. 14.8 (below).

Fig. 14.8: Mosaic corn cobs.

Clearly, kernel color is inherited. The inheritance of colorless and purple seed color does indeed follow Mendelian rules, but the genetics of mosaicism does not. Mosaic color patterns after genetic crosses were not consistent, implying that the mutations responsible for kernel color were not due to mutations in germ cells. Rather, genes

controlling anthocyanin synthesis must be undergoing mutations in somatic cells that would become (or already were) the ones in which the pigments were produced.

242 What Interested McClintock About Maize

14.3.1 Discovering the Genes of Mosaicism; the Unstable Ds Gene

As we describe McClintock's experiments, keep in mind that her research and intuitions about gene regulation and ***epigenetic*** inheritance came long before molecular technologies made it possible to prove and give names to these phenomena.

McClintock was looking for a genetic explanation for seed color variation in the 1940s and early 1950s. DNA structure had only recently been published and gene cloning and DNA sequencing were decades into the future! Her only available technologies were based on understanding Mendelian allelic assortment in traditional breeding studies.

Since seed color is expressed in cells derived from endosperm, McClintock knew that the inheritance of kernel color ***phenotype*** must be studied against a ***triploid*** genetic background. She was also aware of speculations that the variegated color phenotype might result when an *unstable mutation* that produced colorless kernels *'reverted'* in some cells but not others to create a spotted or streaked phenotype. Just what made for an 'unstable mutation' was of course, unknown. McClintock identified three genes involved in seed kernel coloration and ultimately solved this puzzle.

Two of the genes studied by McClintock controlled the presence *vs.* absence of kernel color. These are the **C** and **Bz** genes:

1. **C'** is the dominant *inhibitor allele*, so-called because if even one copy was present, the kernels were ***colorless*** (yellow), regardless of the rest of the genetic background.
2. **Bz** and **bz** are dominant and recessive alleles of the Bz gene, respectively. In the absence of a dominant C' allele, the presence of a Bz allele would lead to purple kernels. If the bz allele was present without *both* C' *and* Bz alleles, the kernels would be dark brown.

The third gene, the one required to get variegated kernel color was the **Ds**, or ***Dissociator*** gene. McClintock knew that without a viable ***Ds*** gene, kernels were either colored or colorless depending on the possible genotypes dictated by the C and Bz alleles. In other words, the ***Ds*** gene must suffer the '*unstable mutations'* that led to variegated kernel color. The mutations occurred at random among aleurone layer

cells. So, the mutations must be occurring in a region of *chromosomal instability* (prone to damage or breakage) in some cells but not others. Let's look at what McClintock did to figure out what was going on in corn kernel color genetics.

Having already demonstrated crossing-over in maize (actually, another remarkable achievement!), McClintock ***mapped*** the C', Bz and Ds genes to *Chromosome 9.* She then selectively mated corn with genotypes shown in the protocol in Fig. 14.9 (below).

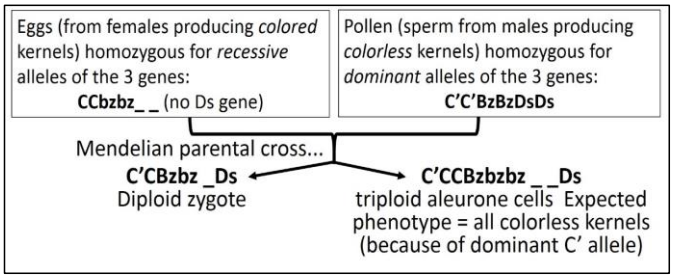


Fig. 14.9: Experimental cross of females with CCbzbz triploid genotype with males having a C'C'BzBzDsDs genotype with progeny genotypes and phenotypes based on Mendelian assumptions.

Remember that triploid cell genotypes are being considered in this illustration! You can refer to the phenotypic effects of the allelic backgrounds of three genes as we follow McClintock's cross. Her cross of a homozygous recessive with a homozygous dominant plant should ring a bell! Let's look more closely at this cross. Fig. 14.10 (below) shows the expected triploid genotypes from the cross. Aleurone cells resulting from this cross should all be colorless (yellow) because of the presence of the dominant C' allele.

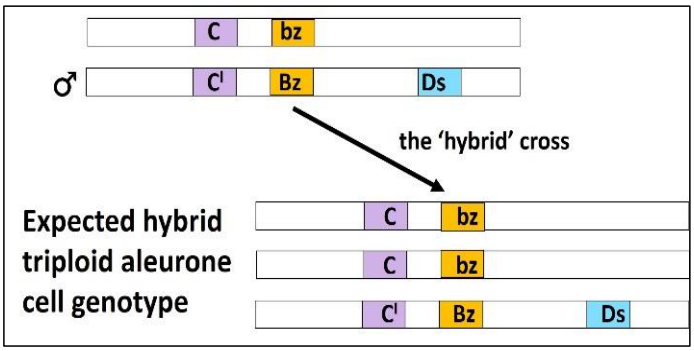


Fig. 14.10: Triploid genotypes expected for the cross in Fig.14.9 (see text for details).

But while there were indeed many ***colorless*** kernels on the *hybrid* cob, there were also many ***mosaic*** kernels with dark *spots* or *streaks* against a colorless background. McClintock's interpretation of events is illustrated in Fig. 14.11 (below).

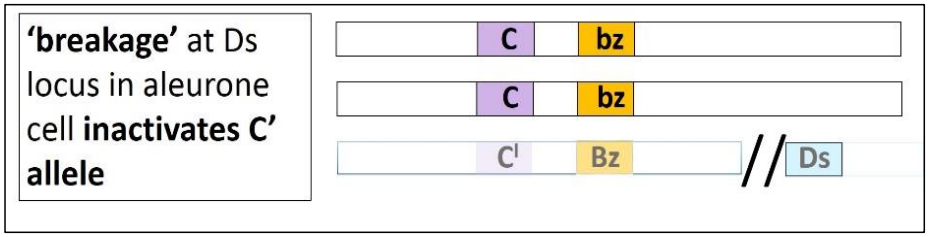


Fig. 14.11: McClintock's interpretation of the results of the triploid cross shown in Fig.14.9 (See text for details).

According to McClintock, if some aleurone layer cells in some kernels suffered chromosome breakage at the ***Ds*** (***Dissociator***) locus (indicated by the double slash, //), the C' allele is inactivated. Without a functional C' allele, the **operative** genotype in the affected cells is **CCbzbz.** These cells then revert to making the brown pigment as directed by the bz allele. When these cells divide, they form clusters of brown cells surrounded by cells with an unbroken chromosome (and thus an active C' allele), creating a mosaic, i.e., the appearance of pigment spots or streaks in the kernel against the otherwise colorless background in the surrounding cells.

243 Variegated Maize Kernels Result from "Loss" of the Ds Gene

14.3.2 The Discovery of Mobile Genes: the Ac/Ds System

The experiments just described were reproducible using a single breeding stock of maize. But when McClintock tried to repeat the experiments by crossing the homozygous dominant males with homozygous recessive females from a *different* breeding stock, all the kernels of the progeny cobs were colorless, as if the Ds gene had not caused any chromosomal damage.

It seemed that the ***Ds*** gene contributed by the male was unable to function (i.e., 'break') in females of this new breeding stock. McClintock hypothesized that the female in the original cross must have contributed a factor that could somehow activate the Ds gene to break, and that this factor, yet another gene, was absent or inactive in the females of the new breeding stock. McClintock called the new factor

the ***activator***, or ***Ac*** gene. Based on the dependence of ***Ds*** on the ***Ac*** locus, McClintock recognized that these 'genes' were part of as a 2-element, ***Ac/Ds system*** influencing mosaicism in maize kernels. She then demonstrated that Ac-dependent Ds 'breakage' was in some cases also associated with inactivation of a normal Bz gene, leading to a loss of purple color kernels. It was at this point that McClintock concluded that far from simply 'breaking' the chromosome at a fragile Ds locus, *the Ds gene had actually moved to (or into) the Bz gene*, disrupting its function. Again, this could not happen in the absence of an active Ac gene. McClintock had discovered the first transposon, earning her the 1983 Nobel Prize in Physiology or Medicine! View an homage, brief history and summary of McClintock's work and impact at *https://www.youtube.com/watch?v=ZHho1c-EbTY&t=46s*.

With the advent of recombinant DNA technologies, we now know that:

1. the **Ds** element is a transposon missing a gene for a ***transposase*** enzyme required for transposition.
2. the **Ac** element has this gene and is capable of independent transposition.
3. **Ac** provides the transposase needed to mobilize itself *and* the Ds element.
4. the sequence similarity of Ds and Ac elements supports their common ancestry.

The basic structural features of the maize Ac/Ds system are:

- Ac is 4563 bp long
- Ds is a truncated version of Ac.
- There are ***eleven bp inverted repeats*** at either end of the Ac and Ds element
- There are ***eight bp direct repeats*** (NOT inverted repeats) of 'target DNA' at the site of insertion of either transposon.

Look for these features as we describe prokaryotic and eukaryotic transposons.

244 Discovery of Mobile Elements and the Ac-Ds System

245 The Ac-Ds System Today

14.4 Since McClintock: Transposons in Bacteria, Plants and Animals

Transposons exist everywhere we look in prokaryotes and they account for much of eukaryotic repetitive DNA. Sometimes called "jumping genes", they can be a large proportion of eukaryotic genomes, including some that no longer even jump (i.e., transpose). Transposons were also once considered useless, described as ***junk DNA***, containing ***selfish genes*** whose only purpose was self-replication to copy useless, junk DNA sequences. But in light of some new evidence, perhaps not!

As you will see, transposition shares many features with DNA replication, recombination and repair, and even viral infection. As you study how transposons move, keep in the back of your mind that transposition is often triggered by cellular stress. Let's begin with a look at some bacterial transposons first and then, at eukaryotic transposons.

14.4.1 Bacterial Insertion Sequences (IS Elements)

Bacterial ***IS elements*** were the first mobile elements described after those in maize. As we'll see, they share some structural features of eukaryotic transposons. Discovered in the late 1960s, many have been identified (IS1, IS2…, IS10 etc.). Some are inserted into well-known genes (e.g., those of the lac operon), but most are not, likely because there is little 'extra' DNA in the compact bacterial genome. Without extra non-coding DNA to serve as a buffer against damaging mutations, few bacterial cells would live to tell a tale of transposition! Perhaps it should surprise us that IS elements can be made to transpose in the lab but are generally silent in nature.

Members of the IS element family vary in length from about 750 to 1425 bp. Within this stretch of DNA lie ***transposase*** and ***resolvase*** genes whose products are necessary for mobility. At either end of the IS element are ***inverted repeats***, and when found in either genomic or plasmid DNA, the IS sequence itself is flanked by ***direct repeats*** of host genome or plasmid DNA that result from the mechanism of transposition. Again, because of their compact genomes, bacteria can only tolerate low copy numbers of IS elements in their genome or on plasmids (less than ten copies and as few as one!). Fig. 14.12 illustrates a typical IS element.

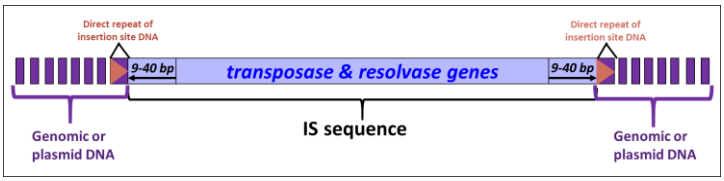


Fig. 14.12: Structure of a bacterial *IS element* (see text for details).

14.4.2 Composite Bacterial Transposons: Tn Elements

If a pair of IS elements should lie close to each other, separated by a short stretch of genomic or plasmid DNA, they can transpose together, carrying the DNA between them as part of a ***composite transposon***, or ***Tn element***. If some of the DNA between IS elements in a Tn element contains antibiotic resistance genes, its transposition can carry and spread these genes to other DNA in the cell. Tn elements (like IS elements) are present in low copy number. Fig. 14.13 (below) illustrates a generic Tn element.

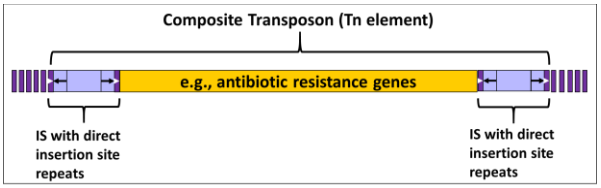


Fig. 14.13: Structure of a bacterial *Tn element* (see text for details).

Antibiotic resistance genes have the medical community worried; their spread has led to antibiotic-resistant pathogens that cause diseases that are increasingly hard and even impossible to treat. Earlier we saw genetic 'transformation' of streptococcal cells that pick up virulence genes in DNA from dead cell. We routinely transform cells with plasmids as part of recombinant DNA experiments. But bacteria can transfer plasmid DNA between themselves quite naturally. During bacterial conjugation, an ***F*** (***fertility***) plasmid normally transfers DNA between compatible bacterial mating types (review bacterial conjugation elsewhere in this text for more details). An F plasmid containing a Tn element harboring an antibiotic resistance gene can thus be passed from donor to recipient during conjugation. The Tn element can then transpose into to the recipient bacterial genome. In this way, transposition is a major pathway for the transfer and spread of antibiotic resistance.

14.4.3 *Complex* Transposons that Can Act Like Bacteriophage

Bacterial ***Complex Transposons*** also contain genes in addition to those required for mobility. Some complex transposons resemble a bacteriophage genome; in the case of ***phage Mu***, it actually is a phage… at least sometimes! In fact, *Mu* can function either as an infectious phage that reproduces in an infected cell, or as a transposon in the bacterial genome. Transposon genes in Mu phage are illustrated in Fig. 14.14.

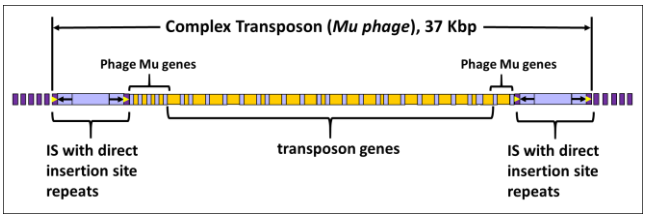


Fig. 14.14: Structure of a complex transposon (*Mu phage* - see text for details).

After infecting a bacterium, Mu can enter the ***lytic*** phase of its life cycle, replicating its DNA, producing and ultimately releasing new infectious phage 'particles' by lysing the host bacterial cell. Alternatively, like other phage, Mu can undergo ***lysogeny***, inserting its

DNA into the host cell chromosome. Integrated copies of Mu can excise and re-enter the lytic phase to produce more phage, usually if some environmental stress threatens host bacterial survival. But, a third lifestyle choice, transposition, is available to Mu once the phage integrates into the bacterial chromosome. The three lifestyle options for Mu phage are illustrated in the next few pages. Fig 14.15 (below) illustrates the lytic and lysogenic lifestyle options for a bacterial virus, and the next illustration (Fig. 14.16) shows the additional lifestyle options of Mu phage; the phage DNA can act as a transposable element while in the lysogenic pathway!

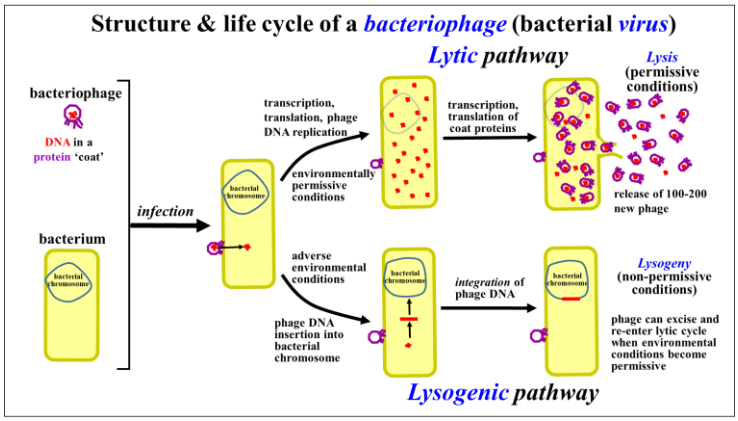


Fig. 14.15: Life cycle options for bacteriophage (see text for details).

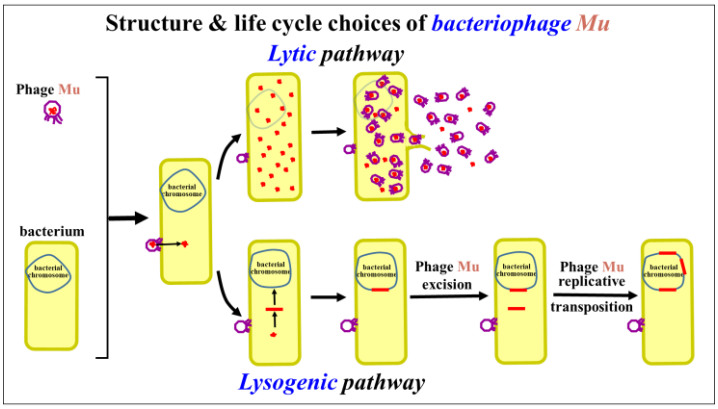


Fig. 14.16: The transposon option for *Mu Phage* (see text for details).

As we turn to a description of eukaryotic transposons, look for similarities to bacterial IS and Tn elements.

246 Bacterial Mobile Elements

14.5 Overview of Eukaryotic Transposable Elements

There are two classes transposons in eukaryotes. ***Class I* (RNA) T*ransposons*** move/'jump' by transcription of RNA at one locus, followed by reverse transcription and integration of the cDNA back into genomic DNA at a different location. Called ***retrotransposons*** they may be derived from (or be the source of) retroviruses since active retroviruses excise from and integrate into DNA much like retrotransposons. ***Retroposons*** are a sub-class of retrotransposons (see below). ***Class II* (*DNA*) *Transposons*** move by one of two mechanisms. In the cut-&-paste pathway, the transposon leaves one locus and integrates at another. In the replicative pathway, the original transposon remains in place while new copies are mobile. Table 14.2 below shows the distribution and proportion of genomes represented by different classes/types of transposable elements.

Organism	Class I (RNA) transposons as a % of all transposons	Class I (RNA) transposons as a % of Genome	Class II DNA) transposons as a % of all transposons	Class II (DNA) transposons as % of Genome	All Transposons as % of genome
Bacteria (e.g., *E. coli*)	-------	-------	100%	~3%	~3%
Yeast	100%	~3.5%	-------	-------	~3.5%
Corn (e.g., *Z. maize*)	>50%	~30-45%	<50%	~40%	~70-95%
Protozoa (*T. Vaginalis*)	-------	-------	100%	~66%	~66%
Frog (e.g., *R. esculenta*)	~25%	~19%	~75%	~58%	~77%
Mouse (*M. Musculus*)	~95%	~38%	~5%	~2%	~40%
Mosquito (e.g., *A. aegypti*)	~30%	~14%	~70%	~33%	~47%
Us! (*Homo sapiens*)	>90%	~40%	<10%	<5%	~42-45%
Flatworm (*C. elegans*)	~5%	~0.5%	~95%	~11-12%	~12%
Fruit fly (*D. Melanogaster*)	<80%	~3%	>20%	~1%	~4%
Rice (e.g., *O. sativa*)	~15%	~1%	~85%	~5%	~6%

The table confirms that in contrast to eukaryotes, bacteria contain relatively few transposons, while eukaryotes vary widely in *transposon load* (transposons as a percentage of genomic DNA). Transposon load can range from as low as 4% to more than 70% in different organisms. Table 14.3 (below) summarizes transposable elements by class, sub-type, size, genomic distribution, mechanism of transposition, etc.

Types of Transposable Elements

Class	Type	Sub-Types	Basis of Mobility	Organismic Distribution (e.g.)	Examples	All, as % of Genome	Length (bp)	Genomic Location; Special Features
PROKARYOTIC Mobile Elements	**DNA transposons*	IS (Insertion Elements)	Cut-&-Paste	*E. coli*	IS1, IS2, IS3…	3%	1000-2000bp	Intergenic DNA; usually insertion-site specific
		Composite Tn	Cut-&-Paste		Tn5		~5900bp	Source of antibiotic resistance genes
		Complex Tn	Replicative, *co*integrate formation		Mu		~37Kbp	Can function as bacteriophage or transposon
EUKARYOTIC Class II (DNA) transposons: **Move via DNA intermediates	**DNA transposons*	----------	Cut-&-Paste	*D. melanogaster*	P-element, Mariner	both = ~1%	1000-7000bp	Dispersed
				C. elegans,	Tc1 (Mariner)	~11-12%		
				Z. maize	Ac, Ds	~40%		
				H. Sapiens (human)	Mariner	~2-5%		
		----------	Replicative, *co*integrate formation	*O. Sativa* (rice) [Also plants, bacteria]	*Miniature Inverted Repeat Transposable Elements* (MITEs)	~6% (rice)	<500bp	Mostly associated with genes; transcribed into small RNAs
EUKARYOTIC Class I (RNA) transposons: **Move via RNA intermediates	**retro-transposons, retroposons*	*LTR retrotransposons*	Reverse-transcription & integration (original copy not excised)	*S. cerevisiae* (yeast)	Ty	~3%	Up to 7000bp	Dispersed; *(retrovirus-like, but no envelope protein genes)*
				D. melanogaster	Copia	~3%		
		LINEs (NON-LTR retrotransposons)		*H. Sapiens* (human)	L1	~5%	~6000bp	Interspersed
				Z. maize	*Cin-1*	~45%		
		SINEs (NON-LTR retrotransposons, or retroposons)		*H. Sapiens* (human)	Alu	~40%	80-400 bp	

* In so-called ***Cut-&-Paste*** transposition, DNA transposons move from one location to another. In ***Replicative*** transposition, DNA transposons replicated a copy of the element that moves. ***Retrotransposons*** are active if their transcripts are translated so that appropriate enzymes can integrate their cDNA copies into genomic DNA.

** Many transposons are inactive, having been silenced by mutations or other factors. Active eukaryotic Class I or II transposons can be ***autonomous*** or ***non-autonomous***. ***Autonomous*** transposons have all of the structural and functional features necessary for transposition (e.g., the maize **Ac** element). ***Non-autonomous*** transposons have all the structural features of autonomous transposons (e.g., inverted repeats and other DNA needed for transposition), except they lack or can't transcribe genes for enzymes required for mobility (e.g., the maize **Ds** element). Therefore, they can only transpose with the assistance of an actively transposing autonomous element that can provide the required enzymes.

Between the two tables (above and below), we can conclude the following:

- *Transposon load* is not correlated with evolutionary complexity of organisms.
- Shared transposons have different evolutionary histories in different organisms.
- Where transposons remain active, they continue to shape genomic landscapes, especially in organisms with a high transposons load.

We will revisit some of these conclusions later, after looking at the structure and mechanism of mobility of different eukaryotic transposable elements.

247 Introduction to Eukaryotic Transposons

14.6 Structure of Eukaryotic DNA (Class II) Transposons

Active eukaryotic *DNA transposons* share structural features with bacterial mobile elements, including genes required for transposition, flanking inverted repeats and flanking insertion-site direct repeats of host cell DNA. As we'll see, Class II transposons can 'jump' by ***cut-and-paste*** or **replicative** mechanisms. Fig. 14.17 (below) illustrates the characteristic structure of a eukaryotic DNA transposon.

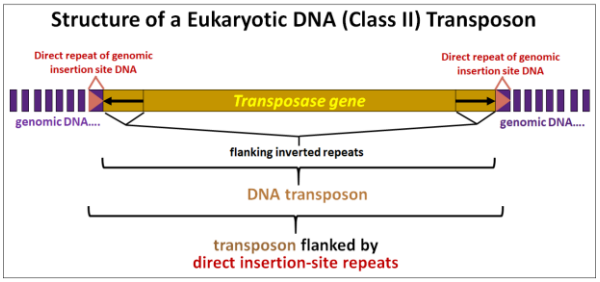


Fig. 14.17: Structure of a eukaryotic *Class II DNA transposon* (see text for details).

14.6.1 Cut-and-Paste Transposition

Cut-and-paste transposition is illustrated below in Fig. 14.18. The mechanism moves a copy from one location and *transposes* it to another location.

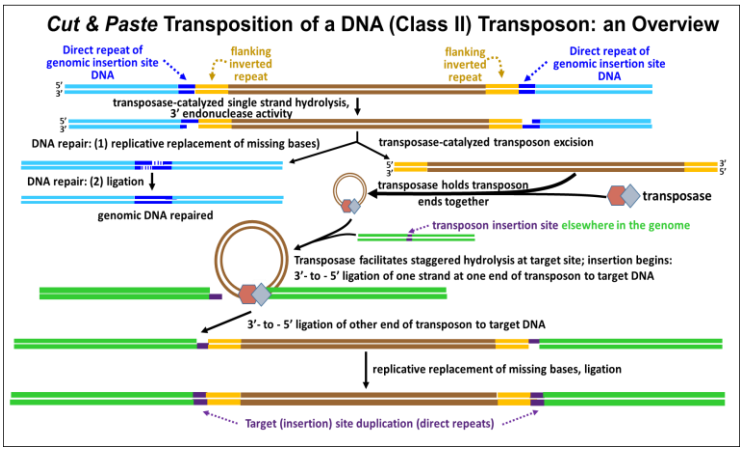


Fig. 14.18: *Cut-&-paste transposition* of a DNA transposon (see text for details).

Note that after transcription of the transposase gene, the enzyme nicks the DNA and trims the 3'OH ends to create a staggered cut to excise the transposon. The transposase actually brings the transposon ends together during the cut step and mediates its insertion at a new DNA site. After ligation of the 3'OH ends of the transposon to the 5'OH at the insertion site, replication replaces the missing bases, generating the direct repeats of host cell genomic DNA at the insertion site. A final ligation step completes transposition.

14.6.2 Replicative Transposition

Fig 14.19 (below) details the steps of replicative transposition.

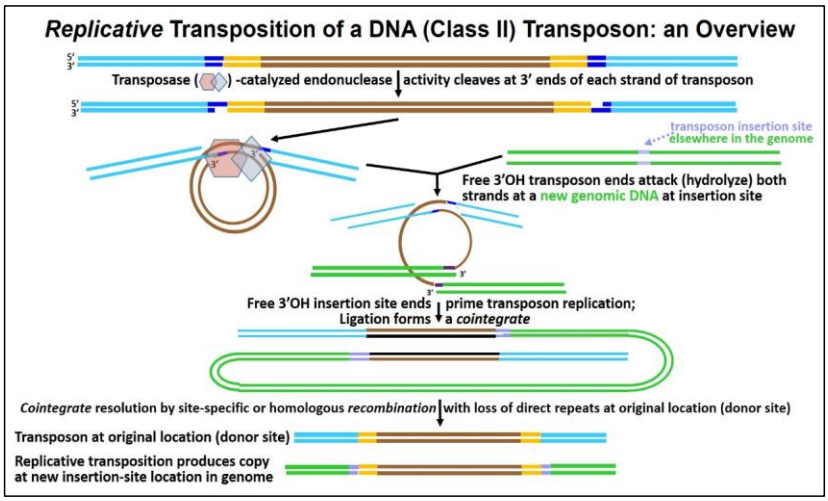


Fig. 14.19: *Replicative transposition* of a DNA transposon (see text for details).

In replicative transposition the transposon is also nicked and trimmed at its source (original) insertion site. But unlike cut-and-paste mechanisms, source transposons are not excised. After nicking the 3' ends of the transposon at the insertion site, transposase holds the transposon ends together while catalyzing a hydrolytic attack of DNA at a new insertion site. This is followed by priming of transposon strand replication from the 3'OH ends of the insertion site DNA strands. A ***cointegrate*** structure forms in which each transposon copy has been made by semi-conservative replication. The *cointegrate* is resolved by one of two recombinational mechanisms. The result leaves copies of the transposon at both the original site and the new insertion site.

Let's compare and contrast the features of cut-&-paste and replicative DNA transposition. The ***common features*** are that (a) a transposon-encoded *transposase* binds, brings transposon ends together and catalyzes single-stranded cleavage (hydrolysis) leaving 'staggered ends', and (b) that the transposase holds the transposon ends together for the remaining steps.

The differences between the two mechanisms are that in c*ut & paste t*ransposition, the transposon is completely excised and then transposed to a new site in genomic DNA. In contrast, after single stranded cleavage in replicative transposition, transposase-bound free 3' ends of the transposon hydrolyze both strands of double stranded DNA at a new insertion site. After ligation of the 3' ends of transposon strands to 5' ends of cut genomic DNA insertion-site ends, the remaining 3' ends of the insertion-site DNA ends prime replication of the transposon to form the ***cointegrate***, the latter then resolved by one of two recombination pathways.

248 Eukaryotic Class II (DNA) Transposition

14.7 Structures of Eukaryotic RNA (Class I) Transposons

Like DNA transposons, all RNA transposons leave insert-site footprints, i.e., *direct repeats* of genomic DNA flanking the element. Unlike DNA transposons, active eukaryotic Class I transposons move via an RNA intermediate. Also, unlike DNA transposons, they lack terminal inverted repeats.

The mobility of the RNA intermediate of all retrotransposons requires a ***promoter*** that recognizes a ***reverse transcriptase*** enzyme as well as endonuclease and integrase enzymes (to be described below). ***Autonomous*** Class I RNA transposons characterized by **L**ong **T**erminal **R**epeats (***LTRs)*** as well as ***Non-LTR retrotransposons*** (that lack LTRs). Non-LTR retrotransposons include the ***autonomous L****ong Interspersed* ***N****uclear* ***E****lements* (***LINEs***) and the ***non-autonomous S****hort Interspersed* ***N****uclear* ***E****lements* (the **SINEs**). Both the *autonomous LTR* and *Non-LTR LINEs* contain and express genes needed for enzymes required for transposition. On the other hand, the *non-autonomous* **SINEs** (a sub-class of Non-LTR retrotransposons) lack genes for enzymes required for transposition and therefore cannot transpose independently. *Non-autonomous* retrotransposons thus rely on "true" (*autonomous*) retrotransposon activity for mobility. SINEs are sometimes called ***retroposons*** to distinguish them from the autonomous retrotransposons.

249 Introduction to Features of Retrotransposition

14.7.1 LTR retrotransposons: The Yeast Ty element

Here we look at retrotransposon structures, genes and enzyme activities required for *retrotransposition*. Fig. 14.20 (below) illustrates that the yeast Ty LTR retrotransposon encodes several genes needed for transposition:

- the **Gag** gene encodes *group-specific antigen*, a protein that forms a virus-like particle that will contain reverse-transcribed transposon DNA,
- the **RT** gene encodes the *reverse transcriptase* that will make reverse-transcribed copies of retrotransposon transcript RNAs.
- the **Prt** gene encodes a protease that will break down the virus-like particle as the retrotransposon enters the nucleus.
- the **Int** gene encodes the *integrase* required for integration of the retrotransposon into a genomic DNA insertion site.

The Ty element in Fig. 14.20 below is an autonomous LTR retrotransposon.

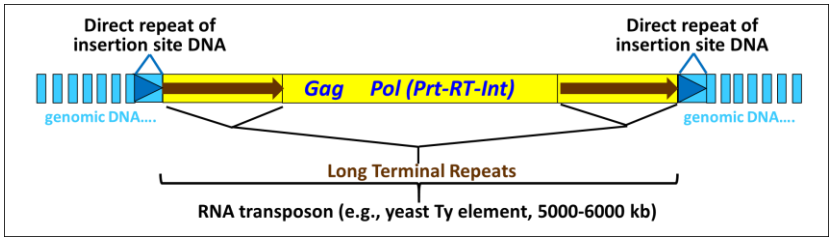


Fig. 14.20: Structure of yeast *Ty*, an RNA *LTR retrotransposon* (see text for details).

In fact, many of the events in Ty transposition occur in the cytoplasmic "virus-like particle" in yeast cells. To see more, click Virus-Like Particles in Ty Transposition. Note that the Pol region in the illustration above consists of overlapping *open reading frames* (***ORFs***) encoding the *Prt*, *RT* and *Int* genes. The ready-to-move transposon consists only of the region of the bracketed region of DNA including the long terminal repeat sequences.

250 LTR Retrotransposons-the Ty Element

14.7.2 Non-LTR Retrotransposons: LINEs

Like LTR retrotransposons, ***LINEs*** (**L**ong **I**nterspersed **N**uclear **E**lements) also encode enzymes needed for transposition and like other transposons, generate target-site direct repeats flanking the inserted element. But they do not have the long terminal

repeats! Instead, their ORFs (genes) are flanked by 5' and a 3' ***untranslated regions*** (***UTRs***). The structure of a typical Non-0LTR LINE retrotransposon is shown below in Fig. 14.21.

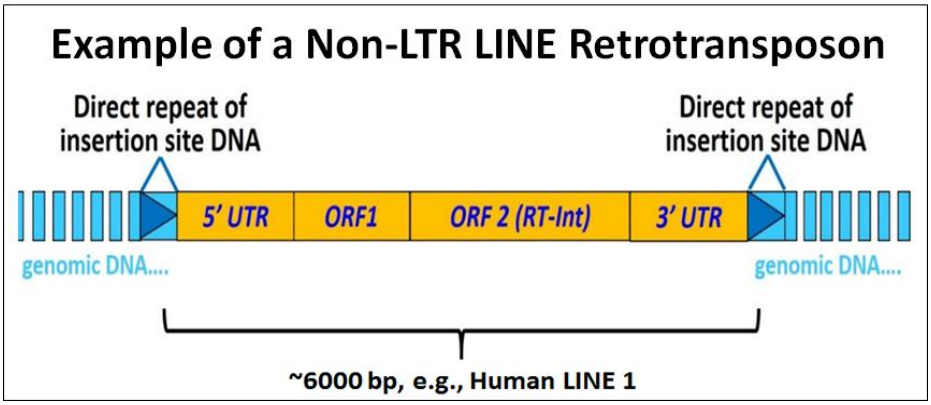


Fig. 14.21: Structure of a *LINE*, a *non-LTR retrotransposon* (see text for details).

The *5' UTR* contains a promoter from which cellular RNA polymerase II can transcribe the downstream genes (see the ***Transcription*** chapter). The second of these (*ORF2*) encodes the reverse transcriptase and an integrase activity essential for transposition of the LINE. All Class I (RNA-intermediate) autonomous transposons share the following features:

- ***a Promoter*** in the *5' UTR* from which they can be transcribed
- ***a Reverse Transcriptase*** that generates a cDNA copy of the transposable element
- ***RNAse H*** (an endonuclease) that degrades the transcript after reverse transcription
- ***Integrase*** (like a transposase) that catalyzes insertion of the retrotransposon copy at insertion sites

251 Non-LTR Retrotransposons: LINEs

14.7.3 Non-LTR Retrotransposons: SINEs

Non-LTR SINE retrotransposons typically lack genes, but their non-genic DNA is nonetheless flanked by 5' and 3' UTRs. RNA polymerase III, which also transcribes transfer RNAs, also transcribes SINEs. However, to transpose, they rely on the concurrent activity of a Non-LTR transposon (a LINE) to provide the requisite enzymatic activities. A typical SINE (e.g., the ***Alu*** element) is shown in Fig. 14.22 (below).

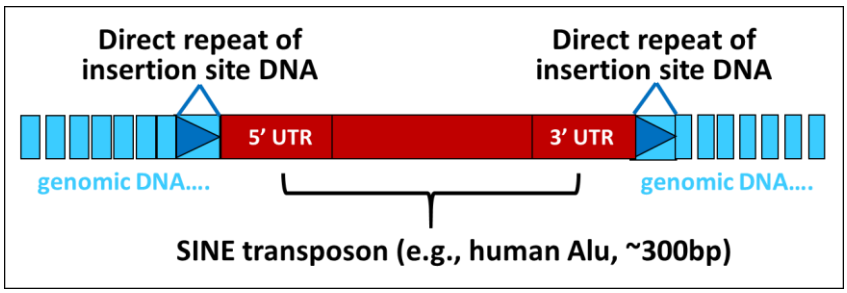


Fig. 14.22: Structure of the *Alu* SINE, a *non-LTR retrotransposon*, **or retroposon** (see text for details).

252 Non-LTR Retrotransposons: SINEs

14.8 Mechanisms of Retrotransposition

There are two mechanisms of retrotransposition: ***Extrachromosomally Primed Retrotransposition*** (LTR retrotransposons for example) and ***Insertion Target-Site Primed Retrotransposition*** (non-LTR Retrotransposons like LINEs and SINEs). We will consider these two mechanisms next.

14.8.1 Extrachromosomally Primed Retrotransposition (e.g., of a LINE)

As its name suggests, in *extrachromosomally primed retrotransposition,* a separate circular reverse transcript of the retrotransposon attacks, nicks and integrates into a genomic insertion site (Fig. 14.23, below). In this mechanism, reverse transcriptase creates a cDNA copy of a transcribed retro-element. Integrase/endonuclease then binds the cDNA copy, holding the ends together, in effect circularizing it. This isolable ribonucleoprotein resembles an ***intasome***, a structure similar to the nucleoprotein complex that catalyzes integration of retroviral cDNAs during lysogeny. The three-dimensional structure of a retroviral *intasome* interacting with DNA and nucleosomes

was recently determined (for more, see Retroviral Intasome 3D Structure). In this form, the retrotransposon attacks DNA at an insertion site, creating staggered ends. After insertion, the gaps in the DNA are filled in. Ligation seals the retrotransposon in its new location, creating direct insertion site repeats.

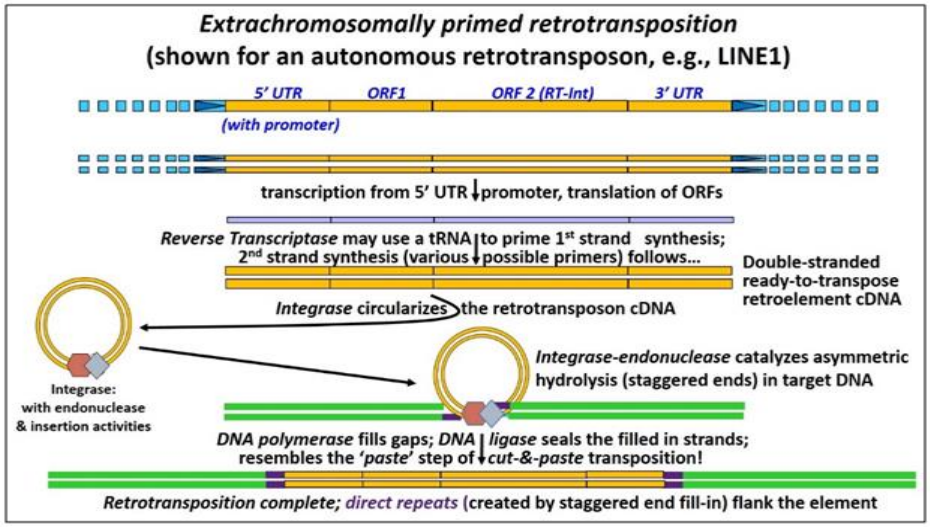


Fig. 14.23: *Extrachromosomally-primed transposition* of a LINE (see text for details).

253 Extrachromosomally Primed Retrotransposition

14.8.2 Target-Site Primed SINE Retrotransposition (e.g., of a SINE)

A key feature of *target-site primed retrotransposition* (*retroposition*) is the absence of an integrase-bound, separate circular double-stranded reverse transcript (Fig. 14.24 below). In SINE transposition, RNA polymerase III (the same enzyme that catalyzes tRNA and 5S rRNA transcription) transcribes the SINE. If a LINE is concurrently transcribed, its enzymes will be made. When its *integrase-endonuclease* catalyzes hydrolysis of one strand of DNA at a new insertion site, the 3'OH end of this strand can prime reverse transcription of the one SINE cDNA strand by the LINE *reverse transcriptase*. After hydrolysis of the second target site DNA strand, its 3'-OH end primes replication of the second strand of the SINE cDNA. Integrase completes insertion of the copy-SINE in its new genomic location.

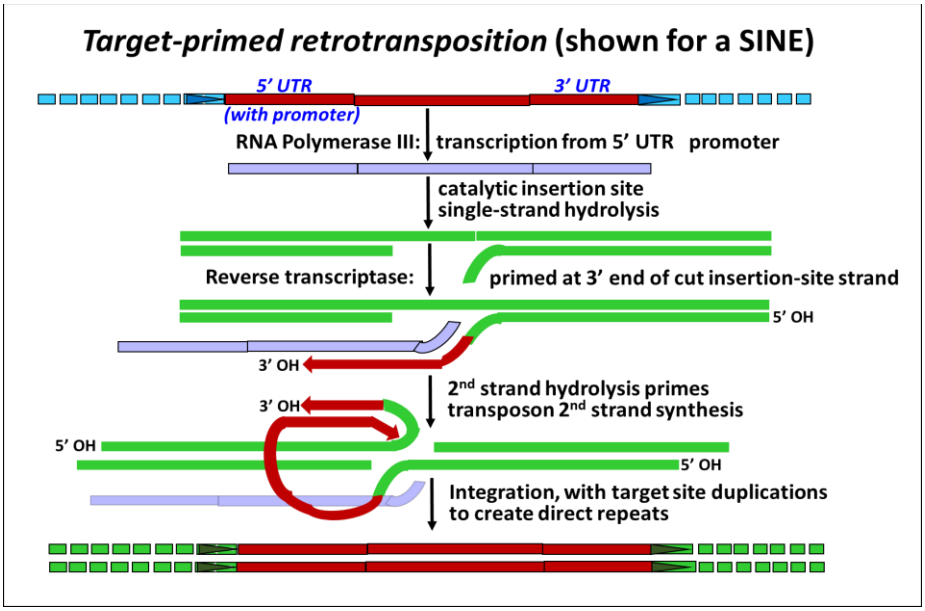


Fig. 14.24: *Target-primed transposition* of a LINE (see text for details).

254 Target Primed Retrotransposition

14.9 On the Evolution of Transposons, Genes and Genomes

We noted that transposons in bacteria carry antibiotic resistance genes, a clear example of benefits of transposition in prokaryotes. Of course, prokaryotic genomes are small, as is the typical bacterial transposon load. Yeast species also have low transposon load. But, what can we make of the high transposon load in eukaryotes? To many geneticists, the fact that genes encoding proteins typically represent only 1-2% of a eukaryotic genome suggested that the rest of the genome was informationally non-essential. Even though transposons turn out to be much of the non-coding DNA in some eukaryotic genomes, they seemed to serve no purpose other than their own replication. These large amounts of transposon DNA were dubbed selfish DNA and their genes, selfish genes.

So, are transposons just junk DNA, some kind of invasive or leftover *genomic baggage*? Given their propensity to jump around and potential to raise havoc in genomes, how do tolerate and survive them? Is the sole 'mission' of transposons really just to reproduce themselves? Or are transposons in fact neither *selfish* nor junk?

By their sheer proportions and activity in eukaryotic genomes, we will see that transposons have dispersed into, and re-shaped genomic landscapes. Do the consequences of transposition (the relocation and dispersal of transposons in a genome, the mutation of genes, the structural reorganization of genomes…) have any functional or evolutionary value?

All of these questions are reasonable responses to the phenomena of jumping genes. A rational hypothesis might be that, like all genetic change, the origin of transposition was a random accident. But, the spread and ubiquity of transposons in genomes of higher organisms must in the long term, have been selected by virtue of some benefit that they provide to their host cells and organisms. Let's briefly looks at the evolutionary history of transposons to see if this assumption has some merit.

14.9.1 A Common Ancestry DNA and RNA Transposons

Transposases catalyze transposition of bacterial IS (and related) elements and eukaryotic Class II (DNA) transposons. They are structurally similar and may share a common ancestry. The amino acid sequences of the so-called *integration domain* of transposases from different transposons are compared in Fig. 14.25 (below).

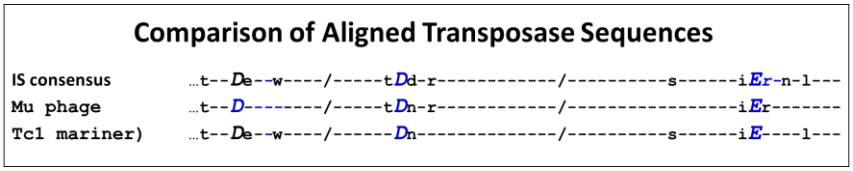


Fig. 14.25: Alignment of *consensus* amino acid sequences of bacterial IS transposases with Mu phage and Tc1 mariner transposases reveals conservation of **D**, **D** and **E** amino acids (upper case) at key positions in the sequence. Other amino acids are shared between some but not all of the sequences (lower case). Slashes are variable gaps in the alignments.

The universally conserved ***D…D…E*** amino acids at key positions in the enzymes define a ***DDE domain***, supporting the common ancestry of bacterial and eukaryotic transposases. This, along with shared structural features of these transposons (e.g., the flanking direct insertion-site repeats) further support a common ancestry of the transposons encoding the enzymes. Other sequence comparisons reveal that transposons themselves comprise distinct families of more closely related elements. This allows us to speculate on the origins of these families in different species. For example, the TC1/*mariner* (DNA) transposon is found in virtually all organisms examined (except diatoms and green algae). Based on sequence analysis, there is even an insertion element in bacteria related to the *mariner* element. Clearly, *mariner* is an ancient transposon.

This amount and diversity of conservation bespeaks an early evolution of the enzymes of transposition, and of transposition itself, within and even between species. ***Linear*** or ***vertical*** descent (the 'vertical" transmission of transposons from parents to progeny) is the rule. However, the presence of similar transposons in diverse species is best explained by interspecific DNA sharing, or ***horizontal transfer***. That is, a transposon in one organism must have been the 'gift' of an organism of a different species! This is further discussed below. Clearly, *moveable genes* have been a part of life for a long time, speaking more to an adaptive value for organisms than to the parasitic action of a selfish, rogue DNA!

14.9.2 Retroviruses and LTR Retrotransposons Share a Common Ancestry

The *integration domain* of retrotransposon transposases and retrovirus integrases also share significant sequence similarities, as shown in the amino acid sequence alignment in Fig. 14.26 below.

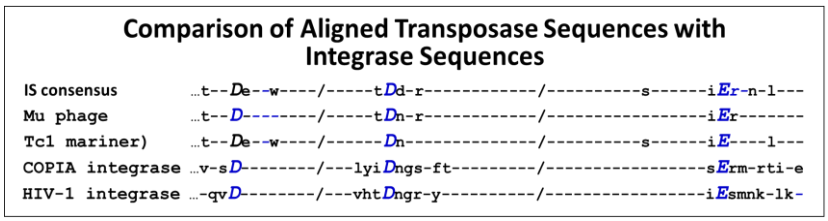


Fig. 14.26: Comparison amino acid sequences of the *COPIA* retrotransposon and a retroviral (HIV) integrase with typical transposase sequences. The alignments reveal conservation of the **D**, **D** and **E** amino acids in the *DDE* domain of the enzymes. Other amino acids are shared between some but not all of the sequences (lower case). Slashes are variable gaps in the alignments.

Here the conserved ***D…D…E*** amino acids at key positions in ***DDE*** *domains* support the common ancestry of Class I (RNA transposon) retroviral integrases and the Class II (DNA transposon) transposases. In other words, all transposons may share a common ancestry. But the common ancestry of retrotransposons and retroviruses raises yet other questions: Did transposons (specifically retrotransposons) arise as defective versions of integrated retrovirus DNA (i.e., reverse transcripts of retroviral RNA)? Or, did retroviruses emerge when retrotransposons evolved a way to leave their host cells. To approach this question, let's first compare mechanisms of retroviral infection and retrotransposition.

LTR retrotransposons and retroviruses both contain flanking long terminal repeats in addition to the structural similarities of the enzymes they encode. However, retrotransposition occurs within the nucleus of a cell while retroviruses must first infect

a host cell before the retroviral DNA can be replicated and new viruses produced (check out Visualizing Retroviral Infection to see how immunofluorescence microscopy using antibodies to single-stranded cDNAs was used to track the steps of HIV infection!).

A key structural difference between retrotransposons and most retroviruses is the ***ENV*** gene-encoded protein envelope surrounding retroviral DNA. After infection, the incoming retrovirus sheds its envelope proteins and viral RNA is reverse transcribed. After the reverse transcripts enter the nucleus, transcription of genes and translation of enzymes necessary for the replication of the viral cDNA leads to the production of new enveloped infectious viruses that will eventually lyse the infected cell…, but here are two curious phenomena:

First, retroviral DNA, like any genomic DNA, is mutable. If a mutation inactivates one of the genes required for infection and retroviral release, it could become an LTR retrotransposon. Such a genetically damaged retroviral *integrate* might still be transcribed and its mRNAs translated. If detected by its own reverse transcriptase, the erstwhile viral genomes would be copied. The cDNAs, instead of being packaged into infectious viral particles, would become a source of so-called *endogenous retroviruses* (***ERVs***). In fact, ERVs exist, making up a substantial portion of the mammalian genome (8% in humans)… and do in fact, behave like LTR retrotransposons!

Second, yeast TY elements transcribe several genes during retrotransposition (see the list above), producing not only reverse transcriptase and integrase, but also a protease and a structural protein called ***Gag*** (*Group-specific antigen*). All of the translated proteins enter the nucleus. Mimicking the retroviral *ENV* protein, the Gag protein makes up most of a coat protein called ***VLP*** (***virus-like particle***). *VLP* encapsulates additional retrotransposon RNA in the cytoplasm, along with the other proteins. Double-stranded reverse transcripts (cDNAs) of the viral RNA are then made within the VLPs. But, instead of bursting out of the cell, encapsulated cDNAs (i.e., new retrotransposons) shed their VLP coat and re-enter the nucleus, where they can now integrate into genomic target DNA.

Compare VLP activities to descriptions of retroviral infection. During infection, retroviral envelope proteins attach to cell membranes and release their RNA into the cytoplasm. There, reverse transcriptase copies viral RNA into double-stranded cDNAs that then enter the nucleus where they can integrate into host cell DNA. When transcribed, the integrated retroviral DNA produces transcripts that are translated in the cytoplasm into proteins necessary to form an infectious viral particle. The resulting viral RNAs are encapsulated by an ENV (envelope) protein encoded in the viral

genome. Of course, unlike VLP-coated retrotransposon RNAs, the enveloped viral RNAs do eventually lyse the host cell, releasing infectious particles. Nevertheless, while VLP coated Ty elements are not infectious, they sure do look like a retrovirus!

In much the same way as early biologists compared the morphological characteristics of plants and animals to show their evolutionary relationships, comparisons of aligned retroviral and retrotransposon reverse transcriptase gene DNA sequences reveal the phylogenetic relationships of genes. A phylogenetic tree of retrotransposon and viral genes is shown in Fig. 14.27 below).

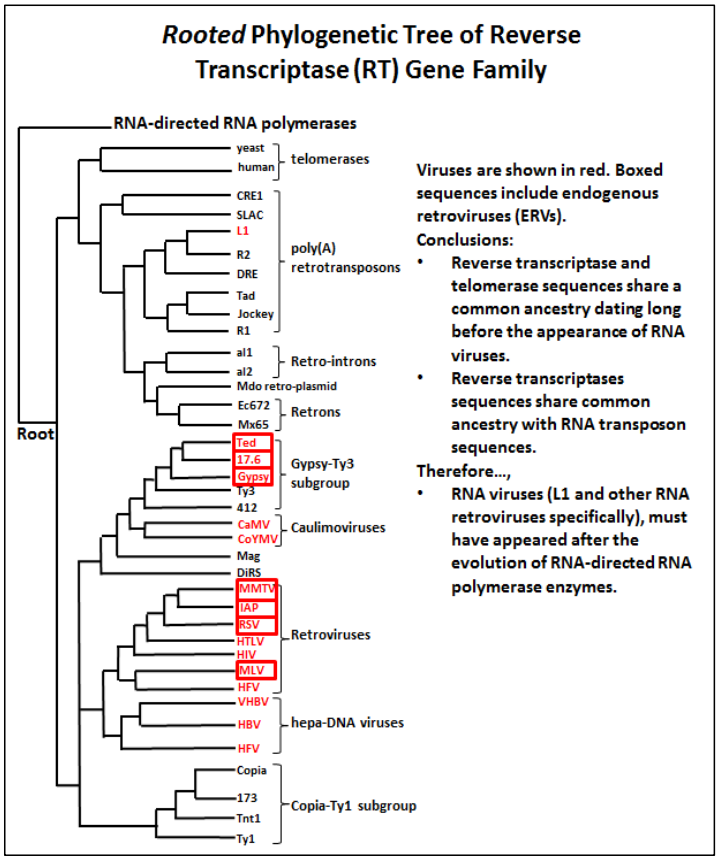


Fig. 14.27: Retroviral and retrotransposon reverse transcriptases share a common evolutionary ancestor.

The data in the analysis supports the evolution of retroviruses from retrotransposon ancestors. From the 'tree', TY3 and a few other retrotransposons share common ancestry with *Ted*, *17.6* and *Gypsy* ERVs (boxed) in the "Gypsi-TY3 subgroup". Further, this sub-group shares common ancestry with more distantly related retroviruses (e.g., *MMTV*, *HTLV*…), as well as the even more distantly related (older, longer diverged!) *Copia-TY1* transposon sub-group. This and similar analyses strongly suggest that retroviruses evolved from a retrotransposon lineage. For a review of retroposon and retrovirus evolution, check Lerat P. & Capy P. (1999, *Retrotransposons and retroviruses: analysis of the envelope gene*. Mol. Biol. Evol. 19(9): 1198-1207).

14.9.3 Transposons Can Be Acquired by *Horizontal Gene Transfer*

As noted, transposons are inherited ***vertically***, meaning that they are passed from cell to cell or parents to progeny by reproduction. But they also may have spread *between* species by ***horizontal gene transfer***. This just means that organisms exposed to DNA containing transposons might inadvertently pick up such DNA and become *transformed* as the transposon becomes part of the genome. Accidental mobility of transposons between species would have been rare, but an exchange of genes by horizontal gene transfer would have accelerated with the evolution of retroviruses. Once again, despite the potential to disrupt the health an organism, retroviral activity might also have supported a degree genomic diversity useful to organisms.


255 Transposon Evolution


14.10 Evolutionary Roles of Transposition in Genetic Diversity

Here we'll see how transposition can affect genes and genetic diversity. We'll also consider parallels between transposition and the generation of immunological diversity, and a provocative notion that our immune system owes at least some of its evolutionary history to transposons, or at least to mechanisms of transposition!

14.10.1 Transposons and *Exon Shuffling*

A role for unequal recombination in moving exons in and out of different eukaryotic split genes was described earlier. This kind of ***exon shuffling*** could happen when short DNA sequences in two different introns misalign during meiotic synapsis, allowing unequal crossing over. Expression of a gene with a 'new' exon produces a protein with a new domain and a new activity. If the new domain is not harmful, especially in only one of two alleles of a gene, the mutation need not be lethal… and genetic diversity and the potential for evolution is increased!

When found in introns, transposons are long regions of DNA similarity that can stabilize synapsis, increasing the chances of unequal recombination and exon shuffling. For example, ***Alu*** (SINE) elements are often found within introns, where they can integrate with no ill effect. The similarity of Alu elements in the introns of unrelated genes does seem to account for exon shuffling by unequal crossing over between the different genes that share domains and specific functions as a result.

Another way in which transposons facilitate germ line cell exon shuffling is more direct. Imagine a pair of transposons in introns of a gene on either side of an exon. Should such transposons behave like the two outer IS elements in a bacterial *Tn element* (discussed above), they might be excised as a single, large transposon containing an exon. The paired transposons flanking the exon might then insert in an intron of a completely different gene! The general pathway of exon shuffling involving paired proximal DNA transposons is illustrated in Fig. 14.28, below.

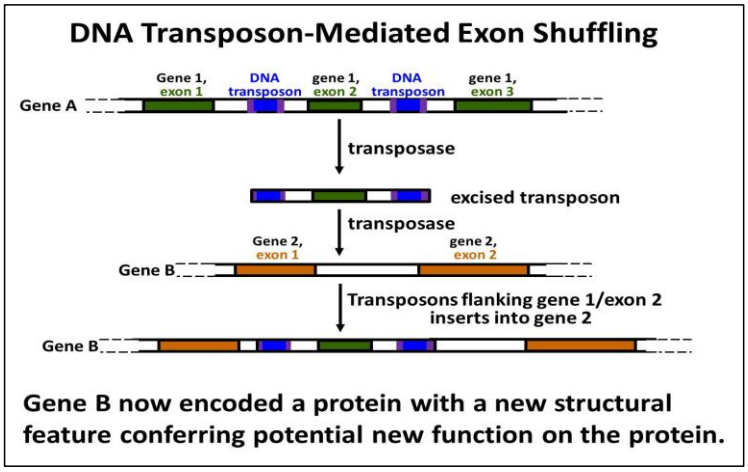


Fig. 14.28: Steps of paired DNA transposon-mediated exon shuffling (see text for details).

In this generic example of exon shuffling shown, exon 2 of gene 1 has been inserted (along with flanking transposons) into another gene (gene 2).

Transposon-mediated exon shuffling can explain insertion of exon-encoded domains of *epidermal growth factor* (***EGF***) into several otherwise unrelated genes. The mitogen EGF was discovered because it stimulated skin cells to start dividing. The gene for ***TPA*** (*tissue plasminogen activator*, a blood-clot dissolving *protease*) shares EGF gene domains. TPA is a treatment for heart attack victims that, if administered

rapidly after the attack, can dissolve the clot and allow coronary artery blood flow to heart muscle to resume. Other genes that contain EGF domains include those for Neu and Notch proteins, both involved in cellular differentiation and development.

Some exon shuffling events may have been mediated by LINE transposition and by a special group of recently discovered transposons called ***helitrons***. Helitrons replicate by a *rolling circle* mechanism. If you are curious about helitrons, do a google search to learn more about them, and what role they may have had in refashioning and reconstructing genomes in evolution.

14.10.2 Transposon Genes and Immune System Genes Have History

Several important eukaryotic genes may have been derived from transposons. Perhaps the most intriguing example of this is to be found in the complex vertebrate *immune system*.

Our immune system includes ***immunoglobulins*** (***antibodies)***. You inherited genes for immunoglobulin proteins from your parents. These genes contain multiple variant ***V***, ***D***, and ***J*** regions linked to a ***C*** region. *V, D, J* and *C* are defined as **V**ariable, **J**oining, **D**iversity and **C**onstant DNA regions, respectively. These regions recombine to create many diverse *V-D-J-C* immunoglobulin antibody molecules (the *D* region is not always included in the final recombined gene). The gene rearrangements occur during the maturation of stem cells in bone marrow that will become immune cells (***B*** or ***T lymphocytes***). In response to challenge by foreign substances called ***antigens***, (e.g., proteins on a bacterial surface or toxins released by invading cells), our immune system responds; *B* or *T lymphocytes* will be selected that contain the ***rearranged immunoglobulin genes*** able to make immunoglobulins that can recognize, bind and eliminate the invading antigens.

A discussion of the molecular biology of the immune system is beyond our scope here. Suffice it to say that the recombinational pathway of immunoglobulin gene rearrangements includes enzymatic activities very similar to those of transposition. In fact, the so-called RAG1 enzyme active in immunoglobulin gene rearrangement is closely related to genes in a family of transposons (*transib*) found in invertebrates and fungi. It looks like immune gene rearrangement might have origins in transposition!

14.11 Coping with the Dangers of Rampant Transposition

Most organisms do not have the high transposon load that we have. Given a general tendency of transposons to insert at random into new DNA loci, how come we exist at all? Isn't the danger of transposition into essential gene sequences magnified by the possibility of multiple simultaneous transpositions of elements generated by cut-and-paste

and especially replicative mechanisms? Indeed, transposons have been found in genes that are inactive as a result. An obvious explanation for our survival of transposon activity is that most transposition is into the >90% percent of the genome that does not code for proteins. Another is that eukaryotic organisms have two copies of every gene, so that if one is inactive, the other may sustain us. Beyond this, several mechanisms exist to silence a transposon after transposition has occurred, mitigating the dangers of rampant transposition. As long as a transposition is not lethal (e.g., because its integration does not disrupt an activity essential to life), the cell and organisms can survive the event. In time, mutations at the ends of transposons or in the genes responsible for transposition would eventually render them inactive. Finally, there may be more direct curbs on transposition. Recall that the *small interfering RNAs* (***siRNAs***) we encountered earlier may complement and target viral RNAs for destruction (see the Transcription chapter for more information on siRNAs). There is some evidence that ***siRNAs*** similarly target transposon transcripts.

Summing up, transposon activity is moderated by mutational loss of function and/or by more direct mechanisms that limit transposition and thus genetic damage. If an accumulation of transposons to a high load were deleterious, they would have been eliminated from genomes. Instead we see persistent transposons in many species, suggesting that frequent transposition is largely neutral, increasing options for diversity in the selection of new genotypes and phenotypic characteristics. We also know now that transposons can function in genetic regulation. Thus, transposons in general are neither selfish nor junk. Check out these links for more: Not junk after all? and Eulogy for Junk DNA.

256 Transposons-Junk or Not

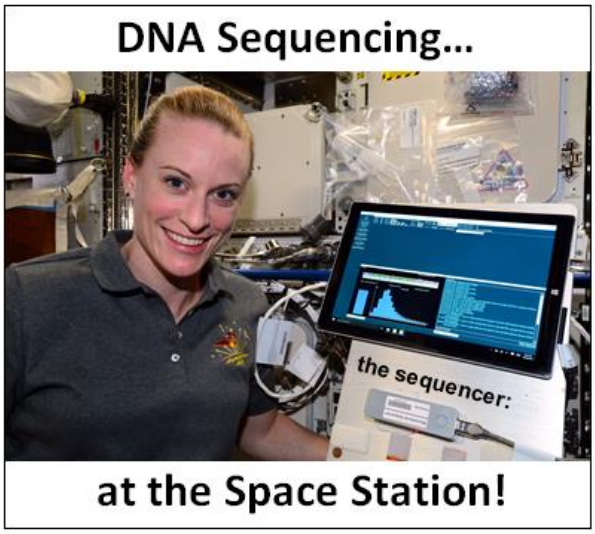
Some iText & VOP Key Words and Terms

Alu	**Gag**	**non-homologous recombination**
anthocyanins	**genomic complexity**	**Non-LTR transposons**
antibiotic resistance genes	**heterochromatin**	**protease**
antibodies	**immune system**	**protein coat**
autonomous transposon	**immunoglobulin**	**prt**
bacterial composite transposons	**integrase**	**renaturation kinetics**
bacterial IS elements	**inverted repeats**	**Repetitive DNA**

bacterial Tn elements	jumping genes	replicative transposition
bacteriophage	L1	resolvase
centromere	LINE	retrotransposon
chromatin	LTR (long terminal repeats)	retrovirus
chromosomes	LTR transposons	RNA transposon
Class I transposon	lysis	satellite DNA
Class II transposon	lysogeny	SINE
cointegrate	lytic pathway of phage	spindle fibers
CoT curves	maize Ac (activator) gene	telomeres
cut-and-paste transposition	maize Ds (dissociator) gene	transposase
density gradient centrifugation	mariner	triploid endosperm
direct repeats	McClintock	Ty
DNA sequence phylogeny	mosaicism	viral infection
exon shuffling	Mu phage	
fertility (F) plasmids	non-autonomous transposon	

Chapter 15: DNA Technologies

Manipulating DNA; cDNA libraries, Genomic Libraries, DNA Sequencing, PCR, Microarrays, Genomics, Transcriptomics, Proteomics



15.1 Introduction

We start this chapter by looking at technologies that led to ***genetic engineering***. The ability of make ***recombinant DNA*** is such a seminal technology that just realizing it could be done and then doing it in a test tube for the first time earned Paul berg a half-share in the 1980 Nobel Prize in Chemistry (the other half was shared by Walter Gilbert and Frederick Sanger for studies that enabled efficient ***DNA sequencing***). First we'll look at cDNA synthesis (the synthesis of DNA copies of RNA), something retroviruses routinely do as part of their reproductive pathway.

The retrovirus first injects its RNA into target cells where it transcribes a ***reverse transcriptase*** enzyme. The enzyme r*everse-transcribes* a copy DNA (cDNA) complementary to the viral RNA. The same reverse transcriptase enzyme then makes a (ds)cDNA (double-stranded cDNA) which then replicates. These cDNAs are transcribed into new viral RNA genomes and mRNAs for viral proteins. The latter encapsulate the RNA genomes into new viruses.

The first steps in retroviral infection are summarized in Fig. 15.1 (below).

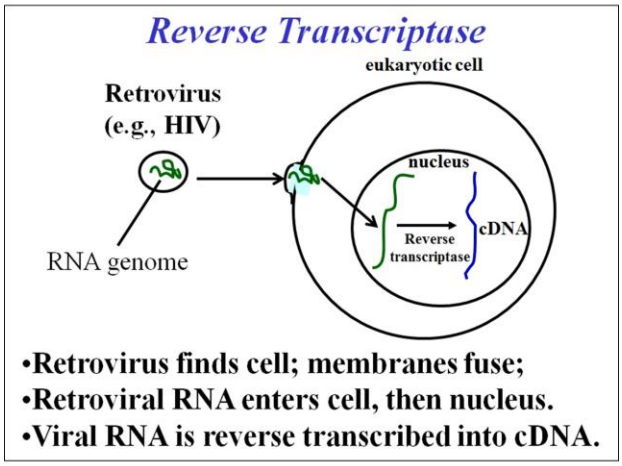


Fig. 15.1: Retroviral infection requires *reverse transcriptase* to make a copy (cDNA) of the viral genome, which will then replicate and reproduce new viruses.

Reverse transcriptases, along with many viral, bacterial and even eukaryotic enzymes and biomolecules, are now part of our recombinant DNA and genetic engineering toolkit.

We will see how a ***cDNA library*** is made and screened for a ***cDNA clone***, and how a cloned cDNA can fish an entire gene out of a ***genomic library***. Next we will see how the ***polymerase chain reaction*** (***PCR***) can produce (*amplify*) millions of copies of a single gene (or other DNA sequence) from as little DNA as is found in a single cell. Apart from its well-publicized use in forensics, *PCR* is another important laboratory tool for fetching, amplifying and studying sequences of interest. These venerable technologies illustrate important principles of cloning and sequence analysis. Of course, the analysis of traditionally cloned and amplified DNA sequences has been used to study the evolution and expression of individual genes.

A cautionary note: despite the realized and future promises of such powerful tools, we are sometimes misled! For example, knowing that a genetic mutation is associated with an illness usually leads to a search for how the mutation might cause the illness. But, as researchers in any discipline keep warning us, *correlation is not causation*! In fact, we know that many phenotypes, including genetic disease, are not the result of a single mutant gene. Autism is just one example. Nevertheless, newer fields of ***genomics*** and ***proteomics*** leverage a growing battery of tools to study many genes and their *regulatory*

networks at the same time. The ***molecular networking*** made possible by genomics and proteomics (and other colorful holistic terms we'll discuss later) promise to get us past naïve and often incorrect notions of causation. We may be soon able to identify *many* correlations that might sum up if not to causation, at least to a propensity to genetic illness. We'll look at some of these *tools of leverage*!

 **257 Overview of DNA Technologies** 

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. suggest molecular techniques to design experiments (e.g., how would you use *cDNA* or a *PCR* product to clone a gene).
2. determine when to make or use a *cDNA library* or a *genomic library.*
3. outline an experiment to *purify rRNA* from eukaryotic cells.
4. outline an experiment to isolate and clone a human cDNA for further study
5. explain why you might want to *clone and express* a human growth hormone gene.
6. list components needed to make a cDNA library using purified *poly(A) RNA*.
7. list the components needed to make a genomic library from isolated *genomic DNA*.
8. compare PCR and genomic cloning as strategies for isolating a gene.
9. outline a strategy for using fly DNA to obtain copies of a human DNA sequence.
10. ask a research question that requires screening a *genomic library* for a specific gene.
11. ask a question that requires using a *microarray* to obtain a gene you want to study.

15.2 Make and Screen a cDNA Library

The first step in making a ***cDNA library*** is to isolate cellular mRNA. This mRNA extract should represent all of the transcripts in the cells at the time of isolation, or the cell's ***transcriptome***. This term is used by analogy to genome. However, a genome is *all* of the genetic information of an organism. In contrast, a transcriptome (usually eukaryotic) reflects all of the genes expressed in a given cell type at a moment in time. Reverse-transcribed cDNAs from an mRNA extract are *also* referred to as a transcriptome.

A cDNA library is a tube full of bacterial cells that have taken up (i.e., been ***transformed*** with) plasmids recombined with cDNAs. cDNA libraries that are made from mRNAs taken from different cell types or the same cells grown under different conditions are in effect, different transcriptomes. Each reflects mRNAs transcribed in cells at the moment of their extraction. When cells in a cDNA library are spread out on a nutrient agar petri dish, each cell grows into a colony of cells; each cell in the colony is the clone of a starting cell. cDNA libraries can be used isolate and sequence the DNA encoding a polypeptide that you are studying.

Recall that the mature mRNA in eukaryotic cells has been spliced. Thus, cDNAs made from eukaryotic cells do not include introns. Introns, as well as sequences of enhancers and other regulatory elements in and surrounding a gene must be studied in genomic libraries, to be discussed later. Here we look at how to make a cDNA library.

15.2.1 cDNA construction

mRNA is only a few percent of a eukaryotic cell; most is rRNA. But that small amount of mRNA can be separated from other cellular RNAs by virtue of their 3' *poly(A) tails*. Simply pass a total RNA extract over an ***oligo-d(T) column*** like the one illustrated in Fig. 15.2 below.

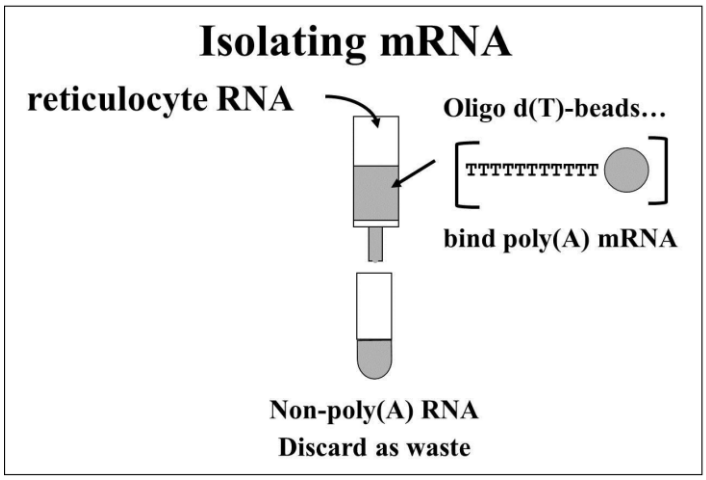


Fig. 15.2: Most eukaryotic mRNAs have *poly(A)* tails; Poly(A) RNA is isolable by *oligo d(T) chromatography*.

The strings of thymidine (T) in the oligo d(T) column H-bond with the *poly(A) tails* of mRNAs, tethering them to the column. All RNAs without a 3' poly(A) tail will flow through the column as waste. A second buffer is passed over the column to destabilize the H-bonds to allow ***elution*** of an mRNA fraction. If 'free' oligo d(T) is then added to the eluted mRNA, it also forms H-bonds with the poly(A) tails of the mRNAs where it can serve as a primer for the synthesis of cDNA copies of the poly(A) mRNAs originally in the cells. Adding four deoxynucleotide DNA precursors and ***reverse transcriptase*** (e.g., from chicken retrovirus-infected cells) will start reverse transcription. Fig. 15.3 (below) shows the synthesis of a cDNA strand complementary to an mRNA.

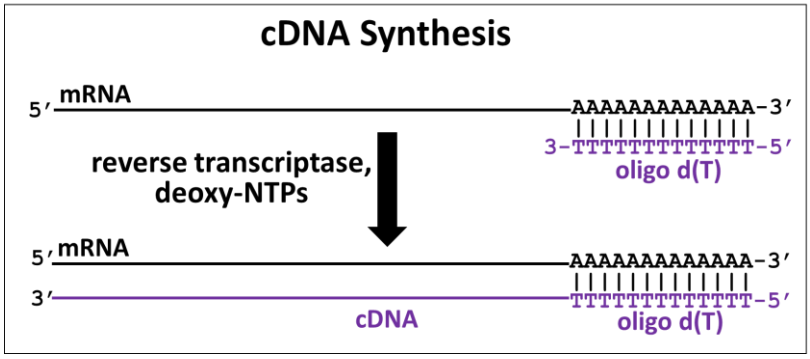


Fig. 15.3: Reverse transcriptase supplied with deoxynucleotides, mRNAs and an oligo d(T) primer will catalyze synthesis of DNA copies (cDNAs) of the mRNAs.

After heating to separate the cDNAs from the mRNAs, the cDNA is replicated to produce double-stranded, or (ds)cDNA (Fig.15.4).

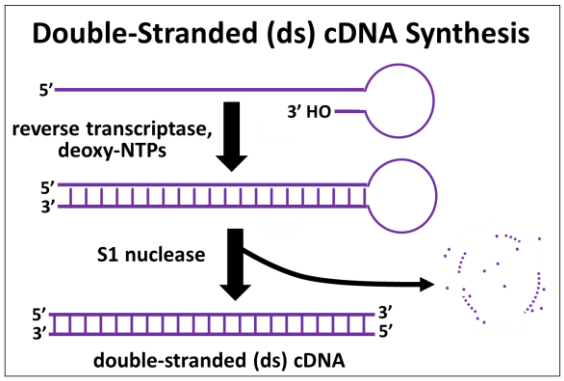


Fig. 15.4: The first cDNA strand often forms a loop at its 3' end that can serve as a primer to synthesize a (ds)cDNA. Reverse transcriptase is also a DNA polymerase, and can catalyze 2nd strand synthesis.

Synthesis of the second cDNA strand is also catalyzed by reverse transcriptase! Second strand synthesis is primed by the 3' end of a stem-loop structure that can form with most mRNAs. Reverse transcriptase is also a DNA polymerase, recognizing DNA as well as RNA templates, with the same 5'-to-3' DNA polymerizing activity as all DNA and RNA polymerases. After 2nd cDNA strand synthesis, an ***S1 nuclease*** (a specifically *single-stranded endonuclease* originally isolated from an East Asian

fungus!) is added to open the loop of the (ds)cDNA structure and trim the rest of the single-stranded DNA. What remains is the (ds)cDNA.

258 Isolate mRNA and Make cDNA 259 Reverse Transcriptase

15.2.2 Cloning cDNAs into plasmid vectors

To understand cDNA cloning and other aspects of making recombinant DNA, let's look again at what's in the recombinant DNA tool kit. In addition to *reverse transcriptase* and S1 nuclease, other enzymes in the 'kit' include ***restriction endonucleases*** (***RE**s* or restriction enzymes for short) and ***DNA ligase***. The natural function of *REs* in bacteria is to recognize and hydrolyze specific ***restriction site*** sequences in phage DNA, destroying the phage DNA and avoiding infection.

Some *restriction enzymes* cut through the two strands of the double helix to leave *blunt ends*. Others make a *staggered cut* on each strand at their restriction site leave behind *complementary* ('sticky') ends (Fig. 15.5).

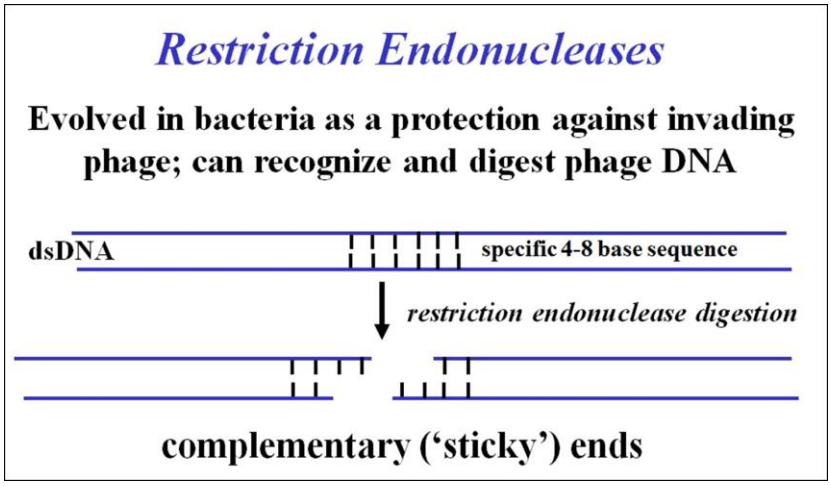


Fig. 15.5: Bacterial restriction endonucleases (REs) recognize and hydrolyze 'foreign' DNA (e.g., phage DNA), blocking infection; most REs cut DNA at specific short DNA sequences; often leaving staggered ends.

If you digest two (ds)DNAs from different sources (e.g. species) with an *RE* that makes a staggered cut, the resulting fragments will have the same sticky ends. If you mix these DNAs, their 'complementary ends will form H-bonds linking the fragments.

If the linked fragments are from different sources, we can add *DNA ligase* to covalently seal the fragments. We say then that the fragments have been recombined. Such a protocol makes it relatively easy to recombine any two different DNAs at will. Let's look at how we recombine plasmid DNAs and cDNA, the first steps in cloning cDNAs.

15.2.3 Preparing recombinant plasmid vectors containing cDNA inserts

Vectors (such as plasmids or phage DNA) are *carriers* engineered to recombine with foreign DNAs of interest. When a recombinant vector with its foreign ***DNA insert*** gets into a host cell, it can replicate many copies of itself, enough in fact for easy isolation and study. cDNAs are typically inserted into *plasmid vectors* (usually "store-bought"). They can be *cut* with an RE at a suitable location, leaving those *sticky ends*. However, it would not do to digest (ds)cDNA with restriction endonucleases since the goal is not to clone cDNA fragments, but entire cDNA molecules. Therefore, it will be necessary to attach ***linkers*** to either end of the (ds)cDNAs. Plasmid DNAs and cDNA-linker constructs can then be digested with the same restriction enzyme to produce compatible *sticky ends*. Fig. 15.6 shows the steps in the preparation of vector and (ds)cDNA for recombination.

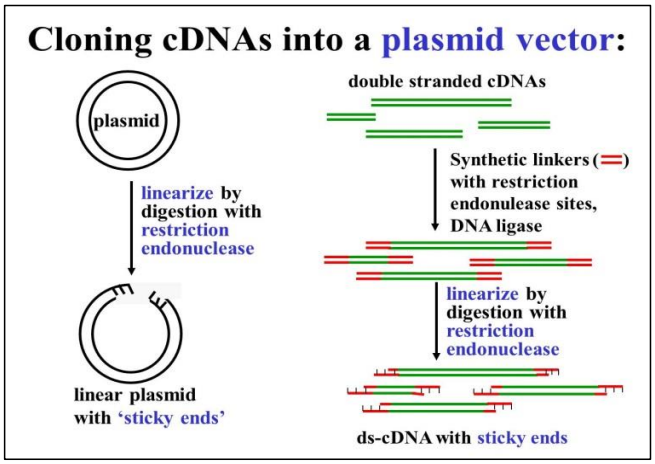


Fig. 15.6: Preparing cDNAs for insertion into plasmid vectors prior to cloning cDNAs sequences (see text for details);

To prepare for recombination, a plasmid vector is digested with a *restriction enzyme* to open the DNA circle. The double-stranded cDNAs to be inserted into the plasmid vector are mixed with *linkers* and ***DNA ligase*** to attach a linker DNA at both ends of

the (ds)cDNA. The *linkers* are short, synthetic double-stranded DNA oligomers containing restriction sites recognized and cut by the same restriction enzyme as the plasmid. Once the linkers are attached to the ends of the plasmid DNAs, they are digested with the appropriate restriction enzyme. This leaves both the (ds)cDNAs and the plasmid vectors with staggered, complementary sticky ends.

260 Restriction Enzymes and Recombinant DNA

15.2.4 Recombining plasmids and cDNA inserts and transforming host cells

The next step is to mix the cut plasmids with the digested linker-ended cDNAs in just the right proportions so that most of the cDNAs form H-bonds with most of the sticky plasmid ends. Once the H-bonds have connected (*annealed*) to the cDNAs to plasmid DNA, adding ***DNA ligase*** forms phosphodiester bonds between plasmid and cDNA insert, completing the recombinant circle of DNA. This process of making recombinant plasmids is shown in Fig. 15.7, below.

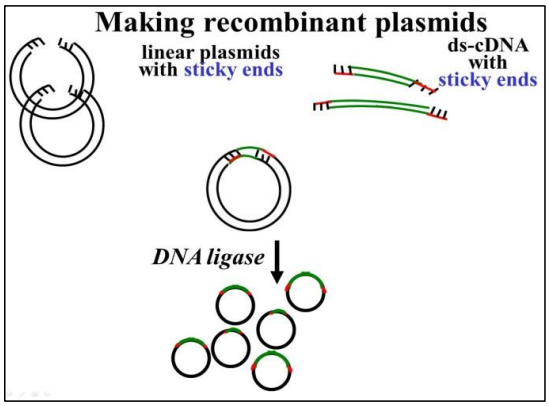


Fig. 15.7: Making recombinant plasmids containing cDNA inserts (see text for details).

In early cloning experiments, an important consideration was how to generate plasmids with only one copy of a given cDNA insert, rather than lots of re-ligated plasmids with no inserts or lots of plasmids with multiple inserts. Using better-engineered vector and linker combinations, this issue became less important.

261 Recombine a cDNA Insert with a Plasmid Vector

15.2.5 Transforming host cells with recombinant plasmids

The recombinant DNA molecules are now ready for the next step, creation of a ***cDNA library***. The recombinant DNAs are added to *E. coli* (or other) host cells, *made permeable* so that they are easily ***transformed***. Recall that transformation as defined by Griffith is the bacterial uptake of foreign DNA leading to a genetic change. The ***transforming principle*** in cloning is the recombinant plasmid! Fig. 15.8 (below) shows the transformation step that results in a tube full of transformed cells that is the *cDNA library*.

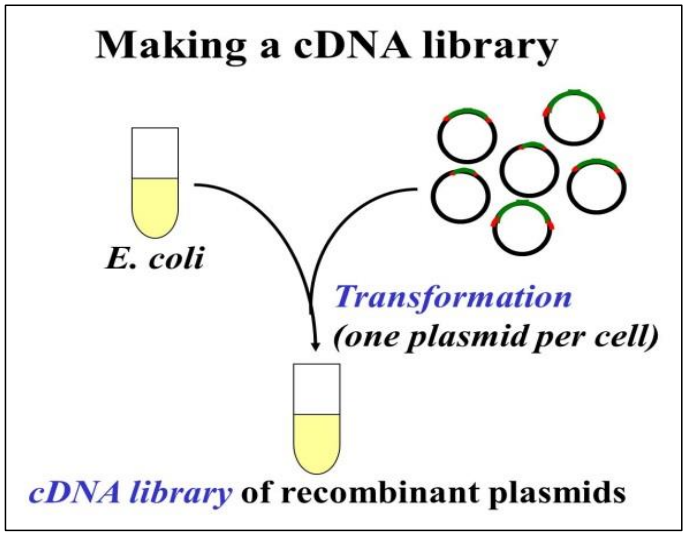


Fig. 15.8: Making the cDNA library (see text for details).

262 Making the cDNA Library

15.2.6 Plating a cDNA Library on Antibiotic-Agar to Select Recombinant Plasmids

After all these treatments, not all plasmid molecules in the mix are recombinant; some cells in the mix haven't even taken up a plasmid. So when the recombinant cells are plated on agar, how do you tell which of the colonies that grow came from cells that took up a recombinant plasmid? Both the ***host strain*** of *E. coli* and plasmid vectors used these days were further engineered to solve this problem. Specifically, a plasmid vector was designed that carried an ***antibiotic resistance gene***. In the

example below, ampicillin-sensitive cells are transformed with recombinant plasmids containing the ***ampicillin resistance gene***. The cells are then plated on media containing ***ampicillin*** (a form of penicillin), with the results shown in Fig. 15.9 below.

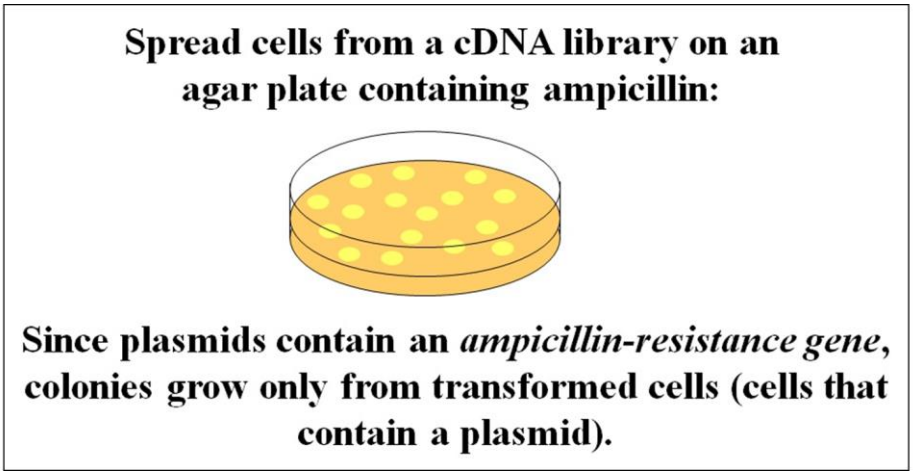


Fig. 15.9: Only cells containing plasmids will grow on agar containing *ampicillin* since the plasmids contain an ampicillin resistance gene (see text for details).

Transformed cells, those that that took up a recombinant plasmid carrying the ampicillin resistance gene, can grow on the ampicillin-agar medium. Untransformed cells (cells that failed to take up a plasmid) lack the ampicillin resistance gene cannot grow on the ampicillin-medium.

But, there is still a question. How can you tell whether the cells that grew were transformed by a recombinant plasmid containing a cDNA insert? It is possible that some of the transformants are non-recombinant plasmids that still have the ampicillin resistance gene! To address this issue, plasmids were further engineered to contain a ***streptomycin resistance gene*** (as well as the ampicillin resistance gene). But in this case, the restriction enzyme sites in the plasmid that were to be used for recombination were placed in the middle of the gene. So, inserting a cDNA in *this* plasmid would disrupt and inactivate the ***streptomycin resistance gene***. This second bit of genetic engineering enabled harvesting *only* cells transformed with recombinant plasmids (i.e., those containing a cDNA insert). We can tell apart *transformants* containing recombinant plasmids from those with non-recombinant plasmids by the technique of ***replica plating*** (Fig. 15.10 below).

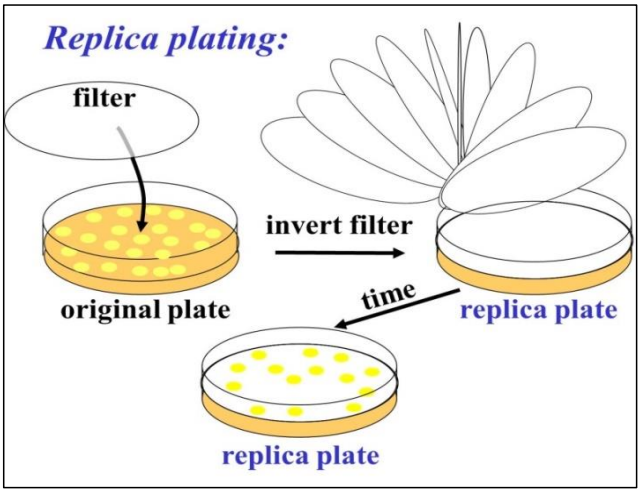


Fig. 15.10: *Replica plating* creates a *filter replica* of colonies from one agar plate to be grown on another plate, e.g., containing 'selective' media containing for example an antibiotic that screens for recombinant plasmids (see text for details).

After colonies grow on the ampicillin agar plate, lay a filter over the plate. The filter will pick up a few cells from each colony, in effect becoming a ***replica filter*** (i.e., a mirror image) of the colonies on the plate. Next, place the *replica filter* on a new agar plate containing *streptomycin*. Any colonies that grow on the filter must be streptomycin-resistant, containing only non-recombinant plasmids. Colonies containing recombinant plasmids, those that did not grow in streptomycin, are easily identified on the original ampicillin agar plate. In practice, highly efficient recombination and transformation procedures typically reveal very few streptomycin-resistant cells (i.e., colonies) after replica plating. When this happens, the ampicillin-resistant cells constitute a good cDNA library, ready for screening.

263 Making a Replica Plate Filter

15.2.7 Identifying Colonies Containing Plasmids with Inserts of Interest

The next step is to ***screen*** colonies in a cDNA library for those containing a specific cDNA. Since cells typically make thousands of proteins at the same time, a cDNA library should contain thousands of cDNAs made from thousands of mRNAs. Finding a single cDNA of interest can require plating the cDNA library in a tube on more than a few agar plates.

Continuing with the example above, actual screening would be done using multiple replica filters of ampicillin-resistant cells. The number of replica filters to be screened can be calculated from assumptions and formulas for estimating how many colonies must be screened to represent an entire *transcriptome* (i.e., the number of different mRNAs in the original cellular mRNA source).

Once the requisite number of replica filters are made, they are subjected to ***in situ* lysis** to disrupt cell walls and membranes. The result is that the cell contents are released and the DNA is denatured (unwound to become single-stranded). The DNA then adheres to the filter *in place* (*in situ*), where the colonies were). The result of *in situ lysis* is a filter with faint traces of the original colonies (Fig. 15.11).

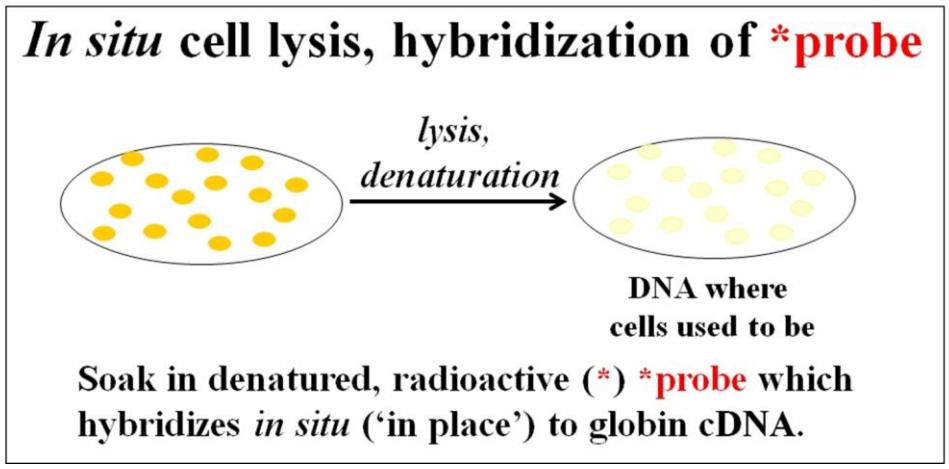


Fig. 15.11 *Replica filters* are lysed *in situ* (in place), leaving partially denatured DNA (including recombinant plasmid DNA) from the colonies where the cells used to be. Filters can be probed for a sequence of interest.

Next, a molecular ***probe*** is used to identify DNA containing the cDNA sequence of interest. A probe can be any DNA sequence that is complementary to the sequence you are looking for. This could be a cloned gene or just as often, a ***synthetic oligonucleotide*** whose sequence was inferred from known amino acid sequences. The probe is made radioactive and placed in a bag with the filter(s). DNA from cells that contained recombinant plasmids with a cDNA of interest will bind the complementary *probe*. The results of *in situ* lysis and ***hybridization*** of a radioactive probe to a replica filter are shown in Fig. 15.12 (below).

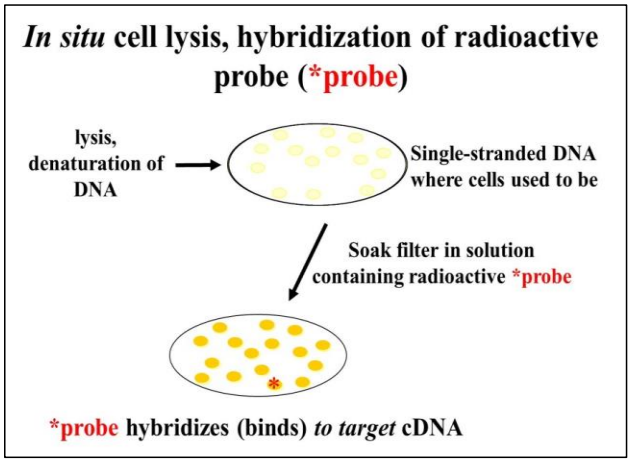


Fig. 15.12: A denatured, sequence-specific probe can be used to probe lysed replica filters for a sequence of interest, here detected as a radioactive colony (see Fig. 15.12 and text for details).

264 Probing a Replica Plate Filter

The filters are rinsed to remove un-bound radioactive oligomer probe, and then placed on X-ray film. After a period of exposure, the film is developed. Black spots will form on the film from radioactive exposure, creating an autoradiograph of the filter. The black spots in the autoradiograph in Fig. 15.13 (below) correspond to colonies on a filter that contain a recombinant plasmid with your target cDNA sequence.

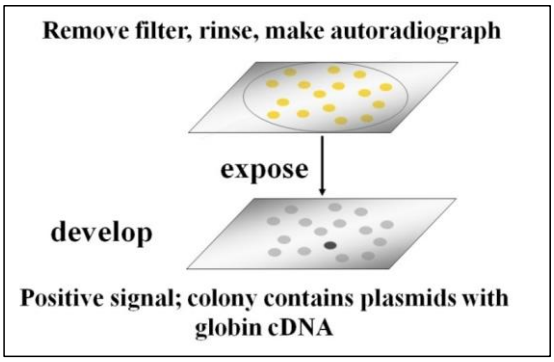


Fig. 15.13:. X-ray exposure of a replica filter incubated earlier with radioactive probe. The developed *autoradiograph* shows a dark spot where probe hybridized to colony DNA (see text for details).

Once a positive clone is identified on the film, the corresponding recombinant colony is located on the original plate. This colony is grown up in a liquid culture and the plasmid DNA is isolated. At that point, the cDNA can be sequenced and the amino acid sequence encoded by the cloned cDNA can be inferred from the genetic code dictionary. This will verify that the cDNA *insert* in fact encodes a protein of interest.

Once verified as containing the desired sequence, recombinant plasmids can be isolated. tagged (made radioactive or fluorescent) and used to

- probe for the genes from which they originated.
- identify and quantitate the mRNA even locate the transcripts in the cells.
- quantitatively measure amounts of specific mRNAs.

Isolated plasmid cDNAs can even be expressed in suitable cells to make the encoded protein. These days, diabetics no longer receive pig insulin, but get synthetic human insulin human made from expressed human cDNAs. Moreover, while the introduction of the *polymerase chain reaction* (***PCR***, see below) has superseded some uses of cDNAs, they still play a role in genome-level and transcriptome-level studies.


265 Pick a Clone From a Replica Filter and Play With It!


15.3 DNA sequencing

Foe some perspective, we should note that RNA sequencing actually came first, when Robert Holley sequenced a tRNA in 1965. The direct sequencing of tRNAs was possible because they are small, short nucleic acids and because many of the bases in tRNAs are chemically modified after transcription. Twelve years late in 1977, two different methods for sequencing DNA were reported. One method, developed by Allan Maxam and Walter Gilbert involved DNA fragmentation and sequencing of the small fragments of DNA, and then aligning the overlapping sequences of the short fragments to assemble longer sequences. This became known as ***Maxam-Gilbert DNA sequencing***. The other method was developed by Frederick Sanger and colleagues in England. This is the DNA synthesis-based ***di-deoxy-DNA sequencing*** technique. Sanger and Gilbert both won a Nobel Prize in Chemistry in 1983 for their DNA sequencing work. However, because of its simplicity, Sanger's method quickly became the standard for sequencing all manner of cloned DNAs, including the complete sequencing of a genome, that of a bacteriophage (bacterial virus) called *φX174*.

At the same time as the advances in sequencing technology were occurring, so were some of the early developments in recombinant DNA technology. Together these led to more efficient and rapid cloning and sequencing of DNA from increasingly diverse sources. The first focus was of course on genes and genomes of important model organisms, such as *E. coli, C. elegans,* yeast (*S. cerevisiae*)…, and of course, us! By

1995, Craig Venter and colleagues at the *Institute for Genomic Research* had completed the sequence of an entire bacterial genome (*Haemophilus influenzae*) and by 2001, Venter's private group along with Frances Collins and colleagues at the NIH had published a first draft of the sequence of the human genome. Venter had proven the efficacy of a whole-genome sequencing approach called *shotgun sequencing*, which was much faster than the gene-by-gene, fragment-by-fragment 'linear' sequencing strategy being used by other investigators (more later!). Since Sanger's di-deoxynucleotide DNA sequencing method remains a common and economical methodology, let's consider the basics of the protocol. Here are the details…

15.3.1 Manual DNA Sequencing

Given a template DNA (e.g., a plasmid cDNA), Sanger used *in vitro* replication protocols to demonstrate that he could:

- replicate DNA under conditions that randomly stopped nucleotide addition at every possible position in growing strands.
- *separate and then detect* these DNA fragments of replicated DNA.

Recall that DNA polymerases catalyze the formation of *phosphodiester bonds* by linking the α *phosphate* of a ***nucleotide triphosphate*** to the free 3' OH of a deoxynucleotide at the end of a growing DNA strand. Recall also that the ribose sugar in the deoxynucleotide precursors of replication lack a 2' OH (hydroxyl) group. Sanger's trick was to add ***di-deoxynucleotide triphosphates*** to his *in vitro* replication mix. The ribose on a *di-deoxynucleotide triphosphate* (ddNTP) lacks a 3' OH, as well a 2' OH (Fig. 15.14).

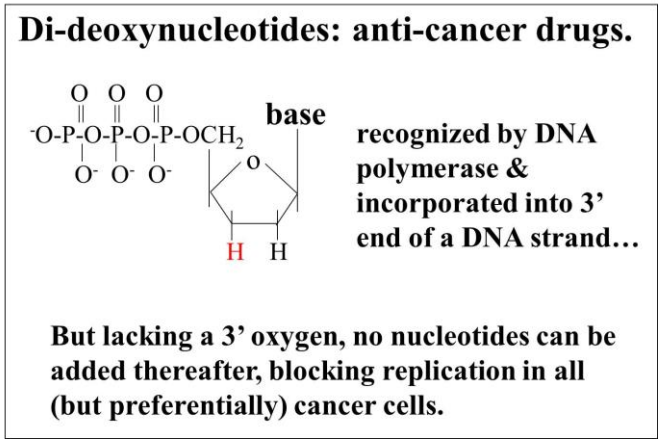


Fig. 15.14: Chemical structure of a di-deoxynucleotide.

Adding a di-deoxynucleotide to a growing DNA strand stops replication. No further nucleotides can add to the 3'-end of the replicating DNA strand because the 3'–OH necessary for the dehydration synthesis of the next phosphodiester bond is absent! Because they can stop replication in actively growing cells, ddNTPs such as *di-deoxyadenosine* (tradename, ***cordycepin***) are anti-cancer chemotherapeutic drugs.

266 Treating Cancer with Dideoxynucleosides

A look at a manual DNA sequencing reveals what is going on in the sequencing reactions. Four reaction tubes are set up, each containing the template DNA to be sequenced. Each tube also contains a *primer* of known sequence and the four required deoxynucleotide precursors necessary for replication. Fig. 15.15 (below) shows the set-up for manual DNA sequencing.

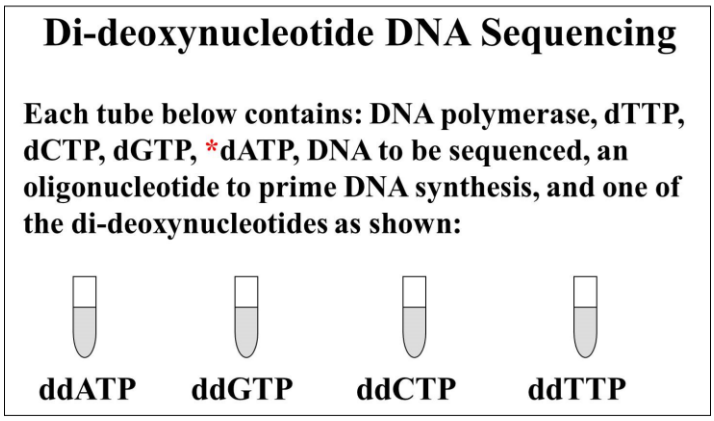


Fig. 15.15: The four tubes in a manual DNA sequencing protocol contain the same ingredients, except that each contains a different di-deoxynucleotide.

A different ddNTP, (ddATP, ddCTP, ddGTP or ddTTP) is added to each of the four tubes. Finally, DNA polymerase is added to each tube to start the DNA synthesis reaction. During DNA synthesis, different length fragments of new DNA accumulate as the ddNTPs are incorporated at random opposite complementary bases in the template DNA being sequenced.

A short time after adding the DNA polymerase to begin the reactions, the mixture is heated to separate the DNA strands and fresh DNA polymerase is added to replace the enzyme destroyed by heating and repeat the synthesis reactions. These reactions

are repeated as many as 30 times in order to produce enough radioactive DNA fragments to be detected. The expectations of these sequencing reactions in the four tubes are illustrated in Fig 15.16 (below).

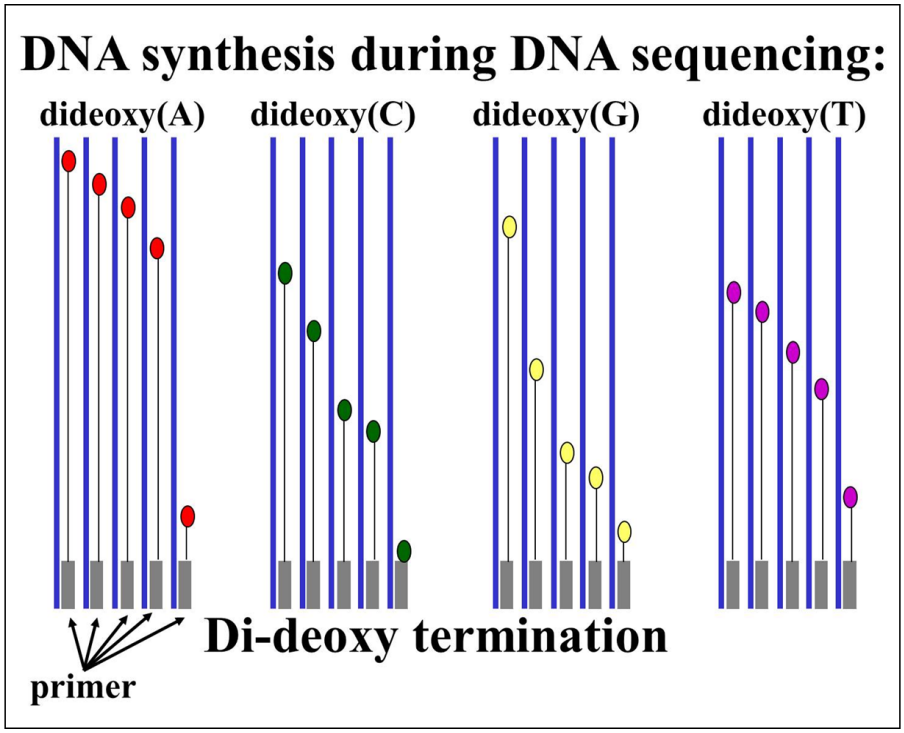


Fig. 15.16: Cartoon of results of the four different manual sequencing reactions. The dideoxy-terminated DNA fragments are electrophoresed to read the sequence of a DNA (see Fig 15.16).

When the ***Taq DNA polymerase*** from the thermophilic bacterium *Thermus aquaticus* became available (more later!), it was no longer necessary to add fresh DNA polymerase after each replication cycle because the heating step required to denature new DNA made in each cycle would not destroy the heat-stable Taq DNA polymerase.

Thanks to Taq polymerase, the many heating and cooling cycles required for what became known as *chain-termination DNA sequencing* were soon automated using inexpensive *programmable thermocyclers*. Since a small amount of a radioactive deoxynucleotide (usually ^{32}P-labeled ATP) was present in each reaction tube, all

newly made DNA fragments are radioactive. After electrophoresis to separate the new randomly terminated DNA fragments in each tube, autoradiography of the electrophoretic gel reveals the position of each terminated fragment. The DNA sequence can then be read from the gel as illustrated in the simulated autoradiograph below (Fig. 15.17).

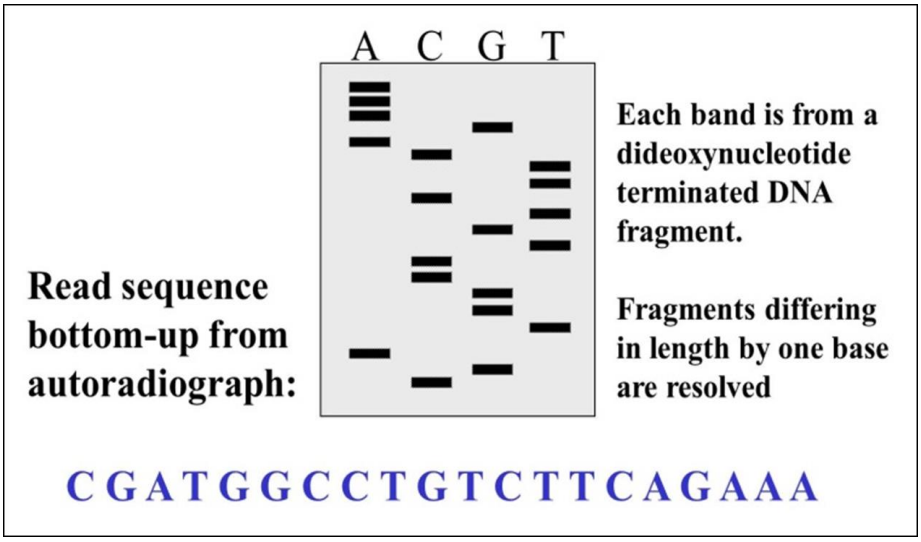


Fig. 15.17: Drawing of a manual dideoxy sequencing autoradiograph with a readable sequence.

The DNA sequence can be read by reading the bases from the bottom of the film, starting with the C at the bottom of the C lane. Try reading the sequence yourself!

267 Manual Dideoxy Sequencing

15.3.2 Automated First Generation DNA Sequencing

The first semi-automated DNA sequencing method was invented in Leroy Hood's California lab in 1986. Though it was still Sanger's di-deoxy sequencing, a radioactive phosphate-labeled nucleotide was no longer necessary. Instead, the four dideoxy-nucleotides in the sequencing reaction were each tagged for detection with a different fluorescent dye. Sequence reaction products are electrophoresed on an ***automated DNA sequencer***. Migrating di-deoxy-terminated DNA fragments pass through a

beam of UV light and a *detector* 'sees' the fluorescence of each DNA fragment. The color and order of the fragments is sent to a computer, generating a colored plot showing the order and length, and thus their sequences (Fig. 15.18).

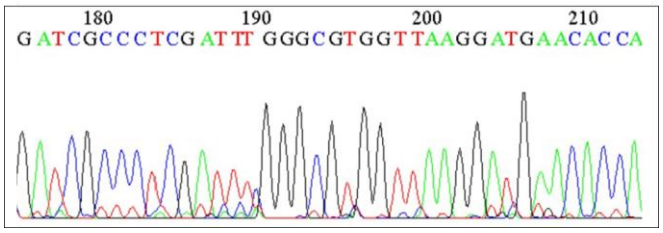


Fig. 15.18: Automated DNA sequence chromatograph readout.

A most useful feature of this sequencing method is that a template DNA could be sequenced in a single tube containing all the required components, including *all* four dideoxynucleotides! That's because the fluorescence detector in the sequencing machine separately sees all the short ddNTP-terminated fragments as they move through the electrophoretic gel. Hood's innovations were quickly commercialized making major (including many genome) sequencing projects possible. Automated DNA sequencing rapidly augmented large sequence databases in the U.S. and Europe. In the U.S., the NCBI (National Center for Biological Information) maintains a sequence database and despite its location, archives virtually all DNA sequences determined worldwide.

Now 'tiny' DNA sequencers have made sequencing DNA so portable that in 2016, one was even used in the *International Space Station* (see a photo of one at the top of this chapter!). New tools and protocols (some described below) are used to find, compare and globally analyze DNA sequences almost as soon as they get into the databases.

268 Automated Sequencing Leads to Large Genome Projects

15.3.3 Shotgun Sequencing

Large-scale sequencing targets entire prokaryotic and typically much larger eukaryotic genomes. The latter require strategies that either sequence long DNA fragments and/or that sequence many short DNA fragments more quickly. We already noted the ***shotgun sequencing*** strategy used by Venter to sequence whole genomes (including our own… or more accurately, his own!). In *shotgun sequencing*, cloned DNA fragments of 1000 base pairs or longer are broken down at random into smaller, more

easily sequenced fragments. The fragments are themselves cloned and sequenced. Non-redundant sequences are then assembled by aligning regions of sequence overlap.

If there are no gaps (due to unrepresented DNA fragments that therefore fail to be sequenced), today's computer software is quite adept at rapidly aligning the available overlapping sequences and connecting them to display long contiguous DNA sequences. Shotgun sequencing is summarized in Fig. 15.19 (below).

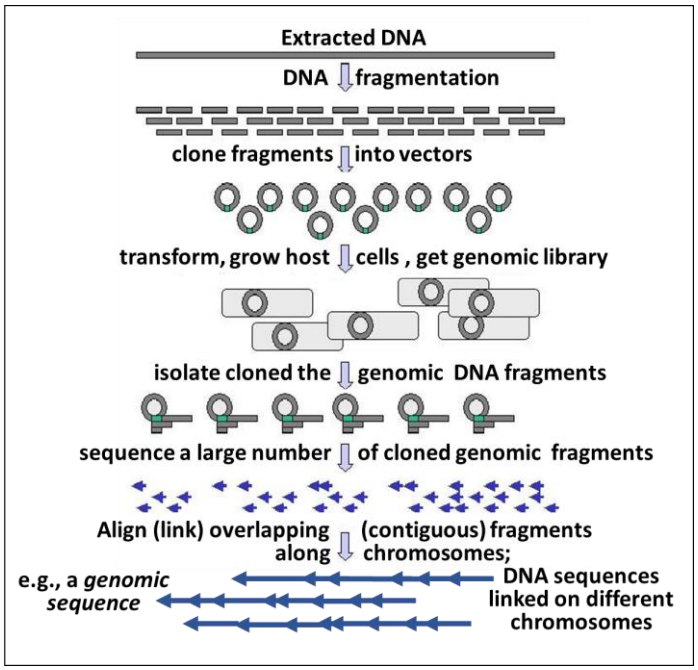


Fig. 15.19:.Overview of shotgun sequencing of DNA.

Sequence gaps that remain after shotgun sequencing can be filled in by ***primer walking***, in which a known sequence near the gap is the basis of creating a sequencing primer to "walk" into the gap region on an intact DNA that has not been fragmented. Another 'gap-filling' technique involves the *Polymerase Chain Reaction* (***PCR***), to be described shortly). Briefly, two oligonucleotides are synthesized based on sequence information on either side of a gap. Then PCR is used to synthesize the missing fragment that is then sequenced to fill in the gap.

15.3.4 Next Generation DNA Sequencing

The goals of "next gen" sequencing methods are to get accurate reading of millions or even billions of bases in a short time. Examples of these sequencing systems include *pyrosequencing*, *ligation-based sequencing* and *ion semiconductor sequencing* and involve ***massively parallel sequencing reactions*** methods. They differ in detail but all require a library (e.g., genomic, transcriptomic) attached to a solid surface, not unlike a microarray. The attached DNAs are amplified *in situ* into separate *clusters* derived from single library clones that then participate in the sequencing reaction; sequences are analyzed and generated by computer. However, accuracy is a problem because longer reads take more reactions and more time that lead to reduced accuracy. Click https://www.youtube.com/watch?v=jFCD8Q6qSTM. for a look at some of these technologies. A more accurate *new* technology is ***nanopore***-based DNA sequencing, outlined in Fig. 15.20 below. *Nanopore* sequencing was first used on the space station in 2016.

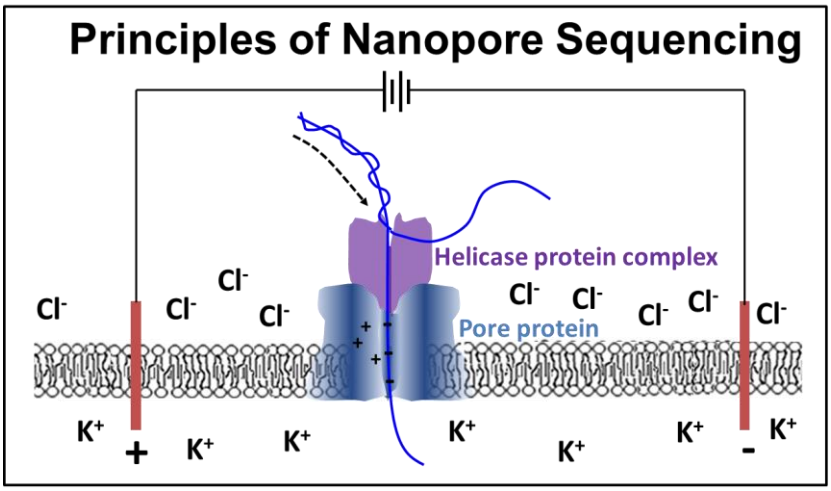


Fig. 15.20: Basics of Nanopore DNA Sequencing, one of several 'next gen' sequencing technologies. See text for details. Click https://www.youtube.com/watch?v=XWdkZhYgMgo to see why one would want to sequence DNA in space.

This device uses an artificial phospholipid membrane in which are embedded an engineered ***helicase/motor-pore protein*** complex. The nanopore itself is derived from the *Mycobacterium smegmatis* porin A (MspA) protein. A voltage is applied across the membrane to create a membrane potential. Double stranded DNA is

propelled into the helicase where it is unwound. The resulting single stranded molecules then are pass through a tiny hole (the *nanopore*) in the MspA porin. As the ssDNA (or RNA) pass through the pore, its charge configurations disturb the electrical field in the porin. These disturbances are different for each nucleotide, generating a unique signal that is detected and recorded and displayed in almost real time on small computer.

First generation automated dideoxy Sanger sequencing is still widely used to analyze individual genes or PCR products (see below). But next generation DNA sequencing and small devices like the one used in the space station can also be used in the field, not only for research, but for example, to quickly identify microbial agents of disease. Next generation sequencing protocols and devices are already providing long and increasingly more accurate data required for research and for clinical applications.

15.4 Genomic Libraries

A tube full of ***recombinant bacteriophage*** is basically a ***genomic library***. Each phage DNA molecule should contain a fragment of foreign cellular DNA. A good genomic library will contain a representation of all of possible fragments of an organism's genome. Bacteriophage are often used to clone genomic DNA fragments because phage genomes are bigger than plasmids and can be engineered to remove large amounts of DNA that are not needed for infection and replication in host cells. The missing DNA can then be replaced by large foreign DNA inserts, fragments as long as 18-20kbp (kilobase pairs), nearly 20X longer than cDNA inserts in plasmids. Purified phage coat proteins can then be mixed with the recombined phage DNA to make ***infectious phage particles*** (i.e., recombinant phage). Infection of host bacteria by these 'particles' leads to replication of the recombinant phage DNA, new phage production, cell lysis and the release of lots of new recombinant phage.

Consider the following bit of math: A typical mammalian genome consists of more than *2 billion* base pairs. Plasmid inserts are short, rarely exceeding 1000 base pairs. Divide *2,000,000,000* by *1000* to get *2 million*, a minimum number of recombinant plasmid clones that must be screened to find a sequence of interest. In fact, you need many more than this number to find even the smaller parts of a gene that add up to a whole gene! Of course, part of the solution to this "needle in a haystack" dilemma is to clone larger DNA inserts in more accommodating vectors. Hence the value of a bacteriophage vector.

From this brief description, you may recognize the common strategy for genetically engineering a cloning vector: determine the minimum properties that your vector must have and remove non-essential DNA sequences. Consider the ***Yeast Artificial Chromosome*** (**YAC**), hosted by (replicated in) yeast cells. YACs can accept humongous

foreign DNA inserts! This is because to be a chromosome that will replicate in a yeast cell requires ***one centromere and two telomeres***… and little else! Recall that telomeres are needed in replication to keep the chromosome from shortening during replication of the DNA. The centromere is needed to attach chromatids to spindle fibers so that they can separate during *anaphase* in *mitosis* (and *meiosis*). So along with a centromere and two telomeres, just include restriction sites to enable recombination with inserts as long as 2000 Kbp - that's the YAC. The tough part of course is keeping a 2000Kbp long DNA fragment intact long enough to get it into the YAC!

Whatever the vector of choice, sequencing its insert can tell us many things. It can show us how a gene is regulated by confirming known and revealing new regulatory DNA sequences. It can show neighboring genes, helping us map genes on a chromosome. Genomic DNA sequences from one species can probe for similar sequences in other species, allowing comparative sequence analysis that can tell us a great deal about gene evolution and the evolution of species. One early surprise from gene sequencing studies was that we share many common genes and DNA sequences with other species, like yeast, worms, flies and of course vertebrates…, including our more closely related mammalian friends. You may already know that chimpanzee and human genomes are 99% similar. And we have already seen comparative sequence analysis showing how proteins with different functions in the same (and even across) species nevertheless share structural domains.

Let's look at how we would make a phage genomic library that targets a specific gene of interest. As you will see, the principles are similar to cloning a foreign DNA into a plasmid, or in fact any other vector, but the numbers and details used here exemplify cloning in phage.

15.4.1 Preparing Specific Length Genomic DNA for Cloning; the Southern Blot

To begin with, high molecular weight (i.e., long molecules of) the desired genomic DNA are isolated, purified and then digested with a restriction enzyme. Usually, the digest is partial, aiming to generate overlapping DNA fragments of random length. The digested DNA is mixed with *ethidium bromide*, a fluorescent dye that binds to DNA. After electrophoresis on agarose gels and exposed to UV light, the DNA appears as a bright fluorescent smear. If we wanted to clone the complete genome of an organism, we could recombine all of this DNA with suitably digested vector DNA. But, since we are only after one gene, we can reduce the number of clones to be screened in order to find our sequence of interest. In the early days of cloning we would do this by creating a ***Southern blot*** (named after Edward Southern, the inventor of the technique). This technique lets us identify the size of genomic DNA fragments most likely to contain a desired gene.

The Southern blot protocol is illustrated in Fig. 15.21 (below).

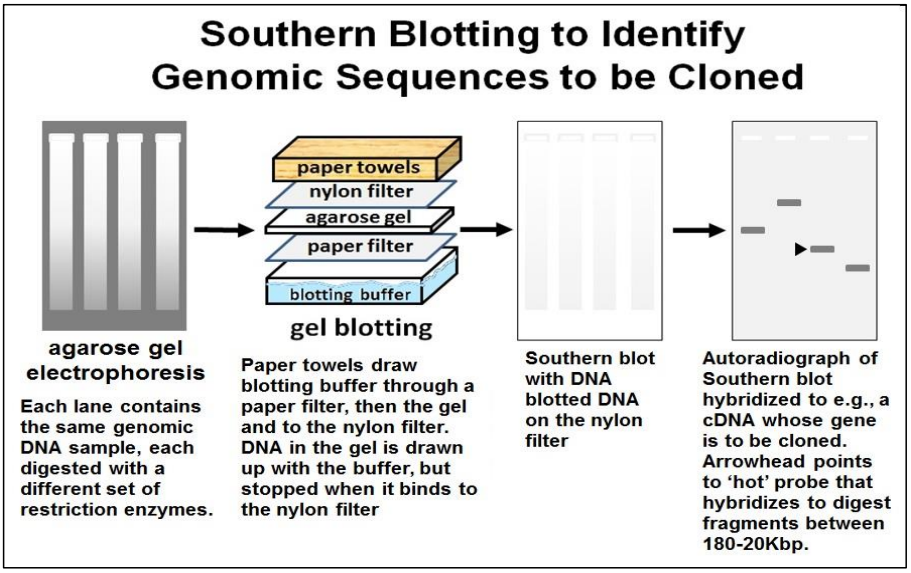


Fig. 15.21:.Overview of Southern blotting, a technique that transfers electrophoresed DNA to a filter for hybridization with a probe (see text for details).

To summarize the steps to make a Southern Blot:

a) Digest genomic DNA with one or more restriction endonucleases.

b) Run the digest products on an agarose gel to separate fragments by size (length). The DNA appears as a smear when stained with a fluorescent dye.

c) Place a filter on the gel. The DNA transfers (blots) to the filter for e.g., 24 hours.

d) Remove the blotted filter and place it in a bag containing a solution that can denature the DNA.

e) Add radioactive probe (e.g., cDNA) containing the gene or sequence of interest. The probe hybridizes (bind) to complementary genomic sequences on the filter.

f) Prepare an autoradiograph of the filter and see a 'band' representing the size of genomic fragments of DNA that include the sequence of interest.

Once you know the size (or size range) of restriction digest fragments that contain the DNA you want to study, you are ready to

a) run another gel of digested genomic DNA, and this time…,

b) cut out the piece of gel containing fragments of the size that that 'lit up' with your probe in the autoradiograph.

c) remove (elute) the DNA from the gel piece into a suitable buffer.

d) prepare the DNA for insertion into (recombination with) a genomic cloning vector.

15.4.2 Recombining Size-Restricted Genomic DNA with Phage DNA

After elution of restriction digested DNA fragments of the right size range from the gels, we mix the DNA with compatibly digested phage DNA at concentrations that favor the formation of H-bonds between the ends of the phage DNA and the genomic fragments (rather than with each other!). Addition of DNA ligase covalently links the recombined DNA molecules. These steps are abbreviated below in Fig. 15.22.

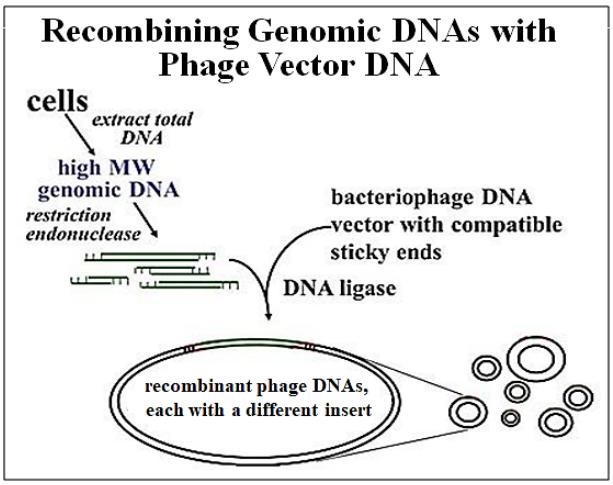


Fig. 15.22:.Genomic DNA restriction enzyme digest is mixed with phage vector with compatible 'sticky' ends and ligated to make recombinant DNAs.

The recombinant phage that are made next will contain sequences that become the genomic library.

15.4.3 Creating Infectious Viral Particles with Recombinant phage DNA

The next step is to ***package*** the recombined phage DNA by adding purified viral coat proteins to make infectious phage *particles* (Fig. 15.23 below).

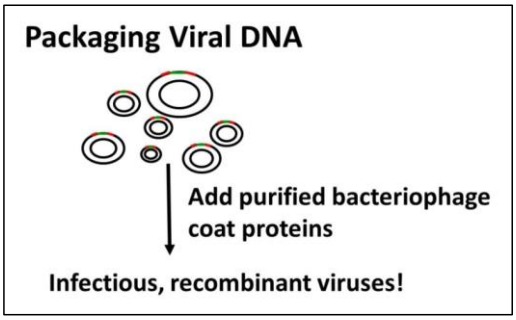


Fig. 15.23:.Mixing recombinant phage DNA with phage coat proteins creates infectious recombinant phage particles.

269 Genomic Libraries: Make and Package Recombinant Phage DNA

Packaged phage are added to a culture tube full of host bacteria (e.g., *E. coli*). After infection the recombinant DNA enters the cells where it replicates and directs the production of new phage that eventually lyse the host cell (Fig. 15.24).

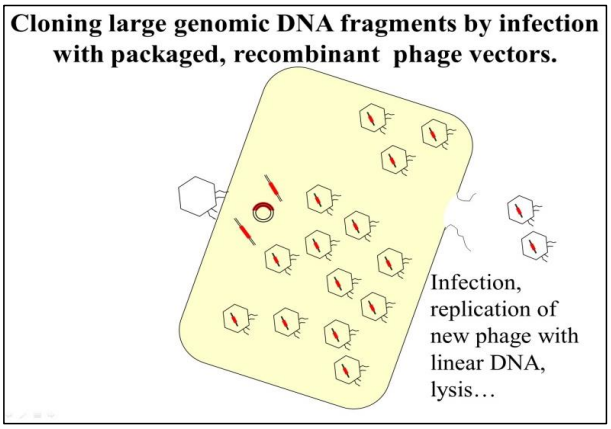


Fig. 15.24:.Recombinant phage infection, the replication of phage DNA and production of new phage leads to host cell lysis, allowing the collection of new recombinant phage.

The recombined vector can also be introduced directly into the host cells by ***transduction***, which is to phage DNA what transformation is to plasmid DNA. Whether by *infection* or *transduction*, the recombinant phage DNA ends up in host cells which produce new phage that eventually lyse the host cell. The released phages go on to infect more host cells until all cells have lysed. What remains is a tube full of ***lysate*** containing cell debris and lots of recombinant phage particles.

270 Infect Host with Recombinant Phage to Make a Genomic Library

Just a note on some other vectors for genomic DNA cloning… For large genomes, the goal is to choose a vector able to house larger fragments of 'foreign' DNA so that you end up screening fewer clones. We've seen that phage vectors accommodate larger foreign DNA inserts than plasmid vectors, and YACs even more… Given a large enough eukaryotic genome, it may be necessary to screen more than a hundred thousand clones in a phage-based genomic library. Apart from size-selection of genomic fragments before inserting them into a vector, selecting the appropriate vector is just as important. Table 15.1 lists commonly used vectors and the sizes of inserts they will accept.

Vector type	Insert size (thousands of bases)
Plasmids	up to 15
Phage lambda (λ)	up to 25
Cosmids	up to 45
Bacteriophage P1	70 to 100
P1 artificial chromosomes (PACs)	130 to 150
Bacterial artificial chromosomes (BACs)	120 to 300
Yeast artificial chromosomes (YACs)	250 to 2000

Click on the links to these vectors if you wish to learn more about them. We will continue this example by screening a phage lysate genomic library for a recombinant phage with a genomic sequence of interest.

15.4.4 Screening a Genomic Library; Titering Recombinant Phage Clones

A ***bacterial lawn*** is made by plating so many bacteria on the agar plate that they simply grow together rather than as separate colonies. If a small amount of phage is evenly plated over the bacterial lawn, each virus will infect one cell. Subsequent lysis of this cell releases many phage, each infecting neighboring cells. After a day or so of

repeated cycled of lysis and infection, ***plaques*** (clearings) appear in the lawn at the site of the first infection. Each plaque shown in the illustration in Fig. 15.25 (below) is a clone of the original phage particle.

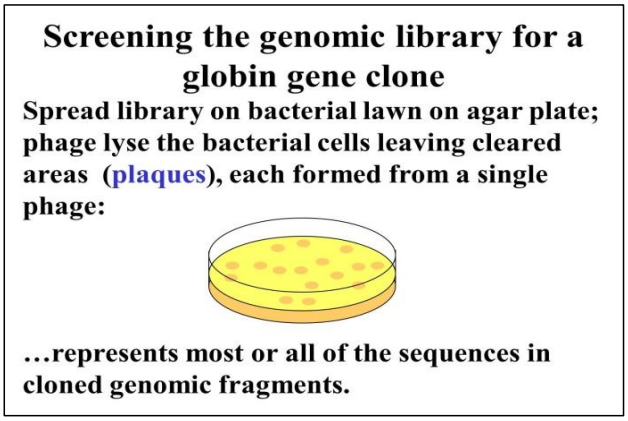


Fig. 15.25:.Phage plaques formed on a bacterial lawn; each plaque is a genomic clone.

To screen a phage genomic library, the phage lysate is ***titered*** on bacterial lawns. In a typical ***titration***, a phage lysate might be diluted 10-fold with a suitable medium. This dilution is further diluted 10-fold… and so on. Each ***serial 10X dilution*** is then spread on a bacterial (e.g., *E. coli*) lawn. The plaques formed on each lawn are then counted. Remember, a plaque is a clone of a single virus.

Let's say that when 10 μl of one of the dilutions are spread on the bacterial lawn, they infect 500 *E. coli* cells on the bacterial lawn. After a day or so, there will be 500 ***plaques*** in this example. If you actually counted 500 plaques on the agar plate, then there must have been 500 virus particles in the 10 μl seeded onto the lawn. And, if this plate was the fourth dilution in a 10-fold serial dilution protocol, there must have been 2000 (4 X 500) phage particles in 10 μl of the original undiluted lysate.

15.4.5 Screening a Genomic Library; Probing the Genomic Library

To represent a *complete genomic library*, you will need many plates of a serial 10X dilutions, containing say, ~500-1000 plaques per plate If size-selected fragments (e.g., identified by Southern blotting) were cloned to make a partial genomic library, then there will be fewer plaques to be screened to find a sequence of interest. Since plaques contain a lot of phage DNA not yet packaged at the time of lysis it is possible

to transfer that DNA directly to filters by to replica plating (similar to replica plating of bacterial colonies). The replica filters are treated to denature the DNA and then hybridized to a probe with a known sequence. In the early days of cloning, the probes were often a sequenced cDNA previously isolated from libraries of the same or different (usually related) species. After soaking the filters in a radioactively-labeled probe, an X-Ray film is placed over the filter, exposed and developed. Black spots will form where the film lay over a plaque containing genomic DNA complementary to the probe. In the example illustrated below in Fig. 15.26, a globin cDNA might have been used to probe our partial genomic library (globin genes were in fact among the first to be cloned!).

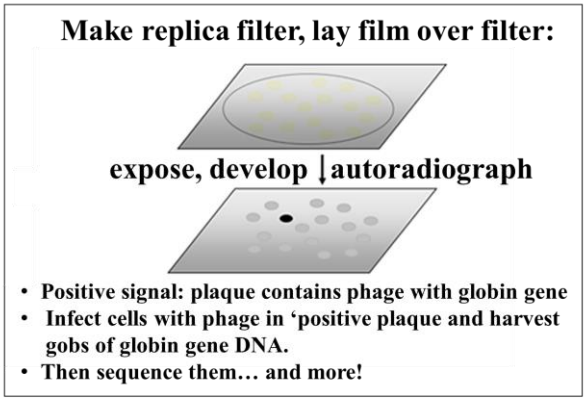


Fig. 15.26:.Replica filters of plaques probed with a sequence of interest (e.g., a globin DNA sequence) locate autoradiographic spots where probe hybridized to unpackaged recombinant phage DNA.

15.4.6 Isolating the Gene

Cloned genomic DNA fragments are much longer than any gene of interest, and always longer than any cDNA from a cDNA library. They are also embedded in a genome that is thousands of times as long as the gene itself, making the selection of an appropriate vector necessary. If the genome can be screened from a reasonable number of cloned phage (~100,000 plaques for instance), the one plaque producing a positive signal on the autoradiograph would be further studied. This plaque should contain the gene of interest. At this point, we seem to have identified a clone containing a globin gene sequence. We can use this clone to infect yet more host cells and grow up much more of the globin gene-containing DNA for further study.

271 Screen a Genomic Library, Pick and Grow a Phage Clone

Given that a gene of interest might be a short sequence embedded in a large genomic insert that is as long as 20kbp, we can further isolate the gene from neighboring DNA. The traditional strategy again involves *Southern blotting*. The cloned DNA is purified and digested with restriction endonucleases, and the digest fragments are separated by *agarose gel electrophoresis*. A Southern blot is made on e.g., a nylon filter and the filter is soaked in a solution that denatures the DNA on the blot. The filter is then probed with the same tagged probe used to find the positive clone (plaque). The smallest DNA fragment containing the gene of interest can then be *subcloned* in a suitable vector and grown to provide enough target sequence DNA for further study.

15.5 The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) can ***amplify*** a region of DNA from any source, like DNA fragments obtained from a fossil or residue left at a crime scene, or even from a single cell. This amplification usually takes just a few hours, generating millions of copies of the desired target DNA sequence. The result is the purification of a specific region of DNA from surrounding sequences in a single reaction! Kary B. Mullis was awarded a Nobel Prize in 1993 for his development of PCR, which is now the basis of innumerable research studies of gene structure, function and evolution as well as applications in criminal forensics, medical diagnostics and other commercial uses. PCR is described in detail below.

272 PCR: Design and Synthesize Opposing Oligonucleotide Primers

273 PCR: The Amplification Reaction

15.5.1 PCR – the Basic Process

Typical PCR relies on knowing just two bits of DNA sequence that will be used to design and synthesize short oligonucleotide sequences (*oligomers*) in the laboratory. The oligomers must be complementary to sequences on the opposite strands of double-stranded DNA containing the gene to be studied, with their 3' ends facing (***opposing***) each other on either side of the sequence to be amplified. This way the two oligomers can serve as *primers* for the replication of both strands of a double stranded target DNA sequence. Check out link #272 below for further explanation. The first step in PCR is to add oligomer primers to the target DNA from which a gene (or other genomic sequence) is to be amplified. The mixture is then heated to denature the target DNA. The mixture is cooled to allow the primers to H-bond to complementary target DNA strands.

Next, the four deoxynucleotide precursors to DNA (dATP, dCTP, dTTP and dGTP) are added along with a small amount of a DNA polymerase. New DNA strands will now lengthen from the oligonucleotide primers on the template DNAs. To make lots of the PCR product, this reaction cycle must be repeated many times. Therefore, after allowing elongation, the mixture is heated to denature (separate) all the DNA strands. When the mixture is again cooled, the oligomers again find complementary sequences with which to H-bond. Early versions of PCR originally relied on an *E. coli* DNA polymerase, which is inactivated by heating, and so had to be re-added to the PCR mixture for each elongation cycle. When the heat-stable DNA polymerase of *Thermus aquaticus* (***Taq polymerase***) became available, it was adapted for PCR because it is active at high temperatures, eliminating the need to add fresh DNA polymerase after each PCR reaction cycle. It also allowed automation of PCR reactions with programmable *thermocylers* that raised and lowered the temperatures during PCR reactions. A PCR amplification with *Taq polymerase* is shown in Fig. 15.27 below.

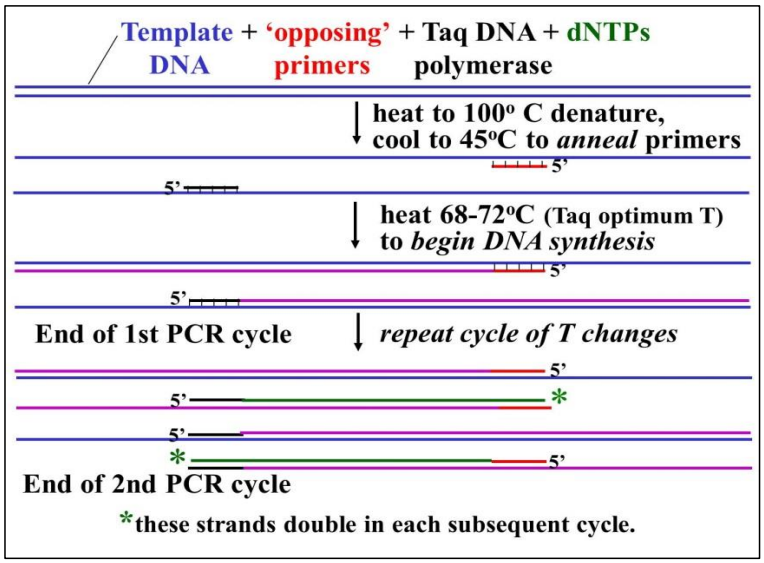


Fig. 15.27: Progress of a PCR reaction: at the end of the second PCR cycle, the strands of DNA that will be geometrically amplified in succeeding PCR cycles have been synthesized.

You can see from the illustration that the second cycle of PCR has generated the two DNA strands that will be templates for doubling and re-doubling the desired product after each subsequent cycle. A typical PCR reaction might involve 30 PCR cycles, resulting in a nearly exponential amplification of the desired sequence.

The products of PCR amplification are in such abundance that they can be seen under fluorescent illumination on an *ethidium bromide*-stained agarose gel (Fig. 15.28, below).

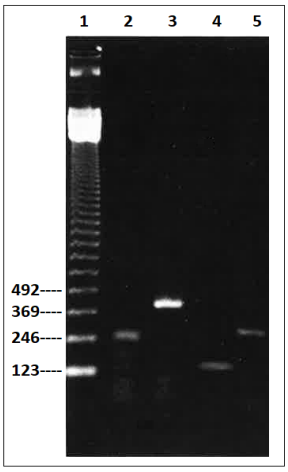


Fig. 15.28: Agarose gel electrophoresis of PCR amplified DNAs, stained with ethidium bromide to be detectable under fluorescent light: PCR products of the amplification of a globin gene clone from the insect *Chironomus thummi*. The sizes (lengths in base pairs) of the 4 PCR products in lanes 2-5 are close to those of the bands in the standard DNA size ladder in lane 1.

Because they are so plentiful, PCR- amplified DNAs can be sequenced and used in many subsequent studies. In this gel, the first lane (on the left) contains *a DNA ladder*, a mixture of DNAs of known lengths that can be used to estimate the size of the bright bands (i.e., PCR products) in the remaining lanes. In this example, the four PCR products were amplified from the same cloned genomic DNA using different combinations of oligonucleotide primers.

15.5.2 The many Uses of PCR

PCR-amplified products can be labeled with radioactive or fluorescent tags to screen cDNA or genomic libraries, to probe where a DNA sequence of interest migrates on a Southern or a blot, or to determine where an RNA sequence of interest migrates on a Northern blot (a fanciful name for RNAs that are separated by size on gels and blotted to filter). In a major PCR advance, ***Quantitative PCR*** was developed to study differential gene expression and gene regulation. The technique allows cDNA amplifications from RNAs under conditions that detect *not only* the presence, but also the relative amounts of specific transcripts being made in cells.

Variant PCR protocols and applications are manifold and often quite inventive! For a list, click Variations on Basic PCR. A recent CRISPR/Cas9 variant amplifies DNA and may lead to PCR at 37^0C, eliminating the need for a thermocycler altogether! This could prove especially useful for conducting PCR in the field. While not yet ready for prime time, check out PCR with Cas9 and without a thermocycler! for more. And in addition to technological advances, PCR has broad applications in research, forensic science, history, anthropology, etc.… and maybe even a study of your own genealogy. Let's look at three of these.

1. **Forensics**

   Another application of PCR is in forensic science, to identify a person or organism by comparing its DNA to some standard (or control) DNA. Fig. 15.29 is an example of one of these acrylamide gel DNA *fingerprints*.

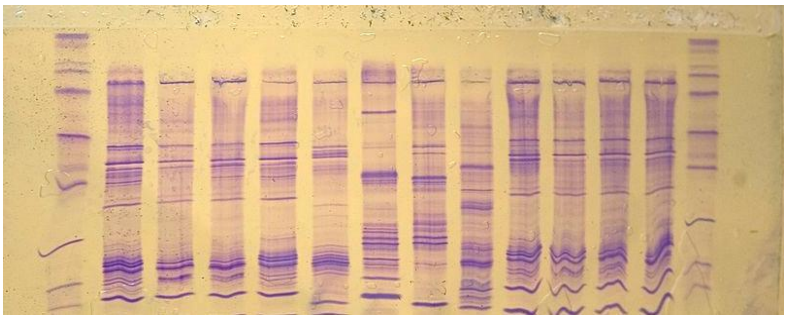


Fig. 15.29: Electrophoretic *DNA fingerprint*.

Using this technology, it is now possible to detect genetic relationships between near and distant relatives (as well as to exclude such relationships), determine paternity, demonstrate evolutionary relationships between organisms and solve recent and even 'cold-case' crimes. *Alu* sequences are ~300bp *short interspersed elements* (**SINES**) that are highly repeated throughout the human genome. DNA fingerprinting is possible in part because each of us has a unique number and distribution of *Alu* SINEs in our genome. To read more about *Alu* sequences and human diversity, click Alu Sequences and Human Diversity. Click Sir Alec Jeffries to learn about the origins of DNA fingerprinting in real life… and on all those TV CSI programs! Check out https://www.future-science.com/btn/news/apr18/07 for a brief history of the birth of DNA fingerprinting and to see how analysis of changes in gene activity that occur after death may even help ID criminals. For a video on DNA fingerprinting, click Alu and DNA fingerprinting.

2. **History**

   Other intriguing examples of the use of PCR for identification include establishing the identities of Egyptian mummies, the Russian Tsar deposed and killed during the Russian revolution (along with his family members), and the recently unearthed body of King Richard the 3rd of England.

3. **Who are your Ancestors?**

   Tracing your ethnic, racial and regional ancestry is related to DNA fingerprinting, in that it relies on PCR amplification of genes and other variable DNA regions and comparison of these your sequences to distinguishing DNA *markers* in large sequence databases. Prices for these services have come down, and as a result, their popularity has gone up in recent years. Typically, you provide spit or a salivary (buccal) swab to the service and they amplify and sequence the DNA in your samples. The analysis compares your DNA sequences to database sequences looking for patterns of ethnic and regional markers that you might share with the database(s). Based on these comparisons, you are provided with a (more…, or less) accurate map of your DNA-based ancestry. Folks who are spending around $100.00 (less when on sale!) often ask how accurate these analyses are and what do they really mean. For example, what does it mean if your DNA says you are 5% native American? In fact, different services can sometimes give you different results! You can get some answers and explanations at DNA Ancestry Testing.

274 The Power of PCR: Some Examples

15.6 Genomic Approaches: The DNA Microarray

Traditionally, when cellular levels of a protein were known to change in response to a chemical effector, molecular studies focused on control of the transcription of its gene. These studies often revealed that the control of gene expression was at the level of transcription, turning a gene on or off through interactions of transcription factors with DNA. However, protein levels are also controlled post-transcriptionally, by regulating the rate of mRNA translation or degradation. Studies of transcriptional and post-transcriptional regulation mechanisms are seminal to our understanding of how the correct protein is made in the right amounts at the right time.

We may have suspected, but now we know that control of gene expression and cellular responses can be more complex than increasing or decreasing the transcription of a single gene or translation of a single protein. Newer technologies make possible the

study of the expression of virtually all genes in a cell at the same time, a broadly defined field of investigation called ***genomics***. Such studies can reveal networks of regulated genes that must be understood to more fully explain the developmental and physiological changes in an organism. Using similar terminology, ***transcriptomics*** looks at a cell's *transcriptome*, allowing the study of 'webs' of interactive RNAs. Let's look at a simulated microarray illustrating how multiple transcripts can be detected simultaneously on one microarray (Fig. 15.30).

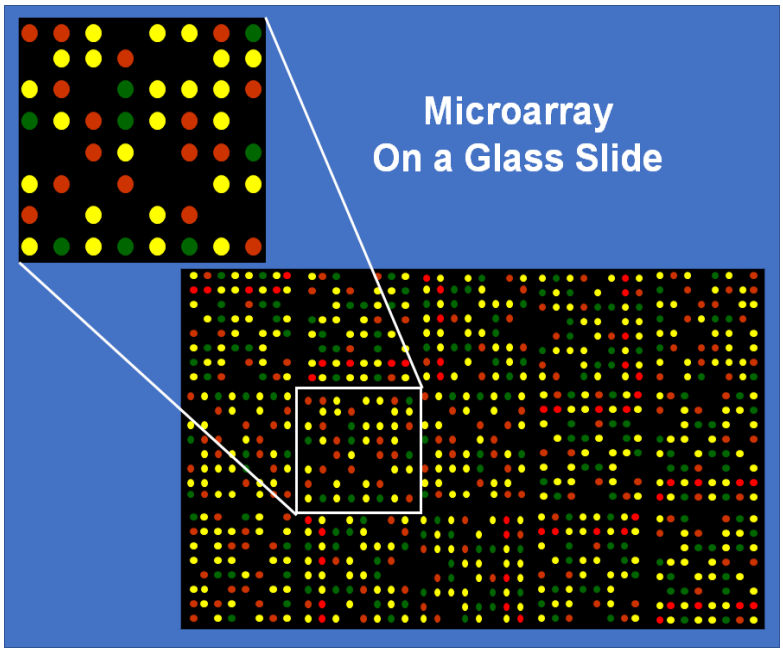


Fig. 15.30: Simulated glass slide microarray showing multiple color fluorescent spots, indicating a hunt for more than one DNA sequence at the same time.

Microarrays are typically made by 'spotting' nucleic acids (e.g., cloned DNA from a genomic or cDNA library, PCR products, oligonucleotides…) on a glass slide, or *chip*. In the language of microarray analysis, the slides are the ***probes***. Spotting a chip is a robotic process. Because the DNA spots are microscopic, a cell-specific transcriptome (e.g., a cDNA library) can fit on a single chip. A small prokaryotic or viral genome microarray might also fit on a single chip. Larger genomes might need several slides. In the simulated microarray above, the three colors would represent different fluorescent

tags on *specific* DNA sequences for three different genes. If the spots were cDNAs synthesized from cellular RNA extracts, the microarray results would indicate that at least three different genes of interest were being actively transcribed in those cells at the time of RNA extraction.

In another use of microarrays, spotted genomic clones could be used to probe a mixture of fluorescently tagged target cDNAs made from cellular mRNAs. This experiment asks not if specific genes are expressed but rather, which and how many genes are being expressed in the cell at the time of mRNA extraction. It is an approach to characterizing a cell's ***transcriptome***). This is a more global question. Identifying the proteins encoded by all those genes would be the next step. Microarrays can be quantitative so that the brightness (intensity) of the signal from each probe can be measured. Quantitative microarrays can be designed to show how global gene expression changes in cells during normal differentiation or in response to chemical signals.

DNA microarrays are also valuable for genotyping, (i.e. characterizing the genes in an organism), and they are so sensitive that they can even distinguish between two genes or regions of DNA that differ by a single nucleotide. Click Single Nucleotide Polymorphisms, or SNPs to learn more.

By analogy to genomics, and transcriptomics, ***proteomics*** is the field of study of global protein interactions. Primary tools of proteomics include mass spectroscopy and more recently, ***Protein Microarrays***. Mass spectroscopy can identify the presence of specific proteins and protein variants in samples, and has even been applied to the analysis of a **Leonardo Da Vinci** painting (*Donna Nuda*) to determine if it was actually painted by the renaissance master, an artist(s) of his school, or someone else entirely (click Proteomics Enlisted to Determine Artistic Provenance to learn the verdict!). Protein microarrays are in some ways similar to DNA microarrays, but can look globally at protein-protein interactions, as well as the different states of proteins under different cellular conditions. Read even more about these exciting developments and their impact on basic and clinical research at Protein Microarrays from the NCBI. Now think about this! Can we create a proteomic library analogous to a genomic library? This would seem a daunting prospect, but efforts are underway. Check out A stab at mapping the Human Proteome for original research leading to the sampling of a tissue-specific human proteome, and click Strategies for Approaching the Proteome for more general information.

As you can see, microarrays and related technologies are powerful tools that can shift our focus from how single molecules influence events, to thinking about webs of biochemical interactions that could more completely explain molecular and physiological causes and effects. Table 15.2(below, adapted from Wikipedia) summarizes different applications of microarrays.

The Power of Microarrays

Application or Technology	Synopsis
Gene expression profiling	In a transcription (mRNA or gene expression) profiling experiment the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression.
Comparative genomic hybridization	Assessing genome content in different cells or closely related organisms, where one organism's genome is the probe for a target genome from a different species.
GeneID	Small microarrays to check IDs of organisms in food and feed for genetically modified organisms (GMOs), mycoplasmas in cell culture, or pathogens for disease detection. These detection protocols often combine PCR and microarray technology.
CHIP; chromatin immunoprecipitation	DNA sequences bound to a particular protein can be isolated by immunoprecipitating the protein. The fragments can be hybridized to a microarray (such as a tiling array) allowing the determination of protein binding site occupancy throughout the genome.
DamID	Analogously to ChIP, genomic regions bound by a protein of interest can be isolated and used to probe a microarray to determine binding site occupancy. Unlike ChIP, DamID does not require antibodies but makes use of adenine methylation near the protein's binding sites to selectively amplify those regions, introduced by expressing minute amounts of protein of interest fused to bacterial DNA adenine methyltransferase.
SNP detection	Identifying single nucleotide polymorphism among alleles within or between populations. Some microarray applications make use of SNP detection, including Genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating ***germline*** mutations in individuals or ***somatic*** mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.
Alternative splicing detection	An exon junction array design uses probes specific to the expected or potential splice sites of predicted exons for a gene. It is of intermediate density, or coverage, to a typical gene expression array (with 1-3 probes per gene) and a genomic tiling array (with hundreds or thousands of probes per gene). It is used to assay the expression of alternative splice forms of a gene. Exon arrays have a different design, employing probes designed to detect each individual exon for known or predicted genes, and can be used for detecting different splicing isoforms.
Tiling array	Genome tiling arrays consist of overlapping probes designed to densely represent a genomic region of interest, sometimes as large as an entire human chromosome. The purpose is to empirically detect expression of transcripts or alternatively spliced forms which may not have been previously known or predicted.

275-2 The Power of Microarrays

15.7 Ome-Sweet-Ome

Early molecular technologies, including the ones described in this chapter, were applied to understanding the structure, function and regulation of specific genes. Some of the more recent technologies (e.g., microarrays) are well adapted to holistic approaches to understanding cell function. Terms we have already seen (genome, epigenome, transcriptome) were coined in an effort to define the different objects of study whose underlying network of molecular interactions can more accurately explain cell function. Distinguishing these *objects of study* can be difficult, and their overlap in cells can be confusing. Here is a short compendium of …*omes* with an attempt at clarification:

- ***Genome*** - total DNA content of a cell, identical in every cell of an organism
- ***Proteome*** –a cell's protein profile and steady state at any given moment
- ***Exome*** - A cell's total coding DNA (excluding non-coding DNA)
- ***Metabolome*** - A cell's metabolic landscape (i.e., metabolic status)
- ***Epigenome*** - A cell's total DNA-modification/chromatin topography
- ***Methylome*** – The pattern of methylation of DNA in the genome
- ***Paleoproteome***: Profile of proteins found in ancient remains by mass spectroscopy
- ***transcriptome***: A cell's RNA transcript profile and steady state at any given moment
- ***epitranscriptome***: a map of chemical modifications of RNAs that inform their function
- ***metabolome***: all the small molecules in a defined sample (cell, organelle, tissue, etc.)

One might be excused for assuming that such cellular profiles would be the same for all cells in a tissue, only changing as gene expression is regulated during development or when signaled by extracellular events or chemical signals. But, even ***genomic*** and ***exomic*** profiles can change! Recall somatic mutations in dividing cells, and genome loss in some cells (e.g., erythrocytes that emerge from our reticulocytes). Studies of large numbers of individual cells suggest profile variation even in cells of the same tissue or cell culture. How and why this is so is a rapidly growing new area of study, made possible by new tools for studying DNA, RNA and proteins molecules in single cells (see Mapping Protein Networks, The Dark Proteome, What on earth is Paleoproteomics?, A Human Transcriptome Study, Nature vs. Nurture: Influences on Epigenome and Methylome, Epitranscriptomics-Functional Chemical Modifications of RNAs. Can you name the next …***ome***? Maybe the ***chondrome*** (see Mining Mitochondrial Genes).

15.8 From Genetic Engineering to Genetic Modification

By enabling us to focus on how genes and their regulation have evolved, genomic, transcriptomic and proteomic technologies have vastly increased our knowledge of how cells work at a molecular level. We continue to add to our knowledge of disease process and in at least a few cases, how we can treat disease. The use of technologies to

genetically modify organisms is more controversial, despite the best of human intentions. Some genetically modified organisms (GMOs) aim to increase food productivity to better feed the world. The introduction 'beneficial' genes into some GMOs have made

- drought-resistant crops to increase the range where major food crops can be grown.
- pest-resistant crops to reduce reliance on environmentally toxic chemical pesticides.
- herbicide-resistant crops that survive chemicals used to destroy harmful plants.

The quest for "improved" plant and animal varieties has been going on since before recorded history. Farmers have been cross-breeding cows, sheep, dogs, and crop varieties from corn to wheat, hoping to find faster growing, larger, hardier, (you name it) varieties. It is the manipulation of DNA (the essence of the genetic material itself) that is at the root of controversy. Controversy is reflected in opinions that GMO foods are potentially dangerous, and that their cultivation should be banned. But the general consensus is that attempting to ban GMOs is too late! In fact, you are probably already partaking of some GMO foods without even knowing it. Perhaps the good news is that after many years of GMO crops already in our food stream, the emerging scientific consensus is that GMO foods are no more harmful than unmodified foods. The current debate is whether or not to label foods that are (or contain) GMO ingredients as genetically modified. In an odd but perhaps amusing take on the discomfort some folks feel about GMOs, a startup company has genetically modified Petunias. When grown in water, their flowers are white, but when 'watered' with beer, they will produce pink flowers or purple flowers depending on how much beer they get (Check it out at Can Beautiful Flowers Change Face?). According to the company, they seek "to bring what it sees as the beauty of bioengineering to the general public" (and perhaps some profit as well?).

Finally, new CRISPR and related tools can precisely edit any DNA sequences. While not yet ready for prime time, one CRISPR-based protocol even promises to amplify genes from DNA without prior denaturation at 37^{o}C, i.e., without the need for a thermocycler (check out PCR with Cas9 and without a thermocycler for more). Unlike the "*quack medicines*" of old, these tools have the real potential to cure disease, destroy disease-carrying vectors, cure cancer, improve crops and… possibly alter the course of evolution. We may also need to mobilize such technologies to cope with the coming effects of climate change! The speed with which one can accomplish such good (or evil) is truly awesome.

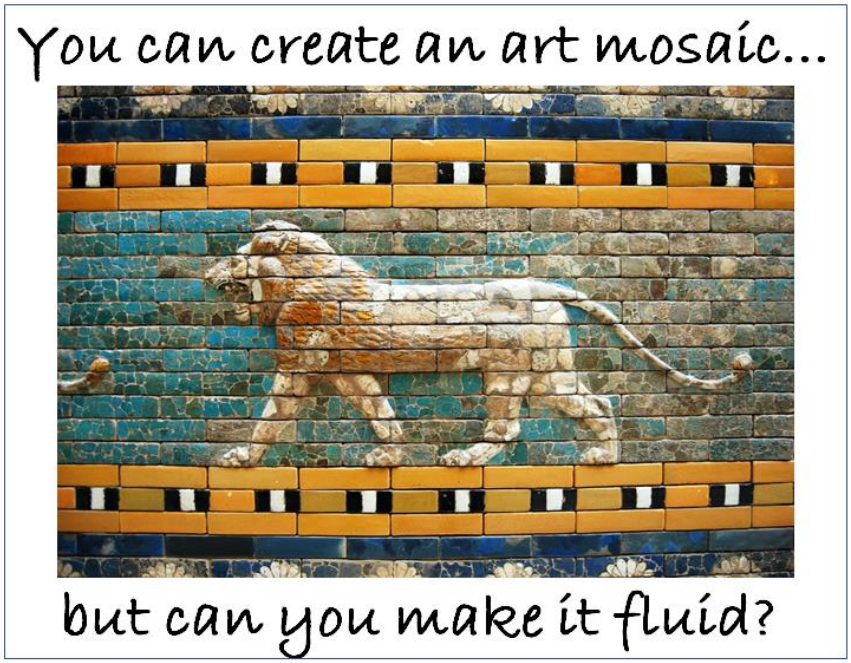
Some iText & VOP Key Words and Terms

2', 3' di-deoxy CTP chemotherapy	**genome**	**regulatory networks**
alternative splicing	**genome projects**	**restriction endonucleases**
automated DNA sequencing	**genomic library**	**reticulocyte**

autoradiography	insert DNA	reverse transcriptase
BACs and YACs	library screening	RNA probes
bacterial artificial chromosome vectors	linkers	RNAse
blunt ends	Northern blot	shotgun sequencing
cDNA	oligo d(T) column	single nucleotide polymorphisms
cDNA hairpin loop	PCR	SNPs
cDNA library	PCR	Southern blot
cDNA probes	PCR primers	sticky ends
chemiluminescence	PCR steps	systematics
cosmid vectors	phage lambda vectors	Taq polymerase
di-deoxy termination	plasmids	thermophilic bacteria
di-deoxy sequencing method	poly(A) tail	thermophilic DNA polymerases
DNA ligase	polymerase chain reaction	*Thermus aquaticus*
DNA sequencing	primer	transcriptome
elution	primer walking	transformation
ethidium bromide	probe hybridization	vectors
fluorescence	proteome	Western blot
forensics	recombinant vector	yeast artificial chromosomes
Genetic (DNA) fingerprint	recombination	

Chapter 16: Membrane Structure

Membrane Structure and Function: the fluid mosaic, membrane proteins, glycoproteins, glycolipids



16.1 Introduction

All cellular membranes share the same ***phospholipid bilayer*** construction, all restrict the flow of substances from one side to the other (i.e., they are ***semipermeable***), and all are a ***fluid mosaic*** of proteins attached to or embedded in the phospholipid bilayer. Specific proteins and phospholipids structurally and functionally differentiate between one kind of cellular membrane and another. ***Integral proteins*** are held in the membrane by a ***hydrophobic*** domain that anchors them to the hydrophobic interior of the membrane. Some integral membrane proteins span the phospholipid bilayer, with ***hydrophilic*** domains on either side of the membrane. In the case of the plasma membrane, the hydrophilic domains of such ***transmembrane proteins*** interact with the watery

extracellular fluid on one side and the aqueous cytoplasm on the other. Once embedded in the fatty acid interior of a membrane, integral membrane proteins cannot escape! In contrast, ***peripheral membrane proteins*** are more loosely held in place by hydrophilic interactions with charged features of the membrane surface (phospholipid heads, hydrophilic surface domains of integral proteins). Integral membrane proteins are often glycoproteins whose sugars face the outside of the cell. Cells thus present a sugar coating, or ***glycocalyx***, to the outside world. As cells form tissues and organs, they become bound to extracellular proteins that they themselves or other cells secrete to form an ***extracellular matrix***. We will spend much of this chapter looking at characteristic structures and biological activities of plasma membrane proteins and their functions.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. distinguish between components of the membrane that can move (diffuse) laterally in the membrane from those that can *flip* (switch) from the outer to the inner surface of the phospholipid bilayer.
2. compare the fluid mosaic membrane to earlier membrane models and cite the *evidence* for and against each.
3. describe how cells might make their plasma membranes and suggest an experiment that would demonstrate your *hypothesis*.
4. distinguish between *transmembrane* and *peripheral* membrane proteins and provide specific examples of each.
5. Determine whether a newly discovered protein might be a membrane protein.
6. predict the effect of *molecular* and *physical influences* on membrane fluidity.
7. suggest how organisms living in *warm tropical waters* have adapted to the higher temperatures. Likewise, fish living under the *arctic ice*.
8. explain how salmon are able to spend part of their lives in the ocean and another part swimming upstream in freshwater, without their cells shriveling or exploding.
9. list the diverse *functions* of membrane proteins.
10. speculate on why only eukaryotic cells have evolved *sugar coated* cell surfaces.
11. compare and contrast the *glycocalyx* and *extracellular matrix* of cells.

16.2 Plasma Membrane Structure

In eukaryotic cells, the ***plasma membrane*** surrounds a cytoplasm filled with ribosomes and organelles. Organelles are structures that are themselves encased in membranes. Some organelles (nuclei, mitochondria, chloroplasts) are even surrounded by double membranes. All cellular membranes are composed of two layers of phospholipids embedded with proteins. All are selectively permeable (***semi-permeable***), allowing only certain substances to cross the membrane. The unique functions of cellular membranes

are due to their different phospholipid and protein compositions. Decades of research have revealed these functions (see earlier discussions of mitochondrial and chloroplast function for instance). Here we'll describe general features of membranes, using the plasma membrane as our example.

16.2.1 The Phospholipid Bilayer

Gorter and Grendel predicted the bilayer membrane structure as early as 1925. They knew that red blood cells (erythrocytes) have no nucleus or other organelles, and thus have only a plasma membrane. They also knew that the major chemical components of these membranes were ***phospholipids***. The space-filling molecular model in Fig. 16.1 shows the basic structure of phospholipids, highlighting their ***hydrophilic*** (polar) heads and ***hydrophobic*** tails.

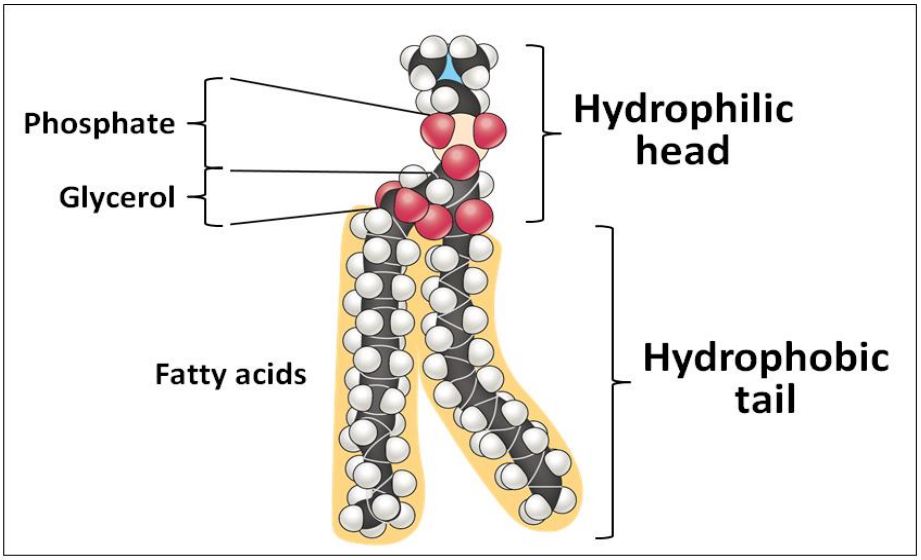


Fig. 16.1: Space-filling model showing components of a phospholipid.

We say that molecules with hydrophilic and hydrophobic domains are ***amphipathic*** molecules. In 1925, E. Gorter and F. Grendel reported blood counts and the surface areas of red blood cells from man, rabbit, dog and sheep, guinea pig and goat. They then disrupted a known number of red blood cells and measured the amount of

phospholipids in the membrane extracts. They next calculated that there were enough lipid molecules per cell to wrap around each cell twice. From these observations, they predicted the ***phospholipid bilayer*** with fatty acids interacting within the bilayer. Fig. 16.2. shows some common membrane phospholipids.

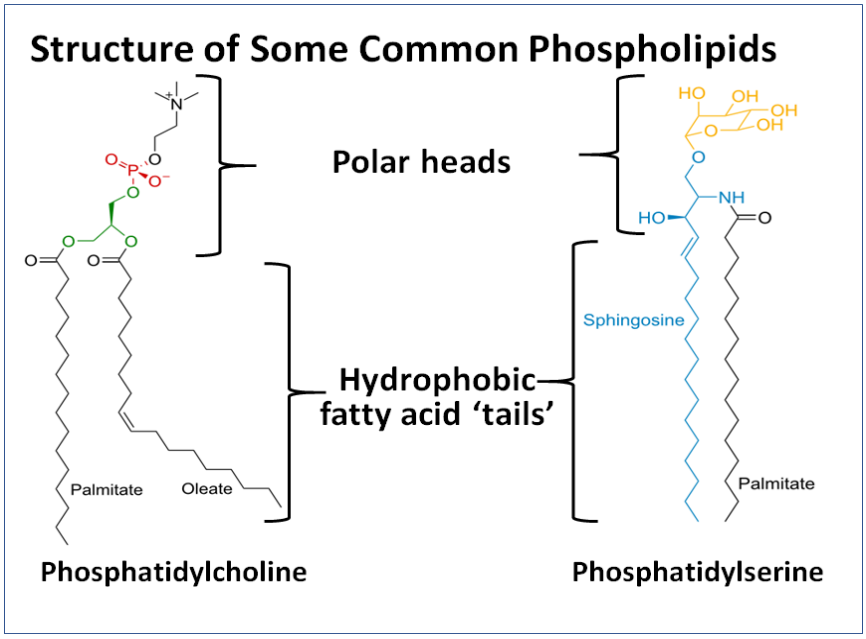


Fig. 16.2: Examples of common membrane phospholipids.

Curiously, Gorter and Grendel had made two calculation errors in determining the amount of phospholipid per cells. Nevertheless, their errors compensated each other so that, while not strictly speaking correct, their conclusion was prophetic! Later, when amphipathic molecules mixed with water, they spontaneously aggregated, hiding their hydrophobic regions from the water, forming actual structures called ***liposomes*** that sediment when centrifuged!

276-2 Membrane Lipids & Phospholipid Bilayer

277-2 Experiments with & Uses of Liposomes

Liposome membrane structure is consistent with the predicted phospholipid bilayer in which the hydrophobic tails interact with each other and the polar heads face away from each other, forming a *phospholipid bilayer*. This led to a picture of membrane architecture based on phospholipid interactions in which the fatty acid tails make up the hydrophobic interior of the membrane with hydrophilic external surfaces facing opposite, aqueous sides of the membrane. The iconic phospholipid bilayer is illustrated in Fig. 16.3 (below)

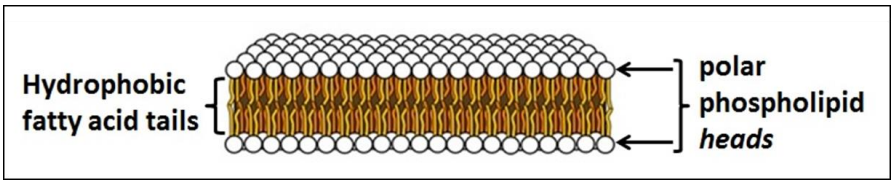


Fig. 16.3: Phospholipid bilayer membrane.

16.2.2 Models of Membrane Structure

In 1935, H. Davson and J. Danielli suggested that proteins might be bound to the polar heads of the phospholipids in the plasma membrane, creating a protein-lipid-protein sandwich. Decades later, J.D. Robertson observed membranes in the transmission electron microscope at high power, revealing that all cellular membranes had a ***trilamellar*** structure. The classic trilamellar appearance of a cellular membrane in the electron microscope is shown in Fig.16.4.

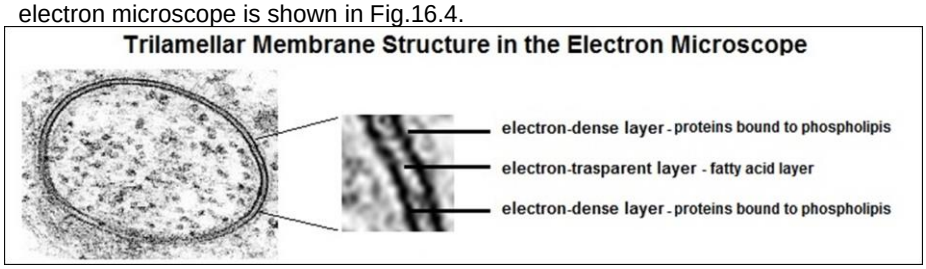


Fig. 16.4: Low and high magnification Transmission electron micrographs of *trilamellar* membrane structure.

A trilamellar structure is consistent with the protein-lipid-protein ***Davson/Danielli model*** of the phospholipid bilayer. Observing that *all* cellular membranes had this trilamellar structure, Robertson further proposed his ***Unit Membrane*** model: *all membranes* consist of a clear phospholipid bilayer coated with electron-dense

proteins. The static view of the implied by the Davson-Danielli or Robertson models of membrane structure was replaced in 1972 by Singer and Nicolson's **Fluid Mosaic** model (see *The fluid mosaic model of membranes*. Science 175:720-731). They suggested that, in addition to ***peripheral proteins*** that bind to the membrane surfaces, many ***integral membrane proteins*** actually span their membranes. *Integral membrane proteins* were imagined as a *mosaic* of protein 'tiles' embedded in a phospholipid medium. But unlike a mosaic of glazed tiles set in a firm, cement-like structure, the protein 'tiles' were predicted to *float in a phospholipid sea*.

In the model, membrane proteins are anchored in membranes by one or more *hydrophobic* domains; with *hydrophilic* domains facing either or both aqueous external and cytosolic environments. Thus, like phospholipids themselves, membrane proteins are *amphipathic*. We know that cells expose different surface structural and functional features to their aqueous environments. Since the exposed domains of membrane proteins would be different on opposite sides of a membrane, we say that cellular membranes are ***asymmetric***. Fig 16.5 (below) is a model of the plasma membrane of a cell including its integral, peripheral proteins and glycoproteins.

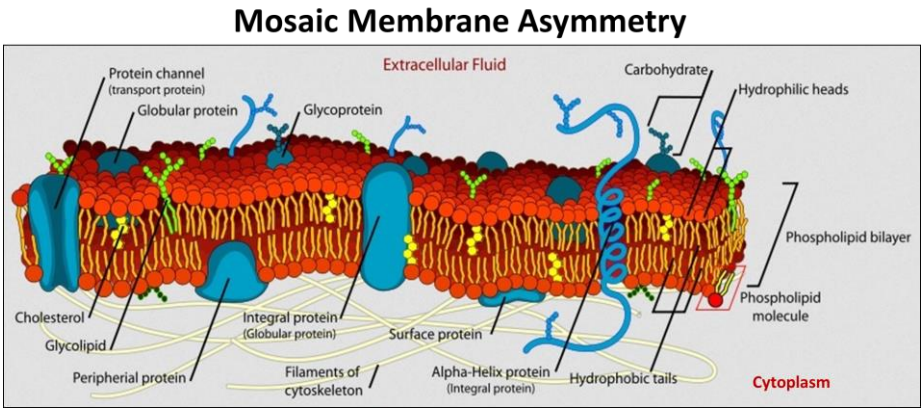


Fig. 16.5: Model of the eukaryotic plasma membrane demonstrating asymmetry, with a sugarless cytoplasmic surface and a glycoprotein rich extracellular surface.

In this model, some integral proteins have a hydrophobic domain that does not span the membrane, but that anchors it to one side of the membrane. Peripheral (or so-called "*surface*") proteins are also shown. These are less firmly bound to the membrane by charge interactions with the polar phosphate groups of phospholipids, or with the polar domains of integral membrane proteins.

Because of their own aqueous hydrophilic domains, membrane proteins are a natural barrier to the free passage of charged molecules across the membrane. On the other hand, membrane proteins are responsible for the ***selective permeability*** of membranes, facilitating the movement of *specific* molecules in and out of cells. Membrane proteins also account for specific and selective interactions with their extracellular environment. These interactions include the adhesion of cells to each other, their attachment to surfaces, communication between cells (both direct and via hormones and neurons), etc. The 'sugar coating' of the extracellular surfaces of plasma membranes comes from ***oligosaccharides*** covalently linked to membrane proteins (as ***glycoproteins***) or to phospholipids (as ***glycolipids***). Carbohydrate components of ***glycosylated*** membrane proteins inform their function. Thus, glycoproteins enable specific interactions of cells with each other to form tissues. They also allow interaction with extracellular surfaces to which they must adhere. In addition, they figure prominently as part of receptors for many hormones and other chemical communication biomolecules. The domains of plasma membrane proteins are not glycosylated. They often attach the membrane to components of the cytoskeleton, giving cells their shape and allowing them to change shape when necessary. Many also have essential enzymatic features, as we will see. Given the crucial role of proteins and glycoproteins in membrane function, it should come as no surprise that proteins constitute an average of 40-50% of the mass of a membrane. In some cases, proteins are as much as 70% of membrane mass (think cristal membranes in mitochondria!).

278-2 Properties of Proteins Embedded in a Phospholipid Bilayer

279 Different Membrane Compositions

16.2.3 Evidence for Membrane Structure

Membrane asymmetry, the different membrane features facing opposite sides of the membrane, was directly demonstrated by the scanning electron microscope technique of ***freeze-fracture***. The technique involves freezing of isolated membranes in water and then chipping the ice. When the ice cracks, the encased membranes split along a *line of least resistance* that turns out to be between the hydrophobic fatty acid opposing tails in the interior of the membrane. Scanning electron microscopy of a freeze-fractured membrane reveals features of the interior and exterior membrane surfaces that confirm membrane asymmetry. Among prominent features in a scanning micrograph of freeze-fractured membranes are the *pits* and opposing *mounds* in the interior of the membrane. Fig. 16.6 (below) illustrates the results of freeze-fracture electron microscopy of a plasma membrane.

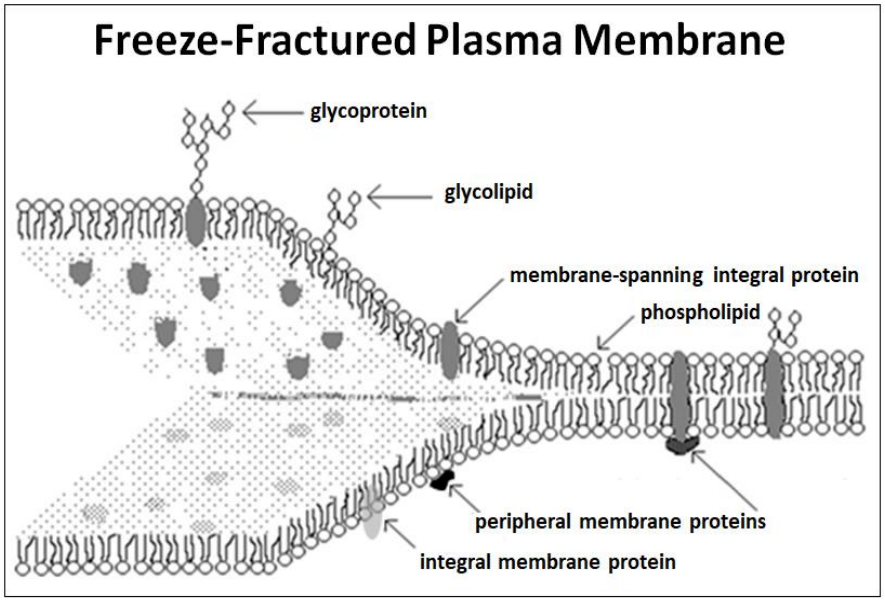


Fig. 16.6: Illustration of a scanning electron micrograph of freeze-fractured plasma membrane with pits and mounds showing on opposing phospholipid layers of the membrane.

Other features shown here are also consistent with phospholipid membrane structure.

280-2 Freeze Fracture Electron Microscopy of Cell Membranes

Cytochemical studies also confirmed plasma membrane asymmetry showing that only the external surfaces of plasma membranes are sugar-coated. Check the link below for more detailed descriptions of the experiments.

281-2 EM Cytochemical Demonstration of Membrane Asymmetry

Finally, membrane asymmetry was also demonstrated biochemically. In one experiment, whole cells treated with proteolytic enzymes, followed by extraction of the membranes and then isolation of membrane proteins. In a second experiment, plasma membranes were isolated from untreated cells first, and *then* treated with the enzymes. In a third experiment, proteins were extracted from plasma membranes

isolated from untreated cells. Electrophoretic separation of the three protein extracts by size demonstrated that different components of integral membrane proteins were present in the two digest experiments, confirming the asymmetry of the plasma membrane. Again, for more details, check the link below.

282-2 Electrophoretic Demonstration of Membrane Asymmetry

The idea that membranes are *fluid* was also tested experimentally. Here is the experiment:

- Antibodies were made to mouse and human cell membrane proteins by isolating their membranes and injection the isolates into a different animal (rabbits most likely). Each rabbit saw the membranes and their associated proteins as foreign and responded by making specific anti-membrane antibody molecules.
- The rabbit antibodies against each membrane source were then isolated and separately tagged with different colored fluorescent labels so that they would glow a different color when subjected to ultraviolet light.
- Next, mouse and human cells were mixed under conditions that caused them to fuse, making human-mouse hybrid cells.
- The tagged antibodies were added to the fused human-mouse cells and examined in a fluorescence microscope.
- At first, the mouse and human antibodies were seen to bind to the mouse and human portions of the fused cell, as shown in Fig. 16.7, below.

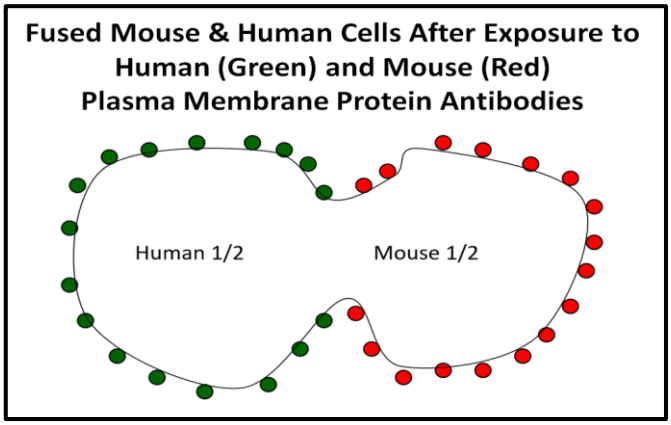


Fig. 16.7: Fluorescent antibodies against human and mouse membrane proteins localize their cell surface antigens (i.e., proteins) on opposite poles of recently fused cells.

- After a short time, the different fluorescent antibodies were seen to mix under a fluorescence microscope under UV light. The fluorescent tags seemed to be moving from their original location in the fused membranes as shown in Fig. 16.8 (below).

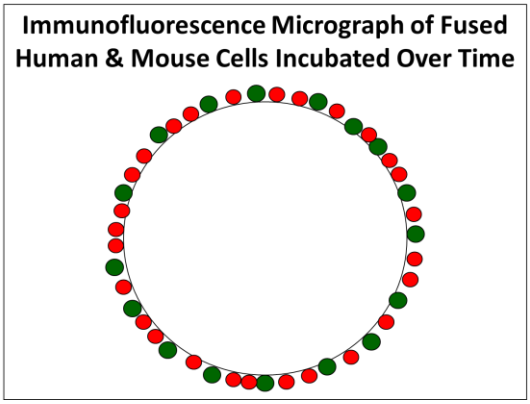


Fig. 16.8: Immunofluorescent human (green) and mouse (red) membrane proteins diffuse and mix over time in fused cells.

Clearly, proteins embedded in the membrane are not static, but are able to move laterally in the membrane, in effect floating and diffusing in a "sea of phospholipids".

283 Two Demonstrations of Membrane Fluidity: The Fluid Mosaic

16.2.4 Chemical Factors Affecting Membrane Fluidity

As you might expect, the fluidity of a membrane depends on its chemical composition and physical conditions surrounding the cell, for example the outside temperature. Just as heating a solution causes dissolved molecules and particulates to move faster, membrane phospholipids *and* proteins are also more fluid at higher temperatures. Fatty acids of phospholipids with more *unsaturated* (C=C) carbon bonds have more kinks, or bends. These tend to push apart the phospholipid tails. With more space between the fatty acid tails, membrane components can move more freely. Thus, higher levels of *polyunsaturated* fatty acids in a membrane make it more fluid. On the other hand, cholesterol molecules tend to fill the space between fatty acids in the hydrophobic interior of the membrane. This reduces the lateral mobility of the phospholipid and protein components in the membrane. By reducing fluidity, cholesterol reduces membrane permeability to some ions.

Factors that affect membrane fluidity are summarized in Fig. 16.9 (below).

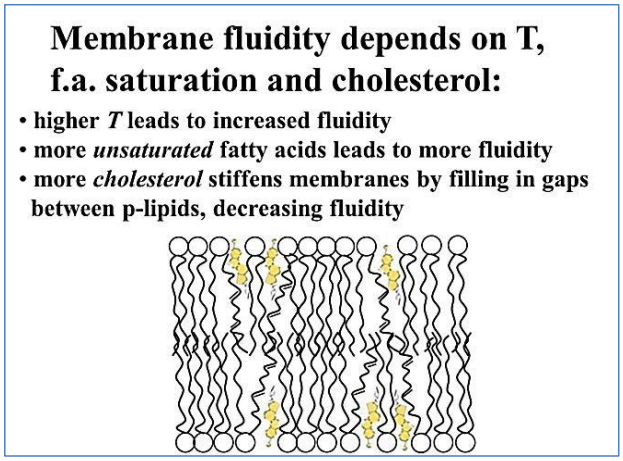


Fig. 16.9: Factors affecting membrane fluidity (i.e. rate of diffusion of membrane components).

Evolution has adapted cell membranes to different and changing environments to maintain the fluidity necessary for proper cell function. ***Poikilothermic***, or cold-blooded organisms from prokaryotes to fish and reptiles, do not regulate their body temperatures. When exposed to lower temperatures, poikilotherms respond by increasing the *unsaturated* fatty acid content of their cell membranes. At higher temperatures, they increase membrane *saturated* fatty acid content. Thus, the cell membranes of fish living under the arctic ice maintain fluidity by having high levels of both monounsaturated and polyunsaturated fatty acids. What about fish species that range across warmer and colder environments (or that live in climates with changing seasons). These fish regulate their cell membrane composition adjust to the demands of their changing environment. The warm-blooded (***homeothermic***) mammals and birds maintain a more or less constant body temperature. Thus their membrane composition is also relatively constant. But there is a paradox! Their cell membranes are very fluid, with a higher ratio of *polyunsaturated* fat to *monounsaturated* fats than say, reptiles. But the paradox is resolved when we understand that this greater fluidity supports the *higher metabolic rate* of the warm-blooded species compared to poikilotherms. Just compare the life styles of almost any mammal to a lazy floating alligator, or a snake basking in the shade of a rock!

284-2 Factors Influencing Membrane Fluidity

16.2.5 Making and Experimenting with Artificial Membranes

Membrane-like structures can form spontaneously. When phospholipids interact in an aqueous environment, they aggregate to exclude their hydrophobic fatty tails from water, forming ***micelles***. Micelles are spherical phospholipid monolayer vesicles that *self-assemble*, a natural aggregation of the hydrophobic fatty acid domains of these amphipathic molecules. Fig. 16.10 illustrates a micelle.

Fig. 16.10: Micelles are phosholipid monolayers that can self-assemble in water.

Micelles can further aggregate into spherical phospholipid bilayer ***liposomes*** (Fig. 16.11).

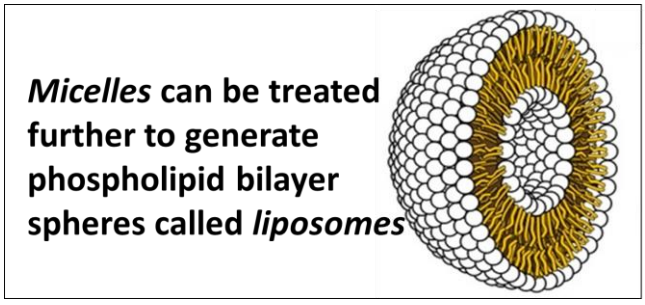


Fig. 16.10: A liposome is a synthetic lipid bilayer that can be formed from micelles.

Liposomes behave somewhat like cells, for example forming a pellet at the bottom of a tube when centrifuged. Liposomes can be custom designed from different kinds of phospholipids and amphipathic proteins that become integral to their membranes. When liposomes are prepared in the presence of specific proteins or other molecules that can't cross the membrane, they become trapped in the vesicles. The trapped molecules cannot get out of this synthetic 'organelle'. Such were the studies that allowed the identification of the mitochondrial respiratory chain complexes. The ability to manipulate liposome content and membrane composition also make them candidates for drug delivery to specific cells and tissues (*google* liposome for more information).

16.2.6 Separate Regions of a Plasma Membrane with Unique Fluidity and Permeability Properties

As we will see shortly, fluidity *does not* result in an equal diffusion of all membrane components around the cell membrane surface. Instead, extracellular connections between cells along with intracellular connections of the membrane to differentiated regions of the cytoskeleton, effectively compartmentalize the membrane into sub-regions. To understand this, imagine a sheet of epithelial like those in the cartoon in Fig. 16.11.

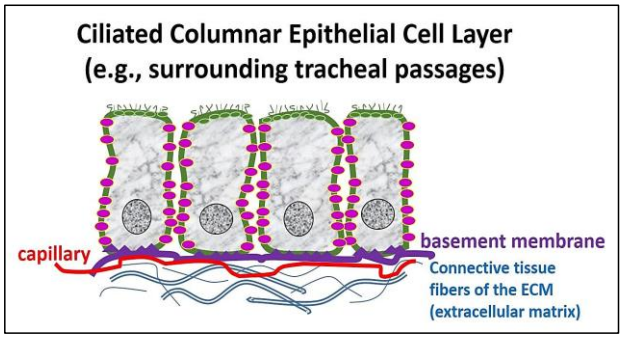


Fig. 16.12: Properties of plasma membranes may be different in ways determined by cell-cell associations during tissue development. Lavender, green and blue regions indicate basic differentiation of a sheet of epithelial cells.

This sheet of cells exposes one surface with unique functions to the inside of the organ they line. It exposes the opposite surface, one with a quite different function, to the other side of the sheet. The lateral surfaces of the cells are yet another membrane compartment, one that functions to connect and communicate between the cells in the sheet. Components, i.e., membrane proteins illustrated with different symbolic shapes and colors, may remain fluid within a compartment. Of course, a tissue-level *macro-differentiation* of cell membranes enabling cell-cell and cell-environmental interactions makes intuitive sense. The new possibility that cellular membranes are even more compartmentalized was perhaps less anticipated. In fact, membranes are further divided into micro-compartments. Within these compartments, components are fluid but seldom move between compartments. Studies indicate that cytoskeletal elements create and maintain these micro-discontinuities. For example, integral membrane proteins are immobilized in membranes if they are attached to cytoskeletal fibers (e.g., actin) in the cytoplasm. Furthermore, when aggregates of these proteins line up due to similar interactions, they form kind of *fence*, inhibiting other membrane components from crossing. By analogy, this mechanism of micro-

compartmentalization is called the *Fences and Pickets* model in which proteins attached to the cytoskeleton serve as the pickets (the usually white pointy boards attached to the fence!). The movement across the fences (i.e., from one membrane compartment to another) is infrequent. Extra kinetic energy is presumably needed for a molecule to 'jump' a fence between compartments. Hence, this kind of motion (*hop diffusion*) differs from the Brownian motion implied by the original fluid mosaic model.

285-2 Regional Differentiation of a Plasma Membrane Domains

16.3 Membrane Proteins

Of course, membrane proteins themselves have domains. These provide catalytic and other activities inside and outside of cells and organelles, and keep them attached to the membrane. Like phospholipids, membrane proteins are amphipathic, with hydrophobic domains that *non-covalently* interact strongly within the fatty acid interior of membranes. Some integral membrane proteins span the entire membrane, with hydrophilic domains facing the cytosol or cell exterior. Peripheral proteins bind to a membrane surface through non-covalent interactions. Different membrane proteins are shown in Fig. 16.13.

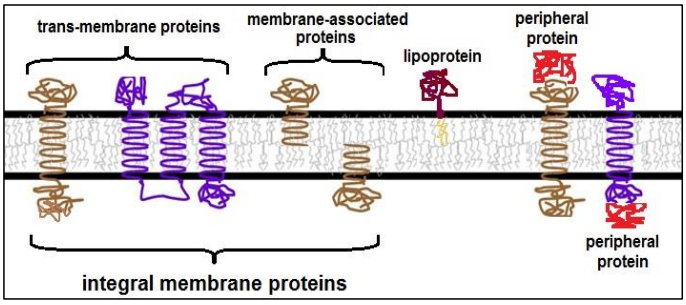


Fig. 16.13: Integral membrane proteins may penetrate or span the membrane; Also shown are peripheral proteins and lipoproteins.

286-2 Domains of membrane proteins

Hydrophobic amino acids of membrane proteins are organized into functional domains. These consist of one or more alpha-helical regions that interact with the fatty acid interior of the membranes. Hydrophilic domains tend to have more tertiary structure. These domains face the aqueous cytosol and cell exterior. Two trans-membrane proteins are cartooned below (Fig 16.14).

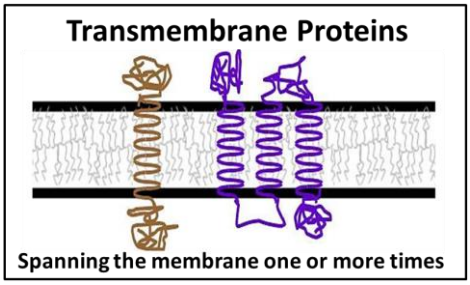


Fig. 16.14: Integral transmembrane proteins cross the membrane one or more times.

The protein on the left in Fig. 16.14 crosses the membrane once, while the one on the right crosses the membrane three times. Regardless of the number of times a polypeptide crosses the membrane, its C-terminus always ends up on the extracellular surface of the cell.

Alpha helical domains that anchor proteins in membranes are mostly non-polar and hydrophobic themselves. For example, consider the amino acids in the alpha-helical domain of the red blood cell membrane protein *glycophorin A*, which prevent red blood cells from aggregating, or clumping in the circulation. Glycophorin A monomers pair to form dimers in the plasma membrane. A single glycophorin A polypeptide with its hydrophobic trans-membrane alpha helix is cartooned below in Fig. 16.15.

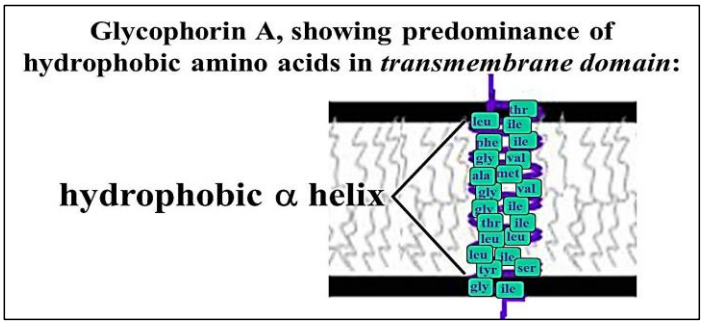


Fig. 16.15: Hydrophobic amino acids of glycophorin form a helix that spans the red blood cell plasma membrane

Proteins that span membranes multiple times may include amino acids with charged polar side chains, provided that these side chains interact between helices so that they are shielded from the fatty acid environment in the membrane. Because of these

hydrophilic interactions, such proteins can create ***pores*** for the ***transport*** of polar molecules and ions. We will see some of these proteins later. Integral membrane proteins that do not span the membrane also have a hydrophobic helical domain that anchors them in the membrane, while their hydrophilic domains typically interact with intracellular or extracellular molecules that can hold cells in place, give cells and tissues their structure, etc.

The very presence of hydrophobic alpha-helical domains in trans-membrane proteins makes them difficult if not impossible to isolate from membranes in a biologically active form. But the peripheral polypeptide *cytochrome c* readily dissociates from cristal membranes, making it easy to purify. The inability to purify other biologically active cristal membrane electron carriers is what slowed our understanding of the structure/function of the mitochondrial electron transport system.

It is possible to determine the primary structure of a polypeptide encoded by a gene before the protein itself has been isolated. Just by knowing the DNA sequence of a gene, we can infer the amino acid sequence of the protein encoded by the gene. Then we can identify all of the hydrophobic amino acids in the inferred sequence and generate a ***hydrophobicity*** (or ***hydropathy***) plot such as the one in Fig. 16.16 below.

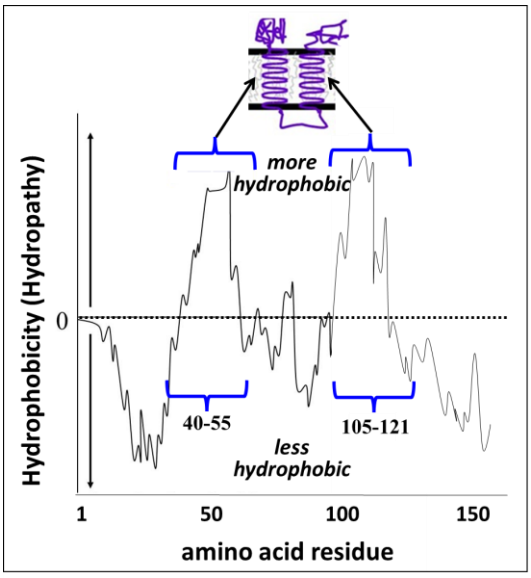


Fig. 16.16: Hydropathy plots correlate amino acid hydrophobicity with its position in a polypeptide. Long regions of hydrophobic amino acids suggest a possible membrane protein domain.

The protein in this hypothetical example has two extended regions, or domains of hydrophobic amino acids. To see how an hydropathy plot can predict whether a protein is a membrane protein, check out the link below.

287-2 Hydropathy Predicts Hydrophobic Membrane Protein Domains

16.4 A Diversity of Membrane Protein Functions

Examples of membrane protein functions include:

- receptors for hormones or neurotransmitters
- antibodies (immunoglobulins) that recognize foreign substances (antigens)
- cell-recognition molecules that bind cells together
- cell membrane structures that directly pass chemical information between cells
- anchoring cells to extracellular surfaces like connective tissue
- anchoring the plasma membrane to proteins of the cytoskeleton
- molecular transport (entry into or exit of substances from cells)
- enzymes that catalyze crucial reactions in cells.

Fig. 16.17 summarizes some membrane protein functions.

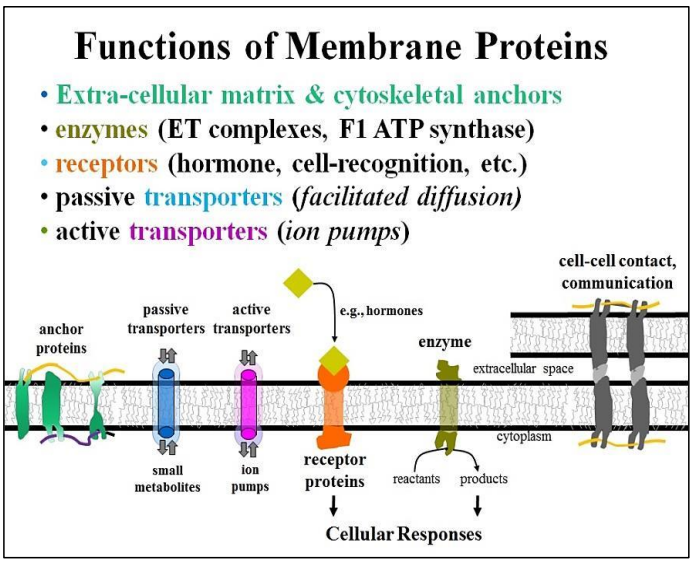


Fig. 16.17: Examples of the many functions of membrane proteins.

Transmembrane proteins perform most of the functions illustrated here. However, peripheral membrane proteins also play vital roles in membrane function. *Cytochrome c* is an example. It is a redox component loosely bound to the rest of the electron transport system in the mitochondrial cristal membrane. Other peripheral membrane proteins may serve to regulate transport or signaling activities of transmembrane protein complexes or may mediate connections between the membrane and cytoskeletal elements. As shown here, peripheral proteins do not penetrate membranes. They bind reversibly to the internal or external surfaces of the biological membrane with which they are associated. Shortly we'll take a closer look at what holds membrane proteins in place and how they perform their unique functions. Check out major membrane protein functions, actions and cellular locations in Table 16.1 (below).

Some Functions of Membrane Proteins

Basic Function	Specific Actions	Examples
Facilitated transport	Regulate diffusion of substances across membranes along a concentration gradient	Ca++ & other ion channels, glucose transporters
Active transport	Use energy to move ions from low to high concentration across membranes	Mitochondrial protein pumps, the Na+/K+ ion pump in neurons
Signal transduction	For e.g., hormones that can't enter cells, these convey information from molecular signals to cytoplasm, leading to a cellular response	Protein hormone and growth factor signaling, antibody/antigen interactions, cytokine mediation of inflammatory responses etc.
Cell-cell interactions	Cell-cell recognition and binding to form tissues	Formation of desmosomes, gap junctions and tight junctions
Anchors to cytoskeleton	Link membrane proteins to cytoskeleton	Give cells their shape, cell movement and response to molecular signals
Enzymatic	Usually multifunctional proteins with enzymatic activities	F1 ATP synthase uses proton gradient to make ATP; adenylyl cyclase makes cAMP during signal transduction; note: some receptor proteins are linked to enzymatic domains in the cytoplasm.

288-2 Diversity of Membrane Protein Structure & Function

289-2 Pore Proteins May Cross the Membrane Many Times

290-2 Red Blood Cell (Erythrocyte) Membrane Protein Functions

16.5 Glycoproteins and Glycolipids

Membrane proteins are often covalently linked to ***oligosaccharides***, which are branched *glycoside-linked* sugars (averaging around 15 sugar residues). As ***glycans***, they are the sugars linked to ***glycoproteins*** and glycolipids.

Glycoproteins are rare in the cytosol, but common on secreted and membrane proteins. Oligosaccharides are typically linked to proteins via the hydroxyl group on serine or threonine amino acids (*O-glycosylation*) and occasionally to modified amino acids (e.g., hydroxylysine, hydroxyproline) or to the *amide nitrogen* on asparagine (*N-glycosylation*). As a major feature of the ***glycocalyx***, oligosaccharide domains of glycoproteins and glycolipids are the 'face' of a plasma membrane that communicates with the extracellular world. Fig. 16.18 illustrates the glycocalyx.

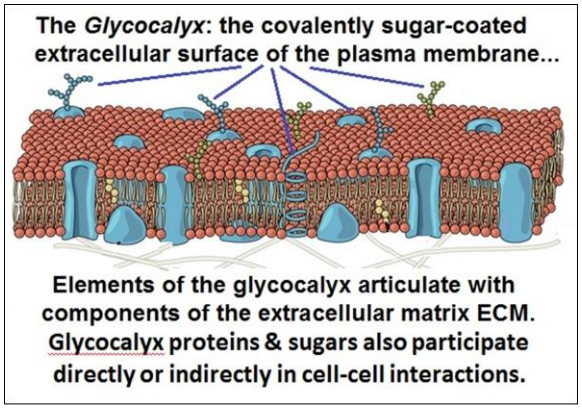


Fig. 16.18: The glycocalyx is the sugar- rich region on the extracellular surface of cells, the result of covalently bound sugars on glycoproteins and glycolipids. It is the basis of many cell functions and is associated with other macromolecules to form an extracellular matrix.

Oligosaccharides begin their synthesis in the rough endoplasmic reticulum (*RER*), with the creation of a ***core glycoside***. These partial *glycans* are enzymatically linked to compatible amino acids of a membrane protein. As these proteins travel through the *Golgi vesicles* of the *endomembrane system*, ***terminal glycosylation*** attaches more sugars to the core glycoside to complete glycoprotein synthesis. When vesicles budding from the trans-Golgi vesicles fuse with the plasma membrane, the sugars on the glycoproteins end up on the exterior cell surface. This is illustrated in the link below.

291 The Path to Sugar Coated Cells

Like glycoproteins, glycolipids are only found on the extracellular surface. They are synthesized in much the same way as glycoproteins. Specific enzymes catalyze initial glycosylation of phospholipids and polypeptides, followed by terminal glycosylation reactions. Glycoproteins, glycolipids and proteoglycans on cell surfaces play critical roles in cell-cell recognition and the formation of tissues. They recognize and bind to carbohydrate receptors (***lectins***) on adjacent cells, leading to cell-cell attachment as well as intracellular responses in the interacting cells. Glycoproteins and glycolipids also mediate the interaction of cells with extracellular molecular signals and with chemicals of the ***ECM***, or ***extracellular matrix*** (Fig. 16.19). The *ECM* includes components of connective tissue, basement membranes, in fact any surface to which cells attach.

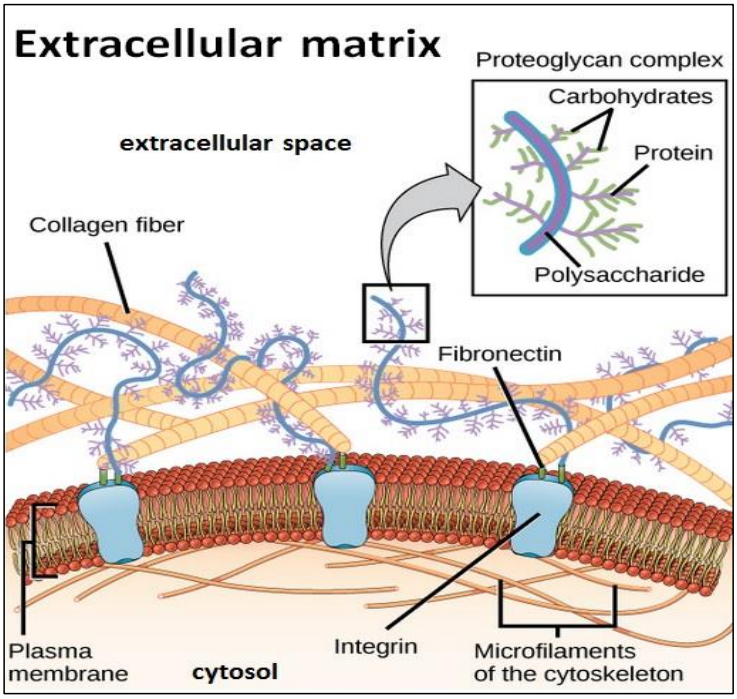


Fig. 16.19: The extracellular matrix (ECM) forms when proteins (e.g., fibronectin, collagen) and other macromolecules (e.g., proteoglycans) associate non-covalently with membrane proteins and elements of the glycocalyx.

292-2 The Extracellular Matrix

16.6 Glycoproteins and Human Health

We'll close this chapter with a few examples of glycoproteins that play crucial roles in human physiology. Let's look first at the major human A, B, AB, O and Rh blood groups that result from the presence or absence of glycoprotein ***antigens*** embedded in red blood cell membranes and the presence or absence in the blood, of ***antibodies*** against the antigens.

Typically an exposure to *antigens* (foreign substances like bacteria, viruses, toxins…) generates ***immunoglobulins***, the *antibody* molecules of our immune system. The *immunoglobulins* are glycoproteins. Since blood group antibodies already in the blood of a healthy person *are not* a response to foreign antigen invasion they are something of a paradox!

You probably know that these blood groups must be compatible for a successful blood transfusion. A mismatch between donor and recipient can be devastating. An interaction of the red cell antigens of one blood group with antibodies in another blood group will cause the red cells to clump, restricting blood flow and ultimately killing the transfusion recipient. Fig. 16.20 summarizes why transfusions with mismatched A, B, AB, O blood groups must be avoided.

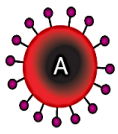
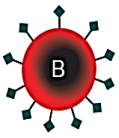
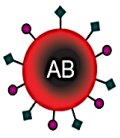
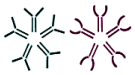
	Group A	Group B	Group AB	Group O
Cell–surface antigens				
Antibodies in the blood	 Anti-B	 Anti-A	None	 Anti-A and Anti-B
Acceptable donor–recipient matchess	Group A or Group O donors	Group B or Group O donors	Universal Recipient (Groups AB, A, B or O donors)	Only Group O donors
Why red cells clump in mismatched blood	Anti-A from Group B donor binds, aggregates recipient red cells; recipient Anti B binds, aggregates donor red cells.	Anti-B from Group A donor binds, aggregates recipient red cells; recipient Anti A binds, aggregates donor red cells.	Recipients have no antibodies to attack donor red cells – neither recipient nor donor cells clump	Antibodies in Group O blood will bind any donor red cell antigens and cause the cells to clump

Fig. 16.20: Blood groups with membrane proteins and elements of the glycocalyx (see text for details).

From the table above, you can see why type O blood is called the *universal donor*, and thus in high demand by clinics and hospitals. Thanks to enzymes produced by members of the *gut microbiome*, the absolute requirement that blood groups must be matched for safe transfusions may someday be a thing of the past! One of these enzymes is a glycosidase that can digest the sugars on A and B antigens on the surfaces A, B and AB blood cells. This effectively converts them to type O red blood cells! Check the *Red Cross* website (http://www.redcrossblood.org/learn-about-blood/blood-types.html) or https://en.wikipedia.org/wiki/Blood_type for more detail about blood groups. For a summary of the research on enzymatically converting all blood types to type O, see www.eurekalert.org/conversion of A, B and AB to O type blood

.

Another red blood cell antigen is the Rh (rhesus) factor. Rhesus factors are antigens originally shown to define blood types in rhesus monkeys, but humans have equivalent antigens, so human Rh blood groups have nothing to do with the monkeys! People either have their own Rh factor and are Rh^+… or they don't have the factor and are Rh^-. When an Rh^- recipient receives blood from an Rh^+ donor, the recipient's immune system makes defensive anti-Rh antibodies in the usual way. This too can cause blood cell clumping with bad consequences.

A word to the wise: While you should be 'typed' in the event you need a transfusion, it's always a good idea to know your own ABO and Rh blood groups…

The last example here involves the cell surface *major histocompatibility complex* (***MHC***) glycoproteins that distinguish *self* from *non-self* in body tissues and organs. Major organ transplantation (liver, kidneys, heart) from donors into patients with failing organs has become if not routine, then at least increasingly common. Before a transplant, *MHC tissue typing* determines donor and recipient compatibility to reduce the chances of the rejection of the transplanted organ. Since available donors are few and good matches even fewer, patients wait on prioritized lists for a matched organ. Even when MHC typing is a match for a patient, the immune systems of transplant recipients are suppressed with hormones to further reduce chances of rejection. Unlike the limited number of blood groups, many MHC proteins are analyzed to determine a match. Thus, it is not practical (or routinely necessary) to 'know' your MHC type! In the next chapter, we look at membrane functions intrinsic to cellular existence itself.

Some iText & VOP Key Words and Terms

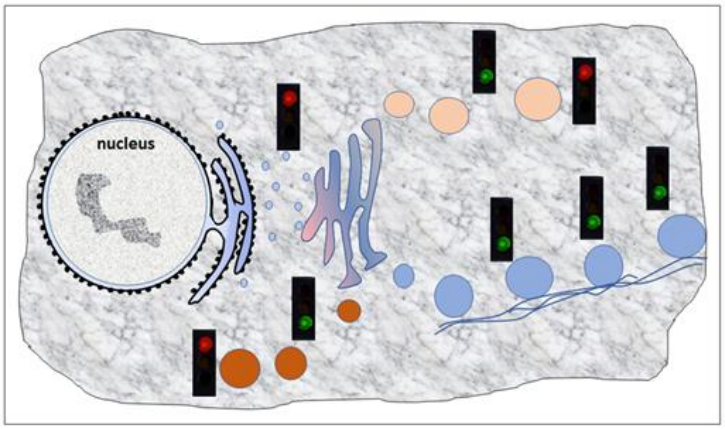
amphipathic molecules	**glycolipids**	**peripheral membrane proteins**
asparagine	**glycosylation**	**phospholipid bilayer**
cell membrane	**Golgi vesicles**	**plasma membrane**

cell-cell attachment	Hydropathy plot	poikilothermic organisms
cytoskeleton	hydrophilic phosphate heads	RER
Davson–Danielli membrane model	hydrophobic fatty acid tails	Rough endoplasmic reticulum
endomembrane system	hydrophobicity plot	saturated fatty acids
exocytosis	hydroxyproline	serine
extracellular matrix (ECM)	hydroxylysine	temperature effects on membranes
fluid mosaic	integral membrane proteins	threonine
freeze fracture method	membrane asymmetry	transmembrane proteins
membrane evolution	membrane proteins	unsaturated fatty acids
glycan	N-glycosylation	
glycocalyx	O-glycosylation	

Chapter 17: Membrane Function

Passive, facilitated and active transport, the traffic of proteins in cells, cell-cell interactions, excitability and signal transduction

GPS? Naahh, cells have a *Cell Positioning System*
CPS!



Telling proteins where to go (nicely of course)

17.1 Introduction

Small molecules like O_2 or CO_2 can cross cellular membranes unassisted; neither the hydrophilic surfaces nor the hydrophobic interior of the phospholipid bilayer are barriers to their transit. On the other hand, most molecules (even water) need the help of *membrane transport proteins* to get in or out of cells and organelles. Transport proteins can act as *gates* that might be open or closed. When open, these gates permit the diffusion of molecules into or out of cells along a concentration gradient so that their concentrations equalize across the membrane. Like the ***passive diffusion*** of small gasses, ***facilitated diffusion*** by membrane proteins does not require an input of energy. In contrast, some transport proteins are actually *pumps*, using chemical energy to move molecules across membranes *against* a concentration gradient. The result of this ***active transport*** is to concentrate solutes on one side of a membrane. For example, pumps that create sodium and potassium ***ion gradients*** are responsible for the e*xcitability of cells*. Recall that this is one of the fundamental properties of life: the ability of cells and organisms to respond to stimuli.

As you read this chapter, look for how allosteric changes regulate membrane function. We'll consider how:

- membrane ***gates*** and ***pumps*** work.
- membrane protein interactions allow cells to ***self-assemble*** into tissues and organs.
- cells direct protein ***traffic*** to the cytoplasm, into membranes themselves, into organelles…, or out of the cell.
- membrane proteins participate in ***direct communication*** between adjacent cells.
- membrane proteins are receptors for more ***long-distance communications***, responding to neurotransmitters, hormones, and other external chemical signals.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain how and why one cell's plasma membrane differs from that of another cell type.
2. explain how and why the plasma membrane differs from other membranes in the same cell.
3. determine if solutes cross a plasma membrane by passive or facilitated diffusion.
4. explain how *salmon* can spend part of their lives in the ocean and part swimming upstream in freshwater to spawn, without their cells shriveling or bursting.
5. explain how *active transport* stores *chemical energy* (recall electron transport and oxidative phosphorylation).
6. explain the role of active transport in maintaining/restoring a cell's *resting potential.*
7. compare and contrast different kinds of *gated channels.*
8. describe the order of ion movements that generate an *action potential.*
9. define and compare *exocytosis*, *pinocytosis*, *phagocytosis* and *receptor-mediated endocytosis.*
10. distinguish between *signal molecules* that enter cells to deliver their chemical message and those deliver their message only as far as the plasma membrane.
11. trace an intracellular response to a *steroid hormone* to a likely *cellular effect.*
12. trace a liver cell response to *adrenalin* from the plasma membrane to glycogenolysis (*glycogen breakdown*) in the cytoplasm of the cell.
13. compare the *signal transduction* activities of different *G-protein receptors* leading to the first active kinase enzyme in a *phosphorylation cascade*.
14. explain how a liver cell can respond the same way to two different hormones (e.g., adrenalin and glucagon)…, and why this should be possible.
15. describe/explain how a phosphorylation cascade *amplifies* the cellular response to a small amount of an *effector* (signal) molecule.
16. discuss the differences and interactions between the *glycocalyx*, *basement membrane* and *extracellular matrix* (ECM).
17. explain *ECM* functions and identify components involved in those functions.

18. describe how the molecular structure of *fibronectin* supports its different functions.
19. describe some structural relationships between cell surfaces and the *cytoskeleton.*
20. compare and contrast the structures and functions of the different cell junctions.
21. distinguish between the structures and functions of *cadherins*, *clathrin*, *COPs*, *adaptin*, *selectins, SNAREs* and *CAMs.*
22. state an hypothesis to explain why some cancer cells divide without forming a tumor.

17.2 Membrane Transport

The first control on the passage of molecules across membranes is the semi-permeable character of the membrane itself. Molecules move in and out of cells in one of three ways: ***passive diffusion***, ***facilitated transport*** and ***active transport***.

Only a few small, relatively uncharged molecules can cross a membrane unassisted (i.e., by passive diffusion). Hydrophilic molecules that must enter or leave cells do so with help, i.e., by *facilitated transport*. Passive and facilitated transport release the free energy inherent in concentration gradients as molecules diffuse across a membrane. In contrast, active transport consumes energy to create concentration gradients of specific solutes. The specificity of *facilitated* and *active transport* lies in integral membrane proteins that recognize and bind specific solutes for transport. As you may predict, allosteric regulation of these proteins controls the movement of their target molecules into or out of cells.

Despite its polarity, many believed that the small water molecules crossed membranes without help. Indeed, it does to a limited extent. However, others suspected that given its highly charged *polar covalent* bonds relative to its small size, water molecules require an assist to get across membranes efficiently. Let's begin with a closer look at passive diffusion and diffusion by facilitated transport, followed by osmosis (a special case of facilitated diffusion of water), and finally at active transport.

17.2.1 Passive Diffusion of Solutes

Diffusion across membranes does not require energy. In fact, diffusion can release energy - recall the movement of protons through the F1 ATPase proton gate that synthesizes ATP during mitochondrial oxidative phosphorylation. Passive diffusion in solution is the movement of molecules over time by random motion (also called *Brownian motion*) from regions of higher concentration to regions of lower concentration. Significant passive diffusion across cellular membranes is limited to a few molecules, mostly gasses like O_2, CO_2, and N_2, that can freely cross the hydrophobic phospholipid barrier. The rapid diffusion of gasses is essential for O_2 and CO_2 exchange between the alveolar capillaries and cells of the lungs during physiological respiration. O_2 and CO_2 exchange also occurs in mitochondria during cellular respiration.

The rate of diffusion of a molecule is dependent only on its own concentration. It is unaffected by the concentration of other molecules. Over time, random motion of solutes within and across compartments results in a ***dynamic equilibrium*** for each different solute over time. At equilibrium, solute molecules continue to diffuse across the membrane, but for each molecule moving across in one direction, another molecule of the same solute crosses in the other direction.

17.2.2 Facilitated Diffusion of Solutes and Ions

Like passive diffusion, f*acilitated diffusion* is the spontaneous (downhill) passage of molecules or ions across membranes through specific transmembrane proteins. The kinetics of passive and facilitated diffusion reveals the differences between the two processes. To understand the latter, recall that the rate of enzyme catalysis is *saturable*. That is, as the concentration of substrate is increased, the rate of the catalyzed reaction approaches a maximum (*Vmax*). This occurs when all enzyme molecules in solution are bound to substrate molecules. The same saturation phenomenon applies to facilitated transport – the rate of diffusion of a solute across a membrane is directly proportional to the concentration of the solute, but is limited by the number of transport proteins in the membrane. Fig. 17.1 plots the kinetics of passive and facilitated diffusion.

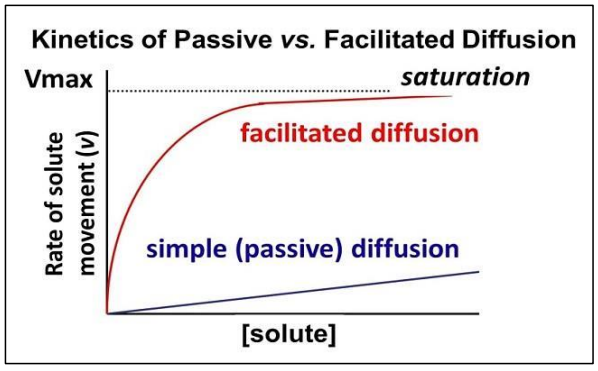


Fig. 17.1: Graph plotting rates of passive and facilitated transport show that facilitated diffusion is a saturable process.

Perhaps you see another similarity between facilitated diffusion and enzyme catalysis in this graph! Relative rates of facilitated diffusion are typically rapid, compared to those of passive diffusion. This is because the allosteric changes that accompany facilitated transport are rapid, just as they are during enzyme catalysis.
Fig. 17.2 (below) illustrates three kinds of ***facilitated transport*** of solutes.

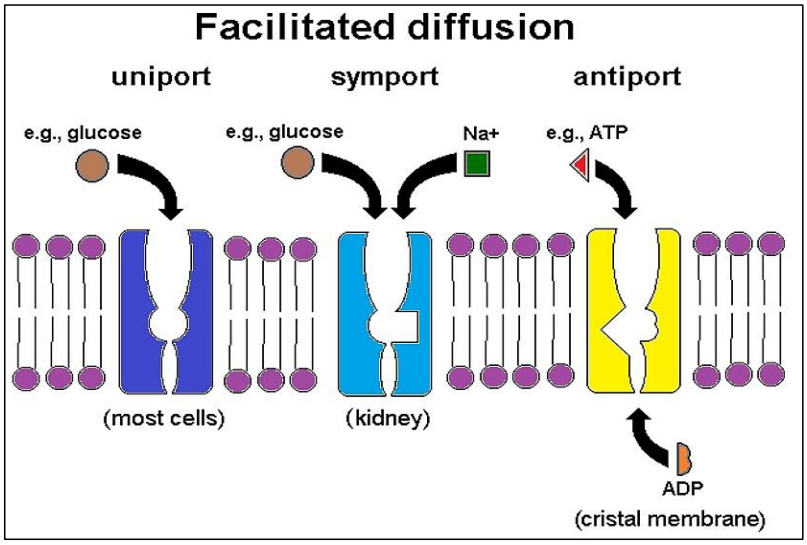


Fig. 17.2: Three kinds of facilitated diffusion (see text for details).

The ***GLUT*** (***GLU****cose* ***T****ransporter*) protein (Fig.17.2, left) allows glucose ***uniport***, the specific transport of a single substance in or out of cells. In ***symport*** (Fig.17.2, middle) glucose transporters couple the simultaneous movement of glucose and sodium ions, for example in kidney cells. The ***SGLT*** (**S**odium-**GL**ucose **T**ransporter) serves a similar function in small intestine cells, enabling absorption of dietary glucose and sodium. ***Antiport*** (Fig.17.2, right) allows the specific exchange of molecules across a membrane. In the example shown, ATP leaves the mitochondrial matrix, crossing the cristal membrane at the same time as ADP enters the matrix. Whether by uniport, symport or antiport, each solute will independently cross a membrane down its concentration gradient, moving from higher concentration to where it is at a lower concentration. Recall that diffusion along a gradient releases free energy that depends on relative concentrations of the solutes.

Proteins mediating facilitated transport are of two kinds: ***carrier proteins*** allow solute transport. Ions, with their high charge-to-mass ratio, need help to cross the hydrophobic membrane barrier. This is the job of ***channel proteins*** that essentially serve as ion pores. Like all transporter proteins, both *carrier* and *channel* proteins undergo allosteric changes during transport. They are also typically subject to allosteric regulation, rather than being in a constant 'open' state. Examples of facilitated diffusion are considered in more detail below.

17.2.2.a Carrier Proteins

When a carrier protein binds a solute that must cross the membrane, it undergoes the first of a series of allosteric changes, as shown in Fig. 17.3

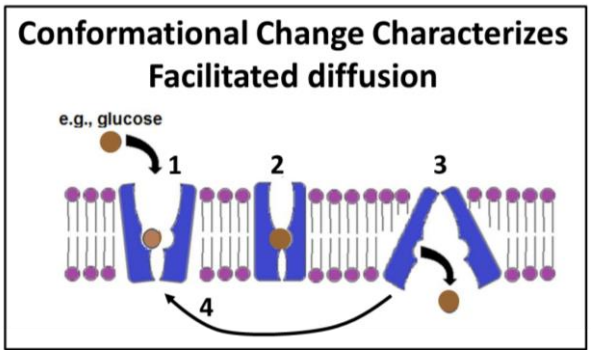


Fig. 17.3: Facilitated transport proteins (e.g., glucose transporter) undergo sequential allosteric changes as they recognize a solute, transfer and release it on the other side of the membrane.

During transport itself, the carrier protein undergoes another change in shape. When the solute reaches the other side of the membrane, it no longer has a high affinity for the carrier protein. After release of the solute, a final allosteric change restores the original conformation of the transport protein.

A given carrier protein is specific for a single solute, or at most a single family of closely related solutes. Thus, ***GLUT1***, a glucose uniport transporter, allows glucose (but not fructose or ribose!) to across membranes. Other specific carrier proteins facilitate the transport of amino acids or charged solutes across cell membranes. Once again, molecules that indicate cell status (i.e., a need to import or export solute) are *allosteric effectors* that regulate carrier proteins. Insulin is a perfect example of the regulation of solute transport, specifically glucose transport into cells. One consequence of insulin released during a meal (or in anticipation of a meal!) is the stimulation of glucose transporters to take up glucose. An inability of those transporters to respond to insulin accounts in part for Type II (adult onset) diabetes.

Water gets across membranes by osmosis; we'll look more closely at how osmosis affects cells in a moment. First, recall that small amounts of water could cross the phospholipid bilayer unassisted. Water can also cross a membrane incidentally, when ions flow through their channel proteins. But most osmosis involves

facilitated diffusion mediated by ***aquaporins***. Some aquaporins only transport water. Others have evolved to co-facilitate the transport of glucose (see above), glycerol, urea, ammonia, carbon dioxide and even ions (protons) along with water.

Like other carrier proteins, aquaporins are allosterically regulated to allow cells to meet their specific water balance requirements. So fundamental was the understanding of water balance that the discovery of aquaporins earned Peter Agre a Nobel Prize in Chemistry in 2003.

Since Agre's discovery (in 1992), several genetic diseases have been linked to aquaporin gene mutations. Kidney cells are critically involved in vertebrate water balance and have many aquaporins in their membranes. In a rare form of diabetes, abnormal aquaporins cause the kidneys to excrete unusually large volumes of water. In another example, aquaporin gene mutations lead to the development of cataracts in both eyes. Since their initial discovery, aquaporins have been described in bacteria and plants. To learn more, click Aquaporins - Wikipedia.

17.2.2.b Ion Channels

Allosteric regulation of ion channel proteins controls ion *homeostasis* in blood and extracellular fluids within narrow limits. Often, multiple integral proteins contribute to the formation of an ion channel. When stimulated, channel proteins rearrange to open a pore allowing specific ion transport. Some ion channels, like the glucose-sodium ion symport system noted above, mobilize the energy of diffusion of one solute (the ion in this case) to rapidly transport another solute through the same channel (acting like an ion channel *and* a carrier protein). Finally, ion channels are responsible for the excitability of cells, where Na^+ , K^+ and Ca^{++} channels collaborate in ion movements into and out of cells leading to neuronal or muscle cell responses (more shortly!).

293-2 Passive & Facilitated Transport

17.3 Osmosis

Osmosis is the diffusion of water across membranes from lower to higher solute concentrations, an essential cellular activity. It allows cells to use water to maintain cellular integrity or to adapt to changes in the solute composition in the extracellular environment. Osmosis relies on the *facilitated transport* of water by ***aquaporins***. The passive diffusion of water molecules can be demonstrated with artificial (e.g., dialysis)

membranes. If solute concentrations are higher on one side of the membrane, water will cross the membrane "trying" to equalize the solute concentrations on both sides of the membrane. In effect, water movement is from the side of a membrane where the ***free water*** molecule concentration is higher (i.e., where the concentration of solute is lower) to the side where the concentration of *free water* is lower (i.e., where the concentration of solute is higher).

17.3.1 Osmosis in Plant and Animal Cells

We could present this section in the context of free water concentrations, but we will do so in the more familiar terms of solute concentrations. Osmosis affects plant and animal cells according to the same principles, but with different effects. The effects of different experimental solute concentrations on animal cells are illustrated in Fig. 17.4.

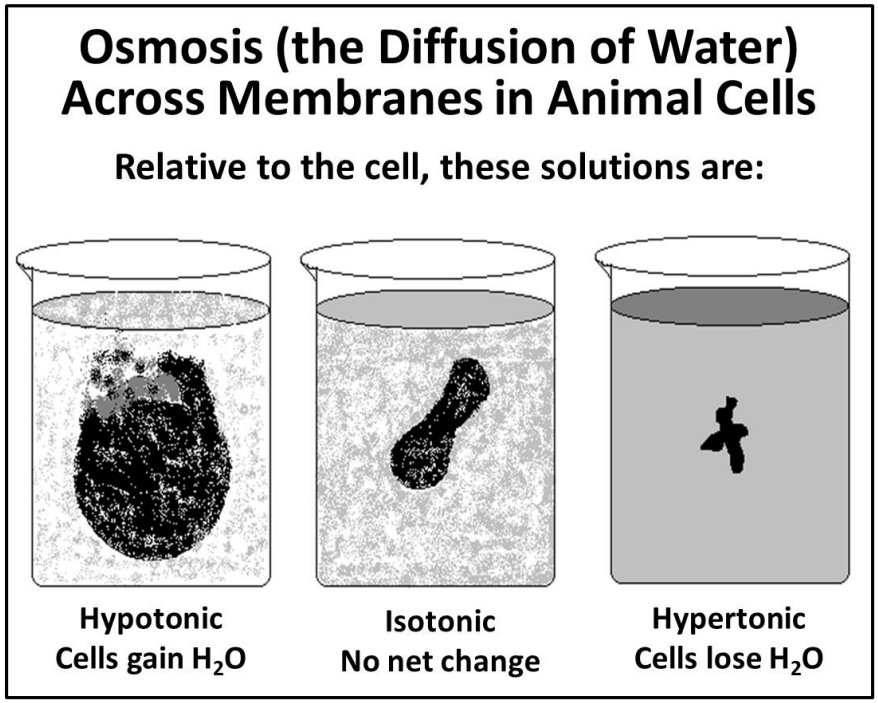


Fig. 17.4: Effects of differences in solute concentrations inside and outside ***animal*** *cells* on the movement of water into or out of the cells, i.e., *Osmosis* (see text for details).

If the solute concentration inside and outside the cell is the same, there is no net water movement into or out of the cells. The extracellular medium and cytosol are said to be **isotonic** to each other. When water diffuses into the cells from a low solute medium, the medium is said to by **hypotonic** to (less concentrated than) the cytosol. In this case, movement of water into a cell lowers the cytosol solute concentration. Animal cells swell and burst in a hypotonic solution. Animal cells in **hypertonic** solutions (with higher solute concentrations than the cytosol) shrivel up as water leaves the cell. From this brief description, you should conclude that *water crosses from the hypotonic to the hypertonic side of a membrane*.

As with animal cells, exposure of plant cells to hypotonic or hypertonic solutions causes the same directional water movements, but with some key differences due to their cell walls. Fig 17.5 shows the effects of different solutions on plant cells.

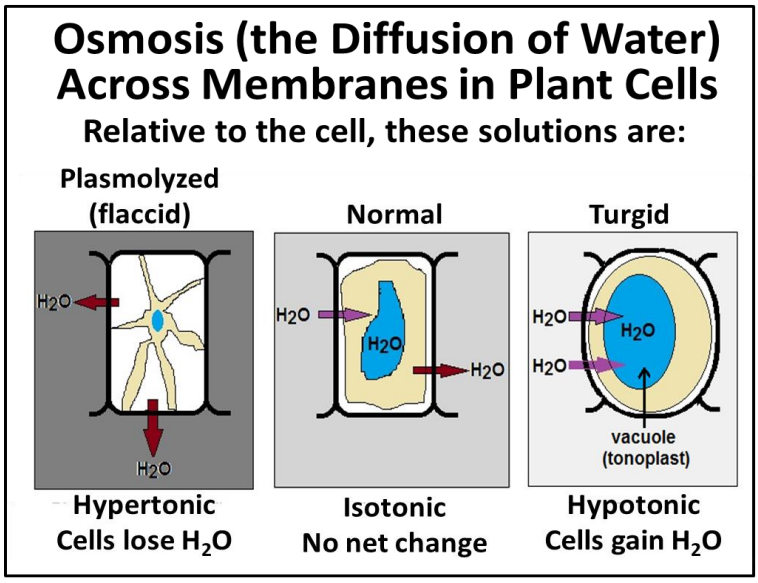


Fig. 17.5: Osmotic effects of differences in solute concentrations inside and outside ***plant*** *cells* on the movement of water into or out of the cells, i.e., *Osmosis* (see text for details). Correct cytoplasmic solute concentrations are achieved by accumulating excess water in the *tonoplast*.

In *hypotonic* solutions, water enters plant cells, moving into the cytosol and then into water vacuoles called ***tonoplasts***. This results in higher ***osmotic pressure*** (water pressure) in the tonoplasts. The expanding *tonoplast* creates ***turgor pressure***, compressing the cytosol against the cell wall. Rather than bursting, the

cells and therefore the plant tissues stiffen and become ***turgid***. Since water cannot enter plant cells indefinitely, water stops entering the cells when the *osmotic pressure* outside the cells and the *turgor pressure* inside the cells are at equilibrium. You've seen this phenomenon if you ever over-watered houseplants. The stiffened leaves and stems become brittle and are easily snapped or broken. In hypertonic medium, plant cells (like animal cells) lose water. The resulting shrinkage of the plasma membrane away from the cell walls is called plasmolysis, in which bits of plasma membrane remain tightly attached to the plant cell wall at several points. You may have seen under-watered plants with floppy or droopy stems and leaves. These have become ***flaccid*** due to loss of water and thus the loss of turgor pressure needed to keep leaves and stems upright. Formally, osmotic or turgor pressure is defined as the force per unit area (i.e., *pressure*) required to prevent the passage of water across a semipermeable membrane from a hypotonic to a hypertonic solution.

17.3.2 Osmosis in Plant Life

While individual plant cells respond to changes in solute concentrations, these changes are rapidly communicated to adjacent cells through ***plasmodesmata***. These structures connect the plasma membranes of adjacent cells through their cell walls, allowing rapid, direct sharing of physical and chemical information. A ***plasmodesma*** is illustrated in Fig. 17.6.

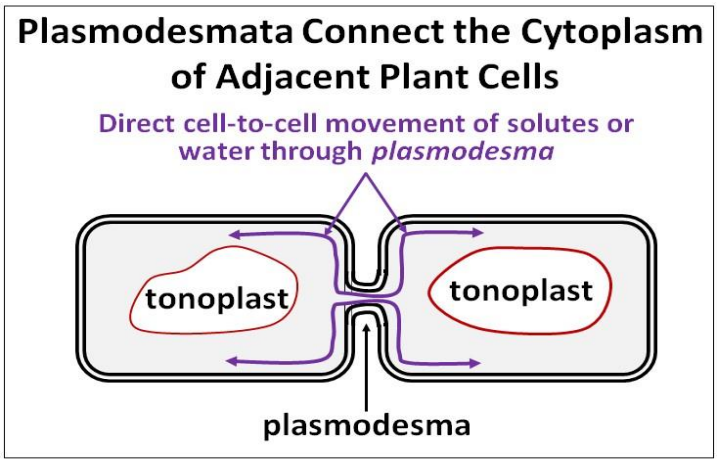


Fig. 17.6: Plasmodesmata are cell wall 'tunnels' connecting plat cells that allow direct movement of water between cells to transmit and balance changes in osmotic pressure in one part of a plant (e.g., roots) throughout the plant (see text for details).

In this way, effects on osmotic pressure in a few cells created by changes in water availability are transmitted to adjacent cells, affecting turgor pressure in those cells and, ultimately, in plant tissues.

Finally, plant life depends on water! Recall that plant cells require a continual supply of water for use in photosynthesis, to provide hydrogen to reduce CO_2 to glucose. Photosynthesis as well as the loss of excess water from plant tissues (especially leaves) by ***transpiration*** lowers cellular osmotic pressure. As water moves up from the roots to replace water used and lost by leaf cells, the osmotic pressure drops in the *fine root hair cells* (with their high surface area). This draws water into the root cells by osmosis. Thus, osmotic pressure is the main force driving water into plants and, defying gravity, moving it up from the roots to the rest of the plant.

17.3.3 Osmosis in Animal Life

Changes in osmotic environment can stress or kill an organism. For example, freshwater organisms (protozoa or fish) placed in sea water will die. Likewise, salt-water fish placed in freshwater. But cells and organisms can ***osmoregulate*** (control the osmotic pressure in their cells), at least to a point. For example, as a *Paramecium* take on water, a ***contractile vacuole*** expels excess water to prevent the cell from bursting (Fig. 17.7).

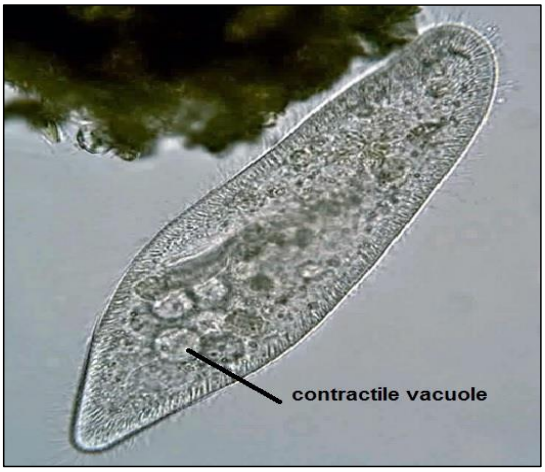


Fig. 17.7: Light micrograph of Paramecium highlighting a contractile vacuole, an organelle that pumps out excess water from the cell, protecting it from osmotic shock.

Water constantly enters these freshwater protists because the solute concentration in the cytosol is always higher than the freshwater water they live in. To cope with a constant uptake of water, their *contractile vacuoles* collect excess water and then contract to expel the water. At a high-energy cost, *Paramecia* constantly pump water out of the cell to maintain water balance (i.e., correct osmotic pressure). Another protist strategy for coping with change in environmental solute concentrations (e.g., salinity) is to pump salts (or suitable salt solute substitutes in or out of the cell, as needed (For some details, see Protist Osmoregulation Genes Acquired by Eukaryotes from Bacteria by Horizontal Gene Transfer.

Larger organisms like freshwater fish cope with their hypotonic environment by urinating a lot! At the other end of the spectrum, salt-water fish cope with the high solute concentration of solutes (salts) in their environment by excreting excess salt. Salmon spend time in seawater growing to maturity and later swim upstream in fresh water to spawn. You can imagine how salmon and similar organisms have to ***osmoregulate*** to adapt to their changing, very different environments. In this case, ***osmoregulation*** begins when hormonal changes respond to changes in living circumstance and dictate a compensatory response.

17.3.4 Summing Up

Osmosis is the movement of water across membranes to where solutes are at high concentration. At the same time, solutes diffuse across membranes, moving in or out of cells to where they are at lower concentration, either passively or by facilitated transport. We've evolved different facilitated transport proteins specific for different solutes. And finally, most water crosses membranes by facilitated diffusion through aquaporin proteins that serve as pores in cellular membranes.

17.4 Active Transport

Excitability (adaptation) is another of the defining properties of life. This property of all cells is based on chemical and electrical reactivity. Neurotransmitters released at a synapse cross the synaptic cleft from a "sending" neuron to a responding cell (another neuron or a muscle cell). The neurotransmitter binds to receptors on the responding cell resulting in a ***membrane depolarization***, a rapid change in the electrical potential difference across the cell membrane. While responses to neurotransmitters occur in fractions of a second, all kinds of cells are responsive, albeit not always as fast as neurons or muscle cells.

Changes in the membrane polarity of any cell depend on *unequal* concentrations of ions inside and outside cells. These ionic differences across membranes are what enable all cells, but especially as neurons and muscle cells to respond to chemical and other (e.g., electrical) signals. Thus, cells have a ***resting potential*** due to a higher **[K+]** in the cytosol and higher **[Cl^-]** and [**Na+]** outside the cell.

The measured resting potential (difference in charge or ***potential difference***) of most cells is typically between -50mv to -70mv. These numbers are negative because there is a higher concentration of negative (i.e. Cl^-) ions inside the cell than outside. Ionic concentration gradients permit physiological response to chemical or other signals. They change when cells are excited, when they (quite normally) leak ions. Whether incidental or intentional, the correct ion balance must be restored to maintain excitability.
The relative concentrations of K+, Cl^- and Na+ ions accounting for a cell's resting potential are shown in Fig. 17.8 (below).

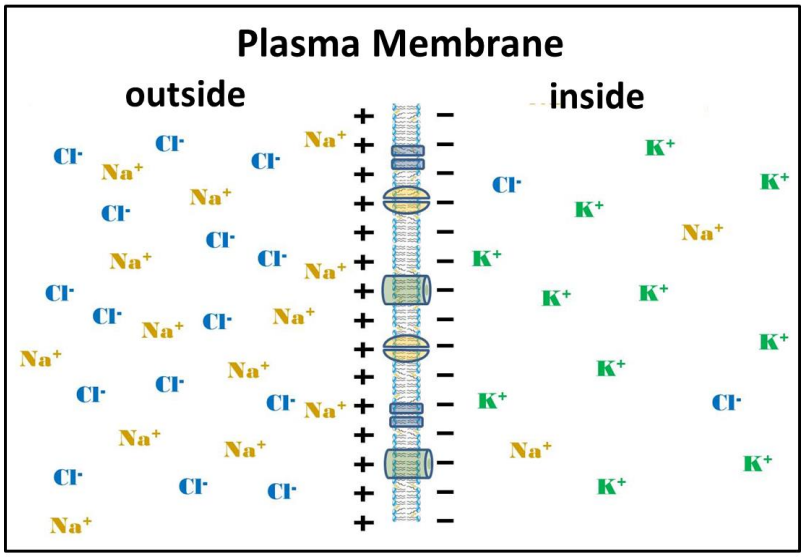


Fig. 17.8: A cell's r*esting potential* results from an ion concentration imbalance across the plasma membrane. Sodium and chloride ions concentrations are higher inside cells while potassium ions levels are higher outside the cell. Thus the cytoplasm is slightly negative compared to the extracellular fluid.

Maintaining a cell's resting potential requires energy is accomplished by the ATP-dependent ***Na^+/K^+ pump***, an ***active transport*** protein complex. Follow the allosteric changes as the Na^+/K^+ pump (Fig. 17.9 below) as it works to restore ion gradients.

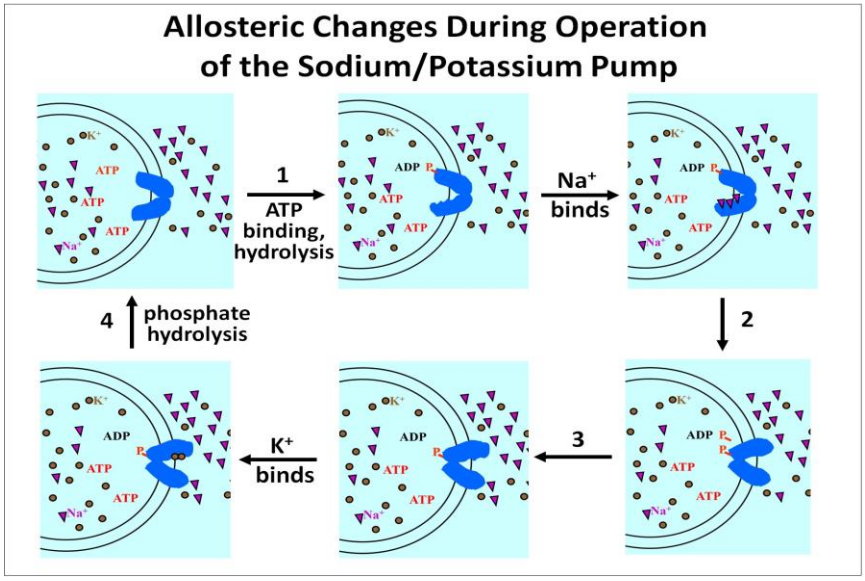


Fig. 17.9: After a change in the resting potential (e.g., depolarization) of a cell, ion balance across the cell membrane is restored by an ATP-powered sodium/potassium pump (see text for details).

In operation, the ATPase domain of the Na^+/K^+ pump protein hydrolyzes ATP, leaving a phosphate attached to the pump and inducing the first of several allosteric changes in the pump proteins (***Step 1*** in the illustration). In its new conformation, the pump binds three Na^+ ions, causing a second conformational change that in turn releases the Na^+ ions into the extracellular fluid (***Step 2***). The release of Na^+ ions outside the cell causes a third allosteric change (***Step 3***), after which two K^+ ions from the extracellular fluid are able to bind to the pump protein. K^+ binding causes the hydrolysis of the phosphate from the pump protein, returning it to its original conformation (***Step 4***) and releasing the two K^+ ions into the cytosol. The Na^+/K^+ pump is ready for action again!

295 Potassium Leakage Helps to Maintain Cellular Resting Potentials

296 Active Transport by the Sodium/Potassium Pump

For his discovery of the ATPase-powered sodium/potassium pump and his studies of how it works to maintain intracellular ion balance, Jens C. Skou earned a share of the Nobel Prize in Chemistry 1997. Read more at Nobel_prize_chemistry_skou.

17.5 Ligand and Voltage Gated Channels in Neurotransmission

Ligand- and voltage-gated channels play a major role in neurotransmission and muscle contraction by regulating a flow of ions into and out of responding cells. With the advent of the ***patch clamp*** device (Fig, 17.5.1, below) it became possible to correlate ion flow with measurements of membrane potential and to define the sequence of electrical and chemical events leading to muscle contraction.

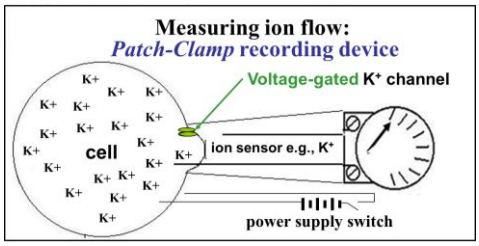


Fig. 17.10: A patch clamp device can measure ion flow through voltage-gated channels in a membrane during a depolarization event.(see text for details).

17.5.1 Measuring Ion Flow and Membrane Potential With a Patch-Clamp Device

When neurotransmitters bind to their receptors, ***ion channels*** in responding neuron or muscle cells open. The resulting influx of Na^+ ions disrupts the ***resting potential*** of the target cell. The effect is transient if the membrane potential remains negative. But if enough Na^+ ions enter the cell, the membrane becomes depolarized. If the cell experiences ***hyperpolarization,*** a localized *reversal of normal membrane polarity* (say from –70 mV to +65mV or more) will generate an ***action potential***. This action potential will travel like a current along the neural or muscle cell membrane, eventually triggering a physiological response, e.g., the excitation of the next nerve cell in a neuronal pathway or contraction of the muscle cell.

In this example of patch clamp measurements, closing the power supply switch sends an electrical charge to the cell, opening a ***voltage-gated ion channel***. A potassium sensor in the device then detects a flow of K^+ ions through the channel, out of the cell. Simultaneously, a volt meter registers the resulting change in membrane potential. In addition to voltage-gated ion channels, the patch clamp device can measure ion flow through ***ligand-gated ion channels*** and ***mechanically-gated ion channels***. The former channels are *receptor-ion gates* that open when they bind an effector molecule. *Mechanically-gated ion channels* sense physical *pressure* or *stress* that results in a local membrane deformation, opening the channel (recall *piezoreceptors*).

297 Patch Clamp Device Records Membrane Potential and Ion Flow

298 Patch Clamp Measures Resting Potential and Depolarization

299 Gated Ion Channels

300-2 Types of Ion Gated Channels

Finally, cells maintain a high intracellular concentration of K^+ ions, causing K^+ ions to slowly leak from the cell, a phenomenon detectable by a patch-clamp. The presence of negative ions (Cl^- ions as well as organic anions) inside a cell limits the leakage. This creates the electronegative interior of a cell relative to outside the cell, i.e., the resting potential across its plasma membrane. The patch-clamp technique has been used to correlate the flow of ions and changes in membrane potential when a neuron fires, causing an action potential in a responding cell (e.g., Fig. 17.11, below).

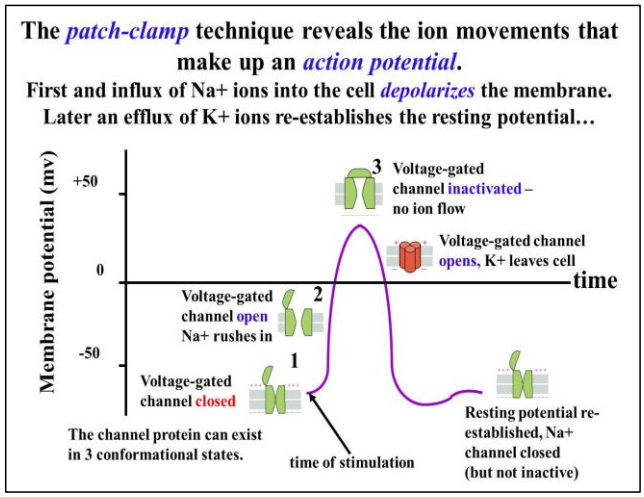


Fig. 17.11: Voltage changes across e.g., a muscle cell membrane during an action potential correlated with the flow of specific ions, determined with the patch clamp device (see text for

In the illustration, follow the opening and closing of ion channels and the resulting flow of ions. A shift from resting potential, and possibly an action potential will result from facilitated diffusion of specific ions into or out of the cell through gated ion channels

that must open and close in sequence. The behaviors of two different *voltage-gated ion channels* are illustrated in the graph. Electrical stimulation opens Na^+ channels and Na^+ ions rush into the cell. This reduces the membrane potential from the resting state to zero. If the Na^+ influx continues, it can make the cytoplasm more positive than the extracellular fluid, which may lead to an *action potential*. If the reversal in polarity is high enough, a voltage-gated K^+ opens and potassium ions rush out of the cell, restoring the *resting potential* of the cell.

A cell can continue to respond to stimuli with action potentials for as long as there is sufficient Na^+ outside the cell and K^+ inside the cell. While active transport of Na^+ and K^+ is not required to re-establish the resting potential, it will eventually be needed to restore the balance of the two cations in the cell. If a nerve or muscle cell fires several times (or even if it just leaks ions), [K^+] inside the cell and [Na^+] outside the cell can drop to a point where the cell cannot generate an action potential when stimulated. Ultimately, it is the role of ATP-dependent Na^+/K^+ pumps to restore the appropriate Na^+-K^+ balance across the responding cell membrane. As we have seen, each cycle of pumping exchanges 3 Na^+ ions from the intracellular space for 2 K^+ ions from the extracellular space. The pump has two effects: It restores Na^+ concentrations in the extracellular space relative to the cytoplasm, and it restores K^+ concentrations in the cytoplasm relative to the extracellular space.

301 Gated Ion Channels Open & Close in Order During an Action Potential

Together with the higher negative ion concentrations in the cytosol, the unequal exchange of Na^+ for K^+ ions maintains the resting potential of the cell over the long term and ensures that nerve and muscle cells remain excitable. Next, we will take a closer look at the role of both *ligand-gated* and *voltage-gated* ion channels in neurotransmission.

17.5.2 Ion Channels in Neurotransmission

Action potentials result in an orderly, sequential opening and closing of *voltage-* and *ligand-gated* channels along the neuronal axon. In the link below, you can see the sequential cycles of voltage-gated channels that propagate a localized ***action potential*** (*membrane depolarization*) along an axon towards a synapse.

302 Propagating an Action Potential Along an Axon

When a propagated depolarization reaches a synapse, gated ion channels either open or close in the neuron and the responding cell. The cooperation of voltage- and ligand-gated channels at a neuromuscular junction is illustrated in Fig. 17.12, below.

As you can see from the illustration, after a neuron fires, an electrical impulse (a moving region of *hyperpolarization*) travels down the axon to the nerve ending. At the nerve ending, the traveling charge difference (electrical potential) across the cell membrane stimulates a Ca^{++}-specific *voltage-gated channel* to open. Ca^{++} ions then flow into the cell because they are at higher concentrations in the *synaptic cleft* than in the cytoplasm. The Ca^{++} ions cause synaptic vesicles to fuse with the membrane at the nerve ending, releasing neurotransmitters into the synaptic cleft that cross the cleft and bind to a receptor on the responding cell plasma membrane. This receptor is a ***ligand-gated channel*** (also called a *chemically-gated channel*). Binding of the neurotransmitter ligand opens the gated channel. The rapid diffusion of Na^{+} ions into the cell then creates an action potential that leads to the cellular response, in this case, muscle contraction. We have already seen both the role of K^{+} channels in restoring the membrane resting potential after an action potential, and the role of the sodium/potassium pump in restoring the cellular Na+/K+ balance.

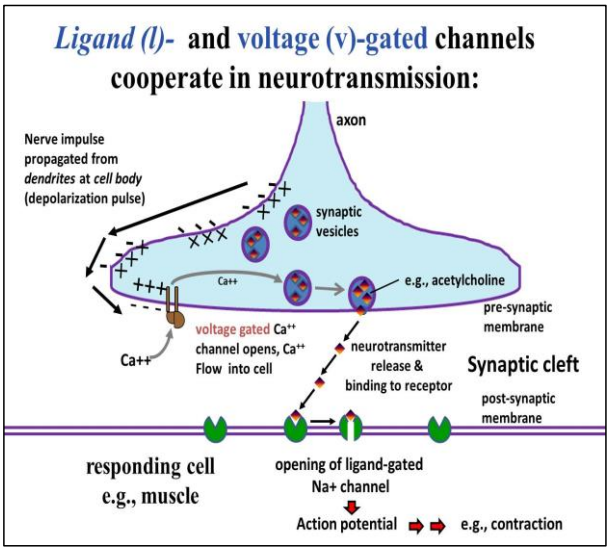


Fig. 17.12: Neurotransmission starts with an *action potential* in the cell body of a neuron. The action potential is propagated along the axon to the nerve terminal *synapse*. The *depolarization pulse* initiates Ca++ flow into the neuron through *voltage-gated channels* that causes neurotransmitter release into the *synaptic cleft*. The neurotransmitter binds to a *ligand gated channel* on the responding cell, causing Na+ ions to flow into the responding cell, leading to an action potential (see text for details).

303-2 The Role of Gated Ion Channels at a Neuromuscular Junction

CHALLENGE: Many neurotoxic venoms (snake, spider) interfere with ion channels to paralyze and immobilize prey organisms. Which of the channels mentioned here would you expect to be affected by these neurotoxins, or for that matter, neurotoxic gasses..., and why?

17.6 Endocytosis and Exocytosis

Endocytosis internalizes extracellular molecules (e.g., proteins), insoluble particles, or even microorganisms. The main pathways of endocytosis are ***phagocytosis***, ***pinocytosis*** and ***receptor-mediated endocytosis***. Pinocytosis of molecules is non-specific. Phagocytosis is also non-specific, internalizing large structures (e.g., bacteria, food particles…). In contrast, receptor-mediated endocytosis *is* specific for substances recognized by a cell-surface receptor. Some endocytotic processes are even used to recycle membrane components. ***Exocytosis*** is the secretion of large molecules like digestive enzymes and peptide/polypeptide hormones, each of which must exit the cell to the extracellular fluid or circulation. Exocytotic pathways also deliver membrane proteins to the cell surface, either new or to replace older, worn out proteins.

17.6.1 Endocytosis

The three main kinds of endocytosis are summarized in (Fig.17.13).

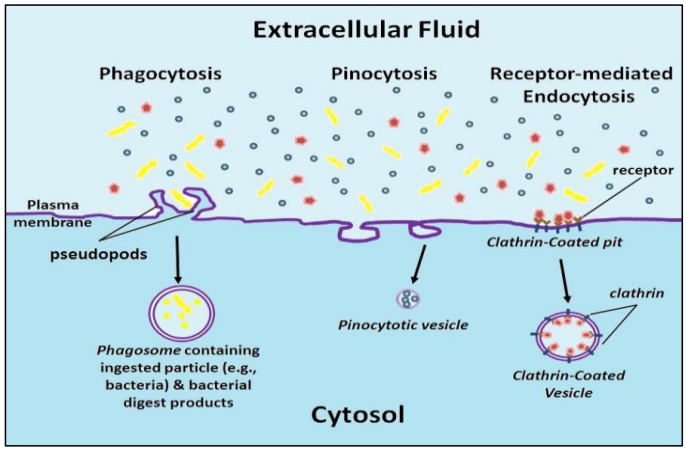


Fig. 17.13: Illustration of three main kinds of *endocytosis*, routes/mechanisms of import of extracellular materials into cells (see text for details).

1. **Phagocytosis** (above left): ***phagocytes*** extend *pseudopodia* by membrane ***evagination***. The pseudopodia of amoeba (and amoeboid cells generally) engulf particles of food that end up in digestive vesicles (***phagosomes***) inside the cytosol. Phagocytes are a class of white blood cells that are part of our immune system. They engulf foreign particles that must be eliminated from the body. A *lysosome* fuses with the phagosome, after which inactive hydrolytic enzymes stored in the lysosomes are activated. The result is the digestion of the engulfed particles. Phagocytosis begins upon contact between the outer cell surface and those particles.

2. ***Pinocytosis*** (above center): pinocytosis is a non-specific, more or less constant pinching off of small vesicles that engulf extracellular fluid containing solutes; they are too small to include significant particulates.

3. ***Receptor-mediated endocytosis*** (above right): this kind of endocytosis relies on the affinity of ***receptors*** for specific extracellular substances. Upon binding their ligands, the membrane receptors aggregate in differentiated regions of plasma membrane called ***coated pits***. The coated pits then invaginate and pinch off, forming a ***coated vesicle,*** bringing their extracellular contents into the cell. After the coated vesicles deliver their contents to their cellular destinations, the vesicle membranes are recycled to the plasma membrane. Receptor-mediated endocytosis is perhaps the best understood mechanism for bringing larger substances into cells. The drawings in Fig. 7.14 (below) are taken from electron micrographs that illustrate the invagination of coated pits to form *clathrin-coated vesicles*.

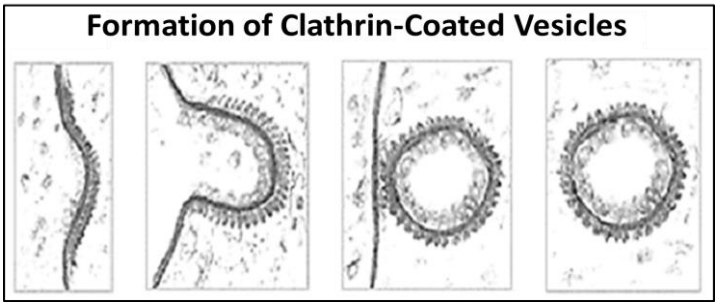


Fig. 17.14: Illustration of the stages of receptor-mediated *endocytosis* (see text for details).

The receptor and coat proteins are clearly visible as larger structures on the inner surfaces of the pits and on the outer surfaces of the clathrin-coated vesicles. ***Clathrin***, a large protein, is the principal protein on the surface of the invaginated

coated pit. Clathrin is linked to specific integral membrane proteins via ***adaptor protein 1*** (**AP1**). *AP1* recruits specific *cargo proteins* to bring into the cell when the coated pits invaginate. Some details of receptor-mediated endocytosis are illustrated below (Fig.17.15).

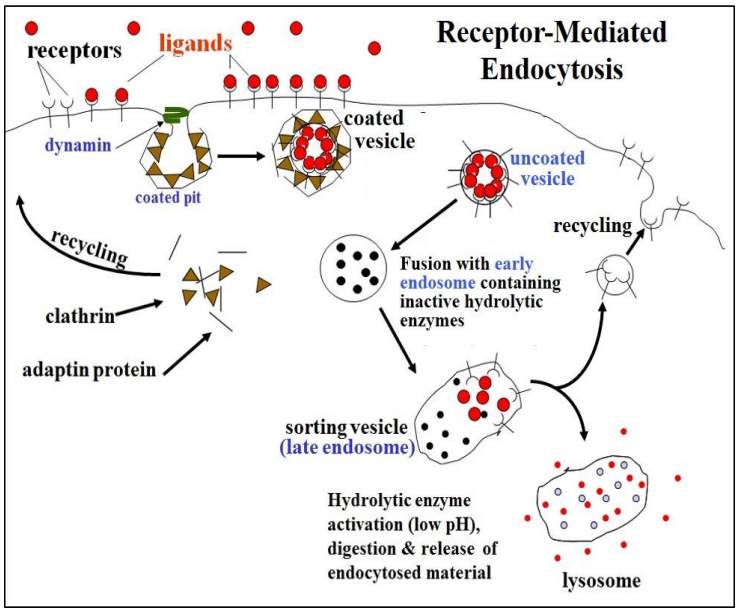


Fig. 17.15: Molecular details of receptor-mediated *endocytosis* (see text for

In the illustration, substances to be internalized have bound to cell membrane receptors. The receptors then cluster to form a ***coated pit***. Assisted by the protein ***dynamin*** (a GTPase), the coated pits invaginate. The final pinch-off of a ***coated vesicle*** requires GTP hydrolysis (not shown). Once internalized, the coated vesicles lose their clathrin and associated adaptor protein coat. The uncoated vesicle fuses with an ***early endosome*** to form a ***sorting vesicle*** (i.e., *late endosome*). Sorting vesicles separate imported content from the receptors. The latter are recycled to the membrane. The vesicle that remains is now a ***lysosome***, containing digestive enzymes that will catalyze hydrolysis of the vesicle contents. The digest products are then released for cellular use. To watch live video of fluorescently labeled proteins (the bright spots) entering cells by receptor-mediated endocytosis at Receptor-mediated endocytosis - esp. watch two left panels.

A well-known example of receptor-mediated endocytosis is the uptake of cholesterol bound to ***low density lipoprotein*** (**LDL**), a complex of phospholipid, protein and cholesterol (Fig. 17.16, below).

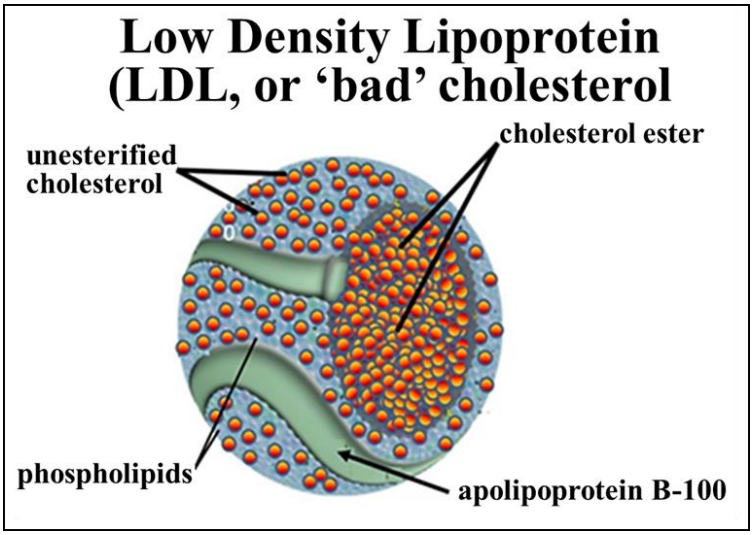


Fig. 17.16: A low-density lipoprotein coated with cholesterol (see text for details).

A single LDL complex carries as many as 15,000 molecules of cholesterol. LDL, sometimes called "bad cholesterol", is not good for you at high levels. On the other hand, high-density lipoprotein (HDL) is "good cholesterol". As one gets older, it is important to monitor one's HDL/LDL ratio; the higher it is the better!

17.6.2 Exocytosis

Maintaining cell size or volume seems to be a built-in component of the machinery of receptor-mediated endocytosis that balances endocytosis with membrane recycling. However, exocytosis is also necessary to restore plasma membrane internalized by pinocytosis and phagocytosis, and for eliminating cellular waste products. It is also the end-point of a complex process of packaging proteins destined for secretion, intracellular storage (e.g., in lysosomes, peroxisomes, etc.) or insertion into the membrane itself. While *endocytotic* and *secretion vesicles* form in 'opposite directions', they both share common structural features with the plasma membrane,

from which they are derived and with which they fuse (*respectively*). The formation of both lysosomes and secretion vesicles begins in the ***rough endoplasmic reticulum*** (***RER***), followed by passage and maturation through Golgi vesicles, as illustrated in Fig. 17.17 below.

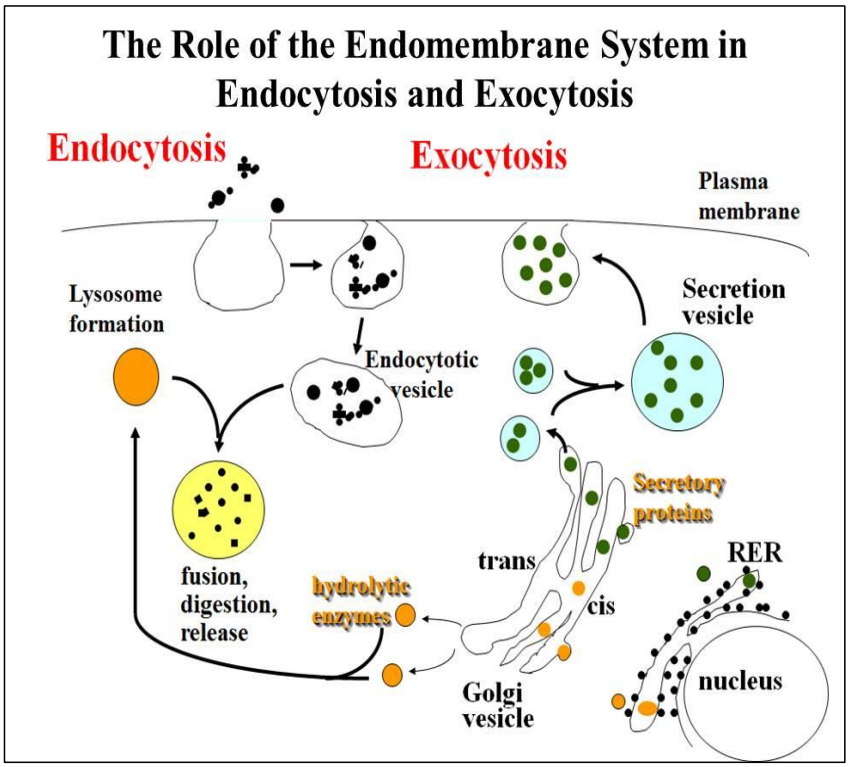


Fig. 17.17: Rough endoplasmic reticulum (RER), Golgi and other vesicles participate in the trafficking of proteins destined for secretion or vesicular packaging/storage in the cell (see text for details).

As we have seen, many secretory and membrane proteins are glycoproteins, and their glycosylation begins in the RER. Check the link below to review the process.

291 The Path to Sugar Coated Cells

A cell often produces many packaged protein at the same time. This requires sorting each protein to the right place – extracellular fluids, lysosomes, peroxisomes and other 'microbodies', and of course, membranes themselves. How do they do it? Some representative packaged proteins are listed in Table 17.1, below.

Cellular Proteins Segregated From the Cytoplasm			
Hormones	**Immune System Proteins**	**Neurotransmitters**	**Other**
insulin	**immunoglobulin G (IgG), a class of circulating antibodies)**	**acetylcholine**	**EGF (Epidermal growth factor)**
growth hormone	**IgM and other cell membrane antibodies**	**dopamine, adrenaline noradrenaline, other monoamines**	**NGF (Neural growth factor)**
follicle stimulating hormone (FSH)	**Major Histocompatibility Complex (MHC) proteins**	**serotonin**	**Fibrinogen (& other blood clotting factors)**
oxytocin		**some amino acids (glutamate, aspartate, glycine)**	**Fibronectin (and other extracellular matrix proteins**
prolactin			**Plant cell wall components**
ACTH (adrenocorticotropic hormone)			**Trypsin, pepsin, etc. (gut digestive enzymes)**
			Chloroplast and mitochondrial proteins
			Nuclear proteins

17.7 Directing the Traffic of Proteins in Cells

Each polypeptide protein translated by ribosomes from a sequence of bases in an mRNA has a specific functional location, either in the cytoplasm, on cellular membranes, inside organelles or in extracellular fluids. In this section we consider the movement and sorting of proteins from RER through vesicles of the ***endomembrane system*** as well as the transport of proteins into and out of organelles.

17.7.1 Proteins Packaged in RER are Made as Larger Precursor Proteins

All protein synthesis begins with the formation of an initiation complex and subsequent elongation cycles of peptide bond formation and carboxyl-terminal amino acid addition. But proteins to be packaged for secretion or into lysosomes, peroxisomes or other microbodies, complete translation elongation directly into the cisternae (enclosed spaces) of the RER. What was the traffic signal that led some proteins to the RER while others went elsewhere or remained in the cytoplasm?

A good model system for studying secretory protein synthesis turns out to be ***mouse myeloma cells***, cancerous lymphocytes of the immune system. The normal cells make immunoglobulin G (IgG) molecules with light and heavy chain polypeptide subunits (IgGs are circulating ***antibodies***). The myeloma cells make only IgG light chains easily isolated from cell culture medium.

In an early experiment mouse myeloma cells were cultured under different conditions and the secreted IgG light chain produced was analyzed (Fig. 17.18, below). The experiment revealed that secreted mouse light chain IgG proteins made in an in vitro translation system were in fact larger than proteins naturally secreted by the cells. Here's how the experiment was done:

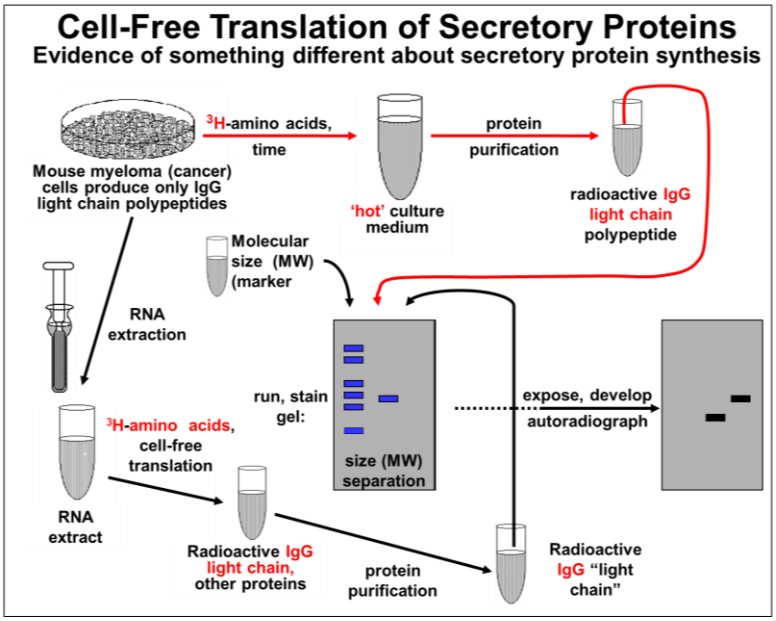


Fig. 17.18: Determining the role of RER in the synthesis of secretory and other packaged protein synthesis (see text for details).

In one part of the experiment described above, myeloma cells were grown in the presence of radioactive amino acids. The resulting radioactive IgG light chain polypeptides were isolated (follow the red arrows). In another part of the experiment, mRNA was separately extracted from another batch of the myeloma cells and added to a cell-free translation system containing radioactive amino acids. The radioactive

polypeptide synthesized *in vivo* and *in vitro* were separated on electrophoretic gels and autoradiographed (follow the blue arrows, above). From the autoradiograph (lower right of the illustration) you see that the mature, secreted polypeptides made *in vivo* had migrated faster on the gel than those translated *in vitro*. So, the cell-free translation product was indeed, larger than the *mature* secreted polypeptide. To explain these results, Gunther Blobel and colleagues extended the Signal Hypothesis, proposing that the signal was a short N-terminal ***signal peptide*** that directs a growing secretory polypeptide to the RER. They further proposed that the *signal peptide* is a temporary 'traffic' signal, removed by an RER-associated enzyme as the polypeptide crossed the RER membrane into the cisternal space.

304 Formulating the Signal Hypothesis: Early Experiments

17.7.2 Testing the Signal Hypothesis for Packaging Secreted Proteins in RER

In the test of the *Signal Hypothesis* (which won Blobel the 1999 Nobel Prize in Physiology or Medicine), isolates of RER membranes were included with mouse myeloma cell mRNA in cell-free protein synthesis systems. This time, when secreted and cell-free synthesized IgG light chain polypeptides were electrophoresed and the gel autoradiographed, both polypeptides were the same size as the mature, secreted polypeptides. Therefore as predicted, RER contains *processing* activity, i.e., a ***signal peptidase*** that removes the signal peptide! The steps of the signal hypothesis that emerged from the experiments of Blobel and his colleagues are illustrated in Fig. 17.19 (below).

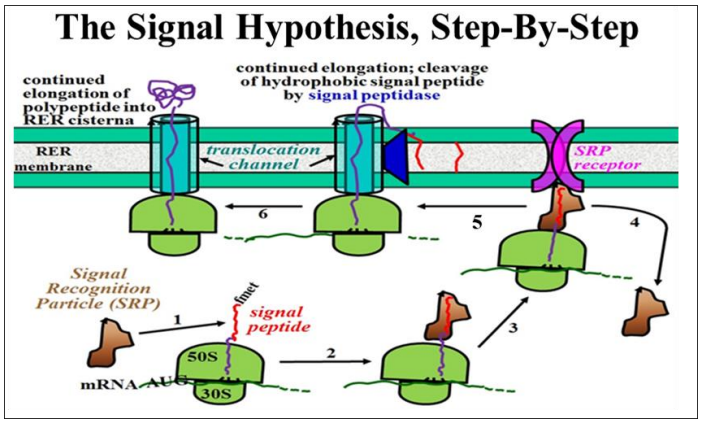


Fig. 17.19: Molecular details (steps) of the Signal Hypothesis (see text for details).

Recall that protein translation starts with assembly of an *initiation complex*, followed by polypeptide elongation. During elongation, the growing polypeptide moves through and emerges from a channel, or groove in the large ribosomal subunit. As the N-terminal ***signal sequence*** (i.e., the signal peptide) of a secretory polypeptide emerges from this groove, it interacts with the RER membrane. Beginning at the lower left in Fig. 17.19, the steps of the process are:

1. An ***SRP*** (***signal recognition particle***) binds to the hydrophobic *signal peptide*.
2. Elongation stops until the SRP-ribosome complex finds the RER membrane.
3. The ribosome-SRP complex binds to an ***SRP receptor*** on the RER membrane.
4. The SRP detaches from the growing polypeptide chain and is recycled.
5. Translation elongation resumes through a ***translocation channel***; a ***signal peptidase*** in the RER membrane catalyzes ***co-translational*** hydrolysis of the signal peptide, which remains embedded in the RER membrane.
6. Elongation continues and the growing polypeptide begins to fold in the RER.

305 Testing the Signal Hypothesis

306-2 Details of the Signal Hypothesis

Step 2 above requires that the SRP find and bind to the signal peptide before the nascent polypeptide gets too long and starts to fold into a 3D (tertiary) conformation. It turns out the ribosome itself may keep the signal peptide available by destabilizing electrostatic interactions that would otherwise lead to premature folding and an undoubtedly incorrect conformation. For more on ribosome involvement in protein folding, check out the link at Protein Folding-Destabilizing One Protein Strand at a Time.

Bacteria secrete proteins that assist in nutrient scavenging as well as cell wall synthesis and they use a secretory mechanism similar to that of eukaryotes, with obvious differences in detail. Partially elongated signal peptides guide mRNA-bound ribosomes to the cytoplasmic side of the plasma membrane, where the ribosomes bind and then pass elongating proteins through the plasma membrane into the space between the cell membrane and wall. As the protein exits the cell, a bacterial signal peptidase (***SPase***) cleaves the signal peptide.

Early on, we discovered that antibiotics stop bacterial growth either by disrupting the cell wall or otherwise killing the cells outright. We now know that some antibiotics (e.g., *arylomycins*) disrupt plasma membrane *SPase* function, preventing proteins required in the space between the cell wall and membrane from ever making it out of

the cell. Once used against ***Staphylococcus aurease***, arylomycins are no longer effective because many strains have become resistant to these antibiotics (click Bacterial Signal Peptidase and Antibiotic Resistance to read about the mechanism of *arylomycin* resistance). As you may already know, *S. aurease* is now resistant to many antibiotics, and illness from untreatable infections has its own name, **MRSA** (Methicillin-Resistant Staph Aurease - dig on your own to see more about methicillin resistance). While named for *methicillin* resistance, the MRSA acronym has also come to describe untreatable *S. aurease* infections in general.

Apparently, the basic mechanism for the secretion of proteins evolved early and is conserved in prokaryotes. We turn now to well-studied mechanisms, for packaging proteins into eukaryotic organelles and into membranes themselves.

17.8 Synthesis of *Integral* Membrane Proteins

Integral membrane proteins may span a membrane one or more times (Fig. 17.20), or may simply be anchored at one end and be embedded in the membrane.

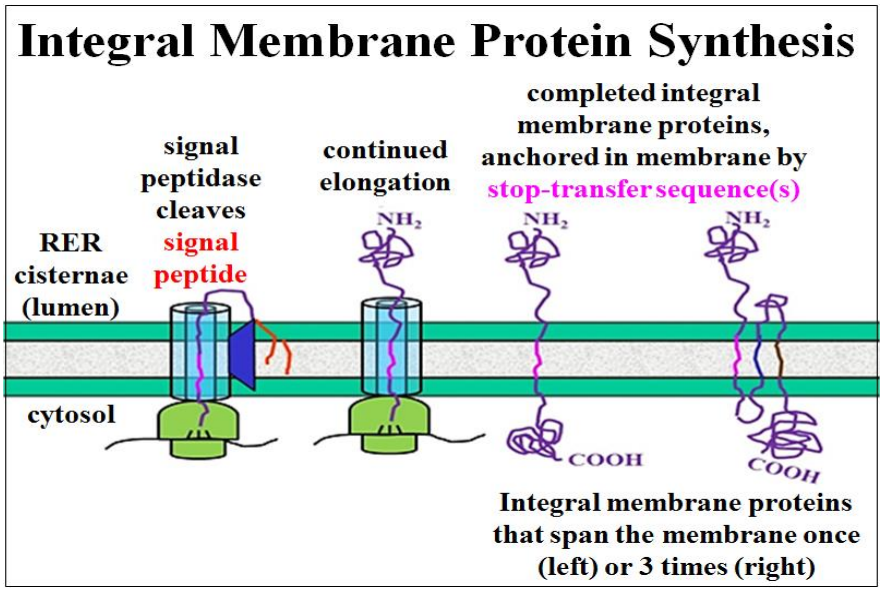


Fig. 17.20: Integral membrane-spanning proteins have one or more very hydrophobic *stop transfer* signals in addition to their signal sequence. Such membrane proteins cross the membranes one or more times during their synthesis.

N-terminal signal sequences guide ribosomes translating *integral membrane proteins* to the RER. But before such a protein can pass completely into the RER cisternae. a ***stop-transfer*** sequence (a hydrophobic domain within the polypeptide chain) traps the protein in the fatty acid interior of the membrane. Multiple stop-transfer sequences account for transmembrane proteins that span a membrane more than once.

308-2 Integral Proteins Have Stop Transfer Sequences

17.9 Moving and Sorting Proteins to Their Final Destinations

Like proteins packaged in RER, those made in the cytoplasm go to different destinations before they become functional. What's more, cells can truly multitask (unlike most of us!), handling the synthesis and distribution of many proteins At the same time! Let's begin with a look at the sorting mechanisms for proteins sequestered by the endomembrane system.

17.9.1 Traffic on the Endomembrane Highway

We have already seen that, once packaged in the RER cisternae, proteins begin post-translational modification (by e.g., 'core glycosylation'). Transport vesicles that bud off from the RER carry packaged and membrane proteins to the *cis* vesicles of the Golgi apparatus. There, vesicle fusion is mediated by the recognition of complementary integral membrane proteins embedded in the two membranes. Later, such packaged proteins are sorted to different organelles or to the plasma membrane.

Sorting starts as proteins move from the *cis* to the *trans* face of the Golgi vesicles, where specific sorting proteins associate with different packaged proteins in the trans Golgi vesicles. The packaged proteins then sort to vesicles that bud off from trans Golgi stacks. These vesicles move to their final destinations, recognizing and then fusing with appropriate membranes.

James E. Rothman, Randy W. Schekman and Thomas C. Südhof won the 2013 Nobel Prize in Physiology or Medicine for their studies of the regulation of vesicle traffic (click 2013 Nobel Prize in Physiology or Medicine for more information). Using Fig. 17.Bb above, let's follow some proteins in and on RER membranes through the cell (also animated at Events in Protein Trafficking). The different pathways of sorting packaged proteins to their cellular destinations begin in the RER and are summarized below in Fig. 17.21.

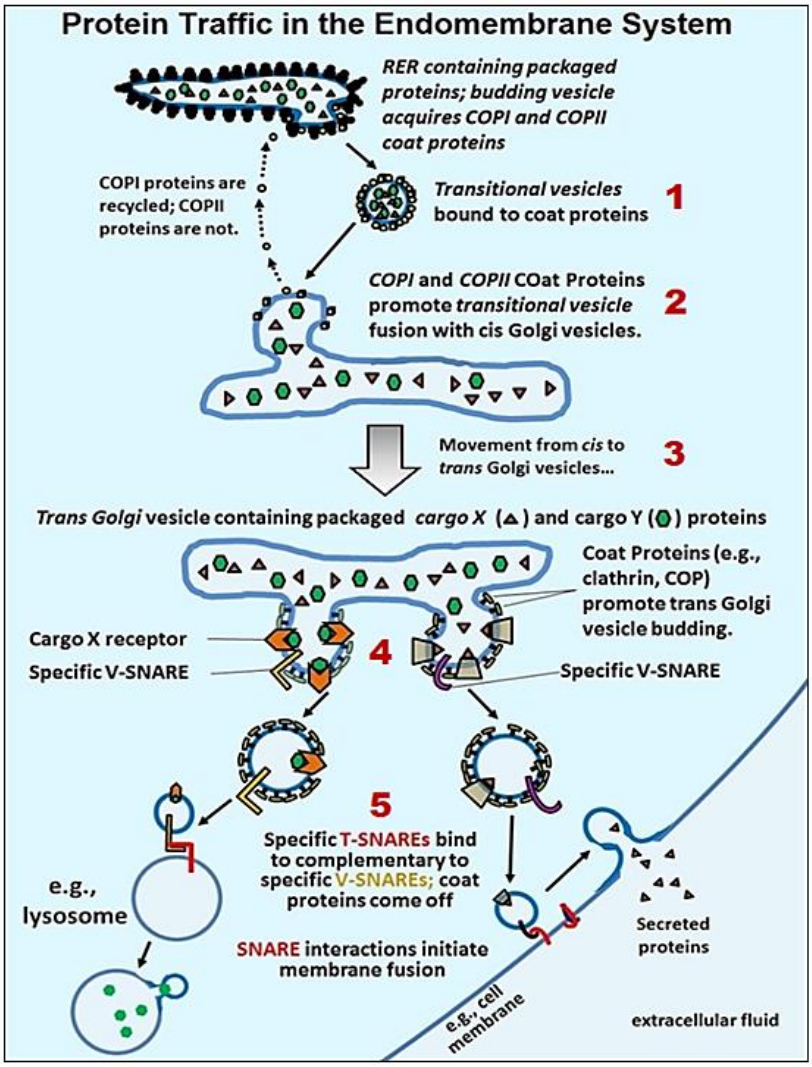


Fig. 17.21: Sorting and directing the traffic of secreted, vesicular and other proteins in cells (see text for details).

Here are the steps:

1. ***Transition vesicles*** carrying their mix of packaged proteins bud off from the RER with the help of ***COPI*** and ***COPII*** coat proteins, dissociating from ribosomes. Transition vesicles however, remain associated with the *COP* proteins.

2. These vesicles fuse with *cis* Golgi vesicles, a process also mediated by COP proteins. COPI proteins detach during or after fusion, recycling back to the RER.

3. Packaged proteins and membrane proteins are further processed as they pass through the Golgi vesicle stack, for example undergoing terminal glycosylation.

4. A key step in sorting packaged proteins occurs as vesicles bud off from the trans Golgi face with the help of ***clathrin*** and other *COP proteins* and specific ***cargo receptor*** proteins. The *cargo receptor proteins* recruit specific packaged proteins (now called ***cargo proteins***), enriching them in their nascent vesicles. ***V-SNARE*** (for vesicle-SNARE) proteins also associate with the vesicles; these will guide them to their ultimate destination.

When ***V-SNARE*** proteins on their vesicles bind to complementary **T-SNARE** (for target-SNARE) proteins on receiving membranes, the membranes fuse. Some vesicles follow a pathway to organelles, fusing with ***lysosomes*** or similar vesicles to stock them with appropriate enzymes and other protein content. Coat proteins come off the fusing vesicle and are recycled, while vesicle contents are transferred into the next vesicle. Other vesicles contain secretory proteins; these typically fuse to form larger ***secretory vesicles***. Secretion vesicles then fuse with the plasma membrane, releasing their contents to the extracellular fluid. Once again, coat proteins and clathrin dissociate from the secretory vesicle during fusion. Secretory vesicles often remain in the cytoplasm, storing their proteins until cells are signaled to release them from the cell.

Other players have been left out of this discussion, notably those that hydrolyze nucleotide triphosphates to provide the energy for protein trafficking. In addition, you may recognize molecular players such as *clathrin* also play a role receptor-mediated endocytosis. Maybe that's not a surprise! After all, endocytosis is at least partly molecular traffic in the opposite direction of vesicle formation and secretion.

17.9.2 Nuclear Protein Traffic

We saw earlier that large molecules (mRNAs, tRNAs) and even whole particles (i.e., ribosomal subunits) cross the nuclear envelope through nuclear pores. What about those large proteins headed for the nucleus? It turns out that these proteins have

nuclear localization signals rich in positively charged amino acids (lysine, proline) that enable binding to the negatively charged domain of a ***nuclear transport receptor*** protein in the cytosol (Fig. 17.22, below).

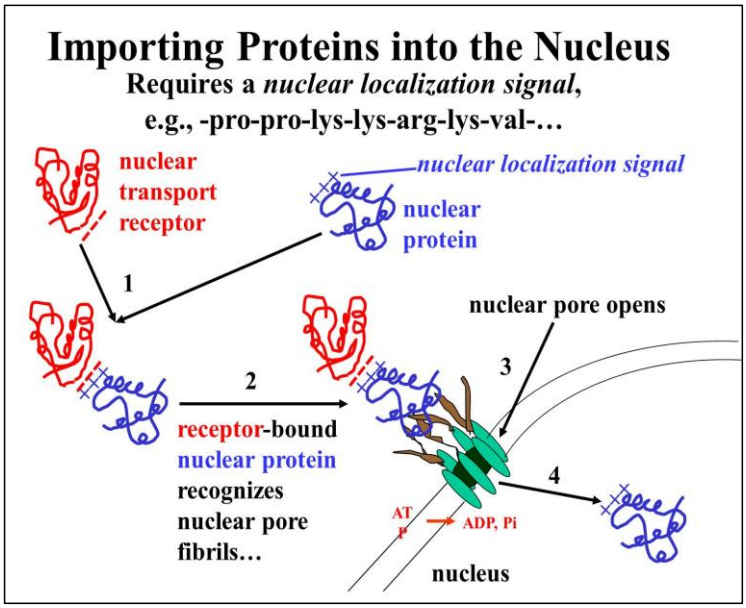


Fig. 17.22: Nuclear proteins made in the cytoplasm contain a positively *charged nuclear localization signal*. This signal binds to the electronegative region of a *nuclear transport receptor* that then binds nuclear pore fibrils, guiding the protein into the nucleus.

As the complex of the two proteins approach a *nuclear pore*, it interacts with ***nuclear pore fibrils***, causing the pore to open. The two bound proteins then cross the double membrane of the nuclear envelope. Once the proteins get in to the nucleus, they begin to accumulate against a concentration gradient. This active transport process requires free energy of course, in this case supplied by ATP hydrolysis as the nuclear proteins cross into the nucleus.

17.9.3 Mitochondrial Protein Traffic

Recall that mitochondria contain their own genome and translational machinery. Thus they transcribe RNAs and translate proteins of their own. However, genes in the nucleus encode many of the proteins found in mitochondria. Fig. 17.23 (below) illustrates how mitochondria import these proteins.

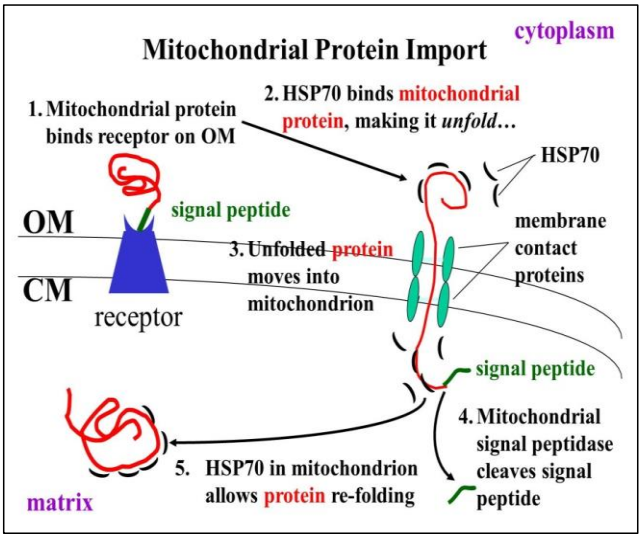


Fig. 17.23: Nuclear proteins destined for mitochondria are synthesized with an N-terminal *mitochondrial signal sequence* that is removed by a *mitochondrial signal peptidase*. Since polysomes do not attach to mitochondria, a different mechanism engages the N-terminal signal with membrane proteins required for transfer (see text and figure for details).

Unlike the co-translational packaging of proteins by the RER, mitochondrial protein transfer is post-translational. This means that mitochondrial proteins formed in the cytoplasm have already folded into a tertiary structure. However, the folded protein exposes an N-terminal ***signal peptide*** on its surface that recognizes and binds to a ***receptor protein*** at the outer mitochondrial membrane. Like pores in the nuclear envelope, mitochondrial receptors, or ***membrane contact proteins***, span *both* the mitochondrial *outer membrane* (OM) and *cristal membrane* (CM). The *membrane contact proteins* act as channels, or pores, through which the mitochondrial protein will cross into the mitochondrial matrix. But there is a problem: the folded protein *cannot* cross the membrane by itself, precisely *because* it is folded! Evolution dealt with this problem by enlisting proteins to assist other, already synthesized and folded proteins to cross membranes!

The entry of a completed mitochondrial protein now sitting in the cytoplasm requires a so-called ***chaperone*** protein, in this case the ***HSP70*** (*heat-shock 70*). *HSP70* controls *unfolding* of the mitochondrial protein as it passes into the matrix. Upon removal of the signal peptide by a mitochondrial ***signal peptidase***, another HSP70 molecule resident in the mitochondrion facilitates *refolding* of the protein into a

biologically active shape. Recall that HSPs were initially discovered as 70 Kd proteins that accumulated in in heat stressed organisms.

309 Protein Traffic to Nuclei and Mitochondria

17.10 How Cells are Held Together and How They Communicate

Proteins and glycoproteins on cell surfaces play a major role in how cells interact with their surroundings and with other cells. We'll look first at some of the proteins in the ***glycocalyx*** of adjacent cells that interact to form different kinds of cell-cell junctions. Then we'll see how some of these proteins interact with extracellular proteins and carbohydrates to form the ***extracellular matrix*** (***ECM***).

Still other membrane proteins are part of receptor systems that bind hormones and other signaling molecules at the cell surface, conveying information into the cell by ***signal transduction***. We shall return to mechanisms of signal transduction shortly.

17.10.1 Cell Junctions

Cell junctions serve different functions in cells and tissues. In healthy cells they serve to bind cells tightly, to give tissues structural integrity and to allow cells in contact with one another to pass chemical information directly between them. As you read about these cell junctions, refer to the illustrations and micrographs in Fig. 17.24 (below).

1. ***Tight Junctions*** (*zonula occludens*) are typical in sheets of epithelial cells that line the *lumens* of organs (e.g., intestines, lungs, etc.). *Zonula* refers to the fact that these structures form a band encircling an entire cell, attaching it to all surrounding cells. *Occludens* refers to the 'water-tight' seal or *occluding barrier* of tight junctions that stops extracellular fluids from crossing to the other side of a sheet of cells by passing between cells. ***Tight junction membrane proteins*** (***TJMP***s) create this waterproof barrier.

2. ***Desmosomes*** (*adherens junctions*) essentially glue (adhere) cells together, giving tissues their strength. ***Belt desmosomes*** (*zonula adherens*) surround entire cells, strongly binding them to adjacent cells. Spot desmosomes (*macula adherens*) act like rivets, attaching cells at *spots*. In both cases, ***cadherin proteins*** cross cell membranes from intracellular ***plaque*** proteins, spanning the intercellular space to link adjacent cell membranes together. Plaques are in turn, connected to intermediate filaments (keratin) of the cytoskeleton, further strengthening intercellular attachments and thus, the tissue cell layer.

3. ***Gap junctions*** enable chemical communication between cells. ***Connexon*** structures made of ***connexin*** proteins are pores that open to allow the flow of ions and small molecules between cells. Communication by ion or molecular flow is quite rapid, ensuring that all cells in a sheet or other tissue in one metabolic state can respond to each other and switch to another state simultaneously. In plants, we have seen the plasmodesmata that perform functions similar to the gap junctions of animal cells.

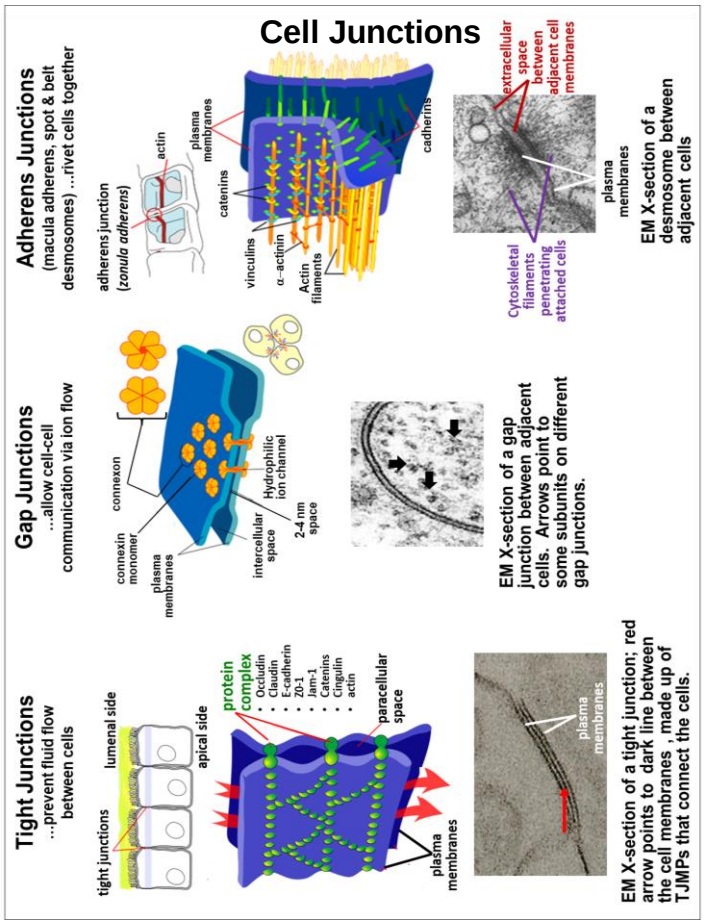


Fig. 17.24: *Tight*, *Gap* and **Adherens** junctions between animal cells involve different membrane proteins (see text for details).

[310-2 Cell Junction Structure and Function]

Many glycoproteins in the glycocalyx are party of cell junctions. Generally, proteins that interact to bind cells together are called ***ICAMs*** (**I**ntercellular **C**ell **A**dhesion **M**olecules). Several of these are described below:

- ***Selectins*** are one kind of *ICAM*. During blood clotting, *selectins* on one platelet recognize and bind to specific receptors on other platelets, contributing to the clot.
- ***NCAMs*** are another kind of ICAM, ones with sugary immunoglobulin domains that interact specifically to enable neural connections.
- We've already seen the calcium-dependent ***cadherins*** involved in forming *adherens junctions* (desmosomes). These are essentially the 'glue' that binds cells together to form strong cohesive tissues and sheets of cells. Cell-Cell binding begins with recognition and connection via the glycocalyx and extracellular matrix.

Fig. 17.25 illustrates several examples of membrane proteins that enable cell-cell recognition and adhesion.

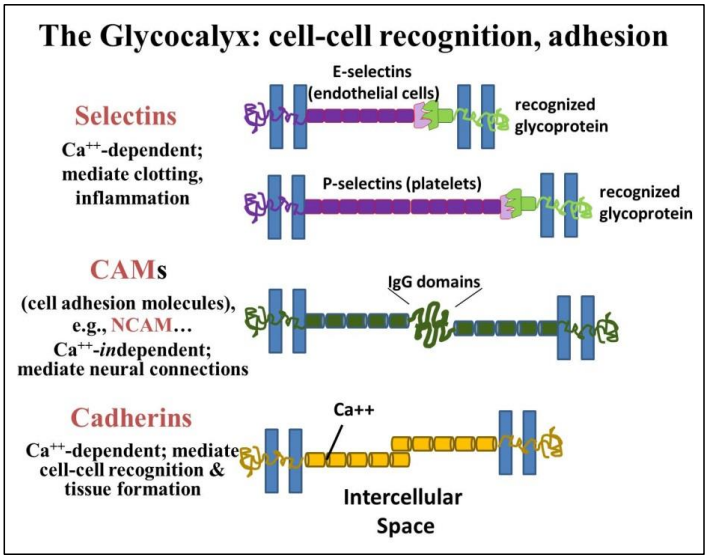


Fig. 17.25: Glycocalyx protein interactions; roles in cell-cell recognition and attachment.

311-2 Glycocalyx-Sugars Covalently link to Plasma Membrane Proteins

312 Cell Adhesion Molecule Functions in the Glycocalyx

17.10.2 Microvesicles and Exosomes

Of the several ways cells take up material from their environment, we saw that *Pinocytosis*, or bulk transport seems to be a random process in which tiny bits of plasma membrane engulf small amounts of extracellular fluids along with their ionic, particle and other solute contents. Pinocytotic vesicles range from 0.5 to 5 mm in diameter. On the other hand, ***exosomes*** and ***microvesicles*** are circular bits of plasma membrane and endosome (respectively) that are shed by cells into the extracellular space. Known by several names, these extracellular vesicles (***EVs***) were first reported in the 1980s as small vesicles released by reticulocytes. At around 1000nm (1 mm), *microvesicles* are similar in size to pinocytotic vesicles. *Exosomes* are about a tenth the size of microvesicles, ranging in size from 40-100nm in diameter (see Fig.17.26 below).

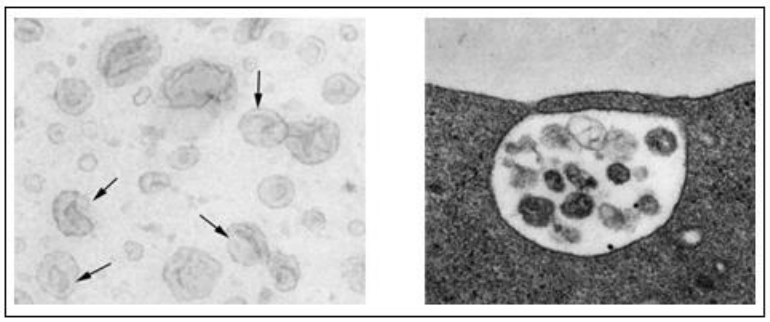


Fig. 17.26: LEFT: Transmission electron micrograph of a Melanoma cell exosomes (bar, 100nm). Some of the larger vesicles show an irregular, cup-like shape (arrows). RIGHT: Transmission electron micrograph of a reticulocyte endosome containing exosomes about to fuse with the plasma membrane.

There is much evidence that microvesicles and exosomes are not artifacts. For example:

- Microvesicles such as those from melanoma cells (Fig. 17.26, left panel) may be normally released, suggesting that they are not cellular waste products.
- reticulocyte endosomal vesicles were caught in the act of fusing with the cell membrane, releasing exosomes with their contents (Fig. 17.26, right panel).
- microvesicles released by *dendritic cells* are able to stimulate *T cells*, both of which are cells of the immune system.

We can conclude that microvesicles are physiologically significant structures. We know that cells talk to each other by releasing chemicals that can act over short or long distances. Familiar examples include information transfer by hormones of the

endocrine system (endocrine glands), chemicals released into the intercellular space and neurotransmitters of the nervous system. Microvesicle activity may be part of another intercellular communication pathway, and they are clearly part of normal reticulocyte maturation to erythrocytes. Perhaps exosomes are yet another unique mechanism of intercellular communication. For a review, see Exosomes and Intercellular Communication.

17.10.3 Cancer and Cell Junctions

During embryogenesis, cells migrate from a point of origin by attaching to and moving along an ***extracellular matrix*** (***ECM***), which acts as a path to the cell's final destination. This ECM (or basal lamina) is made up of secretions from other cells…, or from the migrating cells themselves! One major secretion is ***fibronectin***. One of its functions is to bind to integral membrane proteins called ***integrins***, attaching the cells to the ECM. During development, integrins respond to fibronectin by signaling cell and tissue differentiation, complete with the formation of appropriate cell junctions. An orderly sequence of gene expression and membrane protein syntheses enables developing cells to recognize each other as different or the same (summarized in the drawing in Fig. 17.27).

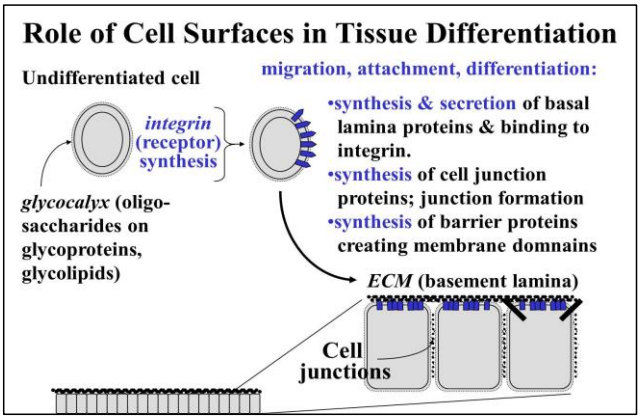


Fig. 17.27: *Migration* and *attachment* of cells during development requires the synthesis of membrane proteins that recognize and bind proteins on the membranes and glycocalyx of other cells, forming cell junctions that enable *tissue formation.*

An early difference between eukaryotic normal and cancer cells is how they grow in culture. Normal cells settle to the bottom of a culture dish when placed in growth medium. Then they grow and divide, increasing in number until they reach ***confluence***, when a single layer of cells completely covers the bottom of the dish.

The cells in this monolayer seem to 'know' to stop dividing, as if they had completed formation of a tissue, e.g., a layer of epithelial cells. This phenomenon, originally called ***contact inhibition***, implies that cells let each other know when they have finished forming a tissue and can stop cycling and dividing. In contrast, cancer cells do not stop dividing at confluence. Instead, they continue to grow and divide, piling up in multiple layers.

Among other deficiencies, cancer cells do not form *gap junctions* and typically have fewer *cadherens* and *integrins* in their membranes. Thus, cancer cells cannot inform each other of when they reach confluence. Neither can they form firm *adherens junctions*. *In vivo*, a paucity of integrins would inhibit cancer cells from binding and responding to *fibronectin*. Therefore, they also have difficulty attaching firmly to an extracellular matrix, which may explain why many cancers ***metastasize*** or spread from their original site of formation. These differences in growth in culture between normal and cancer cells are shown in Fig. 17.28.

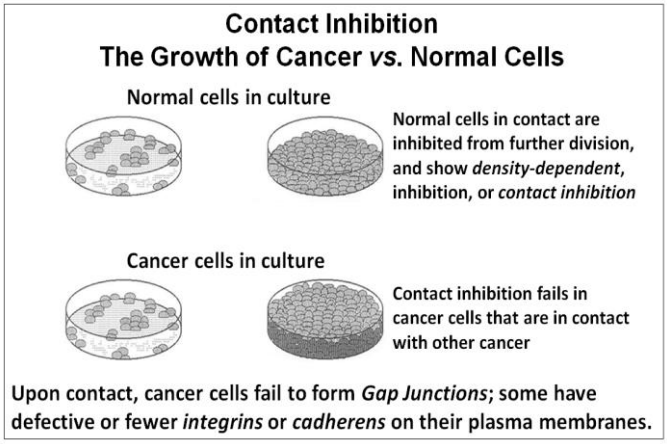


Fig. 17.28: Density-dependent inhibition (aka. Contact Inhibition) occurs when cells multiplying and spreading on a surface cease dividing when there is no more room on the surface. Cancer cells have lost the property of contact inhibition and keep growing over one another in layers. Loss of contact inhibition is correlated with an absence of gap junctions in cancer cells.

313 Formation of a Glycocalyx, Normal Development and Cancer

314-2 Role of the Extracellular Matrix in Cell Migration & Development

17.11 Signal Transduction

When hydrophobic chemical ***effector*** molecules such as steroid hormones reach a target cell, they can cross the hydrophobic membrane and bind to an intracellular receptor to initiate a response. When large *effector* molecules (e.g., protein hormones) or highly polar hormones (e.g., adrenalin) reach a target cell, they can't cross the cell membrane. Instead, they bind to transmembrane protein receptors on cell surfaces. A conformational change initiated on the extracellular domain of the membrane receptor protein induces further allosteric change on its cytoplasmic domain. A sequential series of molecular events then converts information delivered by the external effector into intracellular information, a process called **signal transduction**. A general outline of signal transduction events is illustrated in Fig. 17.29.

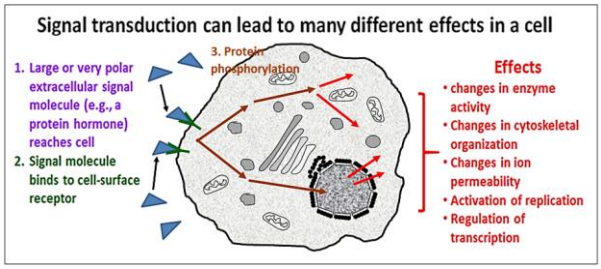


Fig. 17.29: Signal transduction by *effectors* (e.g., hormones) can lead to many different effects in the cytoplasm as well as in nuclei.

Many effects of signal transduction are mediated by a sequence, or *cascade* of protein phosphorylations catalyzed by *protein kinases* inside the cell. Here we will consider ***G Protein-linked*** and ***enzyme-linked receptors***.

315-2 Introduction to Signal Transduction

17.11.1 G-Protein Mediated Signal Transduction by PKA (Protein Kinase A)

GTP-binding proteins (***G-Proteins***) transduce extracellular signals by inducing the synthesis of ***second messenger*** molecules in the cells. When hormones or other effector (signal) molecules bind to a membrane receptor, an allosteric change on the *extracellular domain* of the receptor is transmitted to the cytoplasmic domain of the receptor, increasing its affinity for trimeric G-proteins embedded in the cytoplasmic surface of responsive cell membranes. G- protein *trimers* consist of α, β and γ subunits. Fig. 17.30 (below) illustrates seven steps of G-protein-mediated signal transduction.

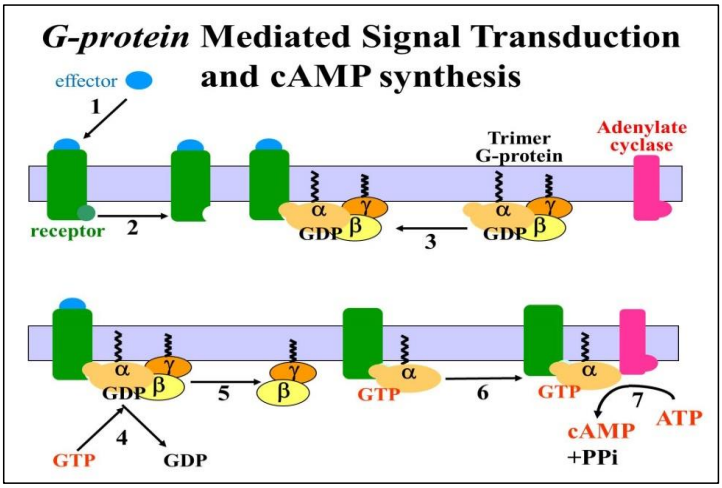


Fig. 17.30: G-proteins cycle between bound GTP and bound GDP. They are intermediates in signal transduction between an active, effector-bound membrane receptor and adenylate cyclase (see text for details of the different steps).

The receptor changes shape upon binding its effector signal molecule (steps 1 and 2). In this altered conformation, the receptor recognizes and binds to the G-protein trimer (step 3). Upon binding of the trimer to the receptor, GTP displaces GDP on the ***α subunit*** of the G-protein (step 4). After a conformational change in the ***α subunit***, it dissociates from the β and γ subunits (step 5). In this illustration, the GTP-α subunit can now bind to a transmembrane ***adenylate cyclase*** enzyme (step 6). Finally, the initial extracellular chemical signal is ***transduced*** to an intracellular response involving a second messenger molecule (step 7). In this case, the second messenger is ***cAMP***.

316-2 G-Protein Signal Transduction

The well-known ***fight-or-flight*** response to adrenalin in liver cells of higher animals is a good example of a cAMP-mediated cellular response. Once formed, cAMP binds to and activates ***protein kinase A*** (***PKA***), setting off a **phosphorylation cascade** leading to a physiological response. In the cascade of phosphorylation reactions, activation of just a few enzyme molecules in the cell results in the activation of many more enzymes referred to as an ***amplification cascade***. The result can be a powerful and almost immediate response. Some details of a G-protein mediated signal amplification cascade are detailed in Fig. 17.31.

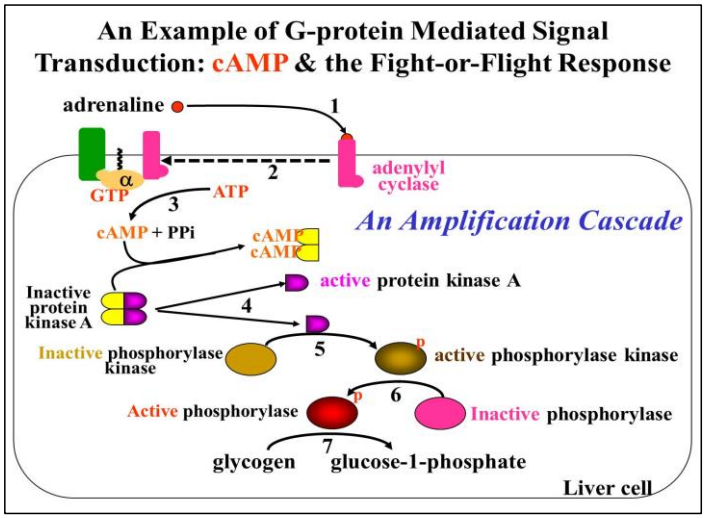


Fig. 17.31: The *fight-or-flight response* to adrenalin (*adrenalin rush*): *cAMP* activates *protein kinase A* and a *phosphorylation cascade* leads to the release of glucose into the circulation (see text for details). cAMP is a second messenger of signal transduction for many different cellular responses.

After activation of adenylate cyclase (steps 1 and 2 in the drawing), cAMP is synthesized. Two cAMPs binds to each of two of the four subunits of an ***inactive PKA*** (step 3). A conformational change dissociates the tetramer into two cAMP-bound inert subunits and two ***active PKA*** subunits (step 4). Each *active PKA* enzyme catalyzes the phosphorylation and activation of an enzyme called ***phosphorylase kinase*** (step 5). In step 6, phosphorylase kinase catalyzes ***glycogen phosphorylase*** phosphorylation. Finally, in the last step of the *phosphorylation cascade*, a now active glycogen phosphorylase catalyzes the hydrolysis glycogen to glucose-1-phosphate (step 7). This results in a rapid retrieval free glucose from liver cells into the circulation. See again how this works by reviewing the conversion of glucose-1 phosphate (G-1-P) to G-6-P in glycolysis and its fate in gluconeogenesis. Of course, the increase in circulating glucose provides the energy for the *fight-or-flight* decision.

317 G-Protein Activation of Protein Kinase A; a *Fight-or-Flight* Response

In addition to activating enzymes that break down glycogen, cAMP-activated PKA mediates cellular responses to different effectors resulting in a phosphorylation cascade leading to

- activation of enzymes catalyzing glycogen synthesis.
- activation of *lipases* that hydrolyze fatty acids from triglycerides.
- microtubule assembly.
- microtubule disassembly.
- mitogenic effects (activation of enzymes of replication).
- activation of transcription factors increases or decreases gene expression.

Of course, when a cellular response is no longer needed by the organism, it must stop producing the signal molecules (hormones or other effectors). As their levels drop, effector molecules dissociate from their receptors and the response stops. This is all possible because binding of signals to their receptors is freely reversible!

17.11.2 Signal Transduction using PKC (Protein Kinase C)

Another G-protein-mediated signaling pathway activates ***protein kinase C*** (***PKC***). The role of G-proteins is similar for ***PKA*** and ***PKC signal transduction***. but *PKC* activation does not involve cAMP and requires two intervening cellular responses to an effector. The net result is to generate different second messengers. These in turn activate different ***phosphorylation cascades***. Like *PKA*, *PKC*-mediated signal transduction also ***amplifies*** the cell's first molecular response to the effector. The events leading to the activation of ***PKC*** are illustrated in Fig. 17.32 (below).

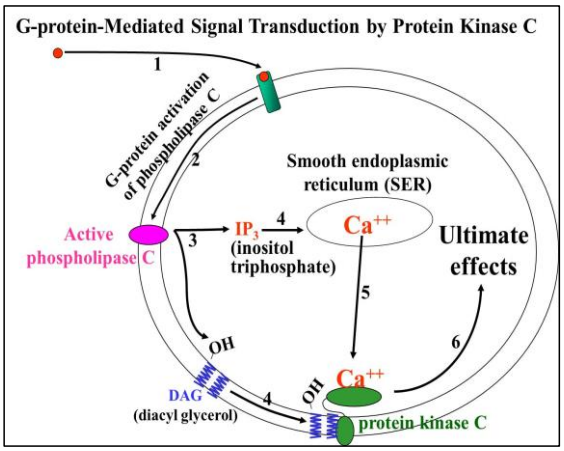


Fig. 17.32 G-proteins also mediate signal transduction through different membrane receptors and different enzymes, as well as *protein kinase C*. These interactions generate different 2nd messenger molecules that lead to a phosphorylation cascade and many different responses in different target cells (see text for details of the steps).

Here are details of the steps leading to ***PKC*** activation. In *step 1*, an effector signal molecule (red) binds to its receptor (green). In *step 2*, G-proteins activation (details not shown) activate an integral membrane ***phospholipase C*** enzyme. In *step 3*, active *Phospholipase C* catalyzes formation of cytosolic ***inositol triphosphate*** (***IP_3***) and membrane bound ***diacyl glycerol*** (**DAG**), two of those other intracellular second messenger molecules. *In step 4*, IP_3 interacts with receptors on smooth endoplasmic reticulum, causing the release of sequestered ***Ca^{++}*** ions into the cytoplasm. Finally, in step 5, Ca^{++} ions together with *DAG* activate ***Protein Kinase C*** (**PKC**) which then initiates a phosphorylation amplification cascade leading to cell-specific responses.

318-2 G-Protein Activation of Protein Kinase C and Phospholipase C

Protein Kinase C mediated effects include:

- Neurotransmitter release.
- Hormone (growth hormone, leutinizing hormone, testosterone) secretion leading to cell growth, division and differentiation.
- Glycogen hydrolysis, fat synthesis.

Additional independent *phospholipase C* effects include:

- Liver glycogen breakdown.
- Pancreatic amylase secretion.
- Platelet aggregation

PKA and PKC are both ***serine-threonine kinases*** that place phosphates on serine or threonine in target polypeptides. Let's consider tyrosine kinases next.

17.11.3 Receptor Tyrosine Kinase-Mediated Signal Transduction

The intracellular activity of ***Tyrosine Kinase receptors*** is in the cytoplasmic domain of the receptor itself. When bound to its effector, receptor-kinases catalyze the phosphorylation of specific tyrosine amino acids in target proteins. While studying the action of ***nerve growth factor*** (***NGF***) and ***epidermal growth factor*** (***EGF***) in stimulating growth and differentiation of nerve and skin, Stanley Cohen and Rita Levi-Montalcini discovered the ***EGF receptor***, the first *enzyme-linked tyrosine kinase*, and won the 1986 Nobel Prize in Physiology or Medicine for their discovery! Watch the animation of receptor kinase signal transduction at the link below (a description is provided in the next few paragraphs).

319 Receptor Kinase Signal Transduction

Monomer membrane receptor kinases dimerize when they bind effector ligands, at which point sulfhydryl group-containing ***SH_2 proteins*** bind to each monomer. This activates the kinase domain of the receptor. After multiple cross-phosphorylations of the receptor monomers, the SH_2 proteins fall away allowing the receptors to interact with other cytoplasmic proteins to continue the response pathway. The characteristic response to EGF and NGF signaling is cellular proliferation. Not surprisingly, the mutations correlated with cancer cells often lie in signaling pathways leading to cell proliferation (growth and division). Cancer-causing genes, or ***oncogenes***, were first discovered in viruses, but as humans, we own our own *oncogenes*! For being the first to show that eukaryotic cells were actually the origin of a chicken retrovirus (the Rous Sarcoma Virus), J. Michael Bishop and Harold Varmus earned the 1964 Nobel Prize in Physiology or Medicine. O*ncogenes* turn out to be mutations of animal genes for proteins involved in mitogenic signal transduction pathways. Under normal circumstances, mitogenic chemical signals bind to their receptors and induce target cells to begin dividing. MAP kinase phosphorylates *transcription factors* and other nuclear proteins affecting gene activity leading to cell proliferation differentiation, as shown in Fig. 17.33.

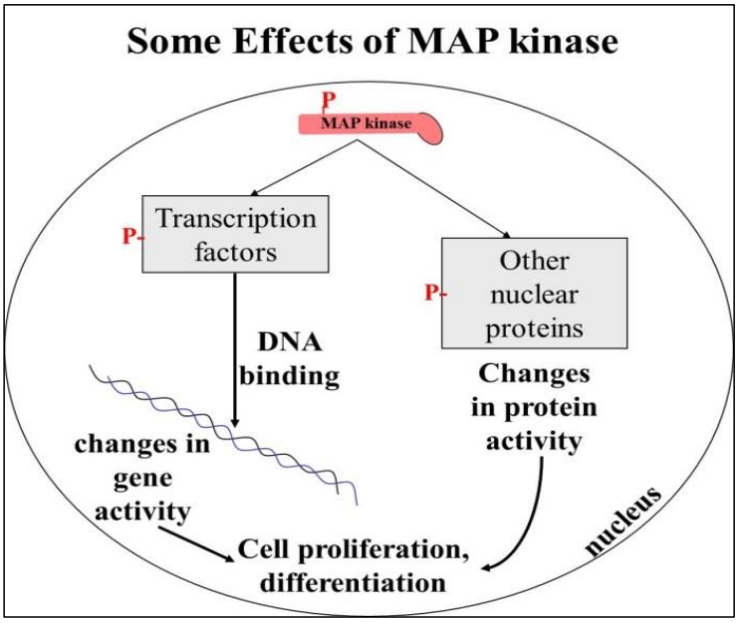


Fig. 17.33: The effects of *MAP kinase* include phosphorylation and activation of DNA-binding transcription factors and other nuclear proteins that lead to cell proliferation.

The ***Ras*** protein-mediated activation of a phosphorylation cascade leading to the **MAP *(mitogen-activated protein)* kinase** is an example of such a signal transduction pathway. This pathway plays a central role in many receptor kinase signaling pathways. The *Ras* gene was one of those originally discovered as an oncogene whose mutation leads to uncontrolled cell division, i.e., cancer. Ras gene/protein activity may in fact be responsible for up to 30% of all cancers!


320-2 The RAS Oncogene-its Normal Mitogenic Effects and Cancer 

17.12 Signal Transduction in Evolution

We saw that signal transduction typically takes a few signal molecules interacting with a few cell surface receptors to amplify a response in a cascade of enzyme-catalyzed reactions (typically phosphorylations) that activate or inactivate target proteins. Amplification cascades can take a single effector-receptor interaction and magnify its effect in the cell by orders of magnitude, making the signaling systems rapid and highly efficient. The range of cellular and systemic (organismic) responses to the same chemical signal is broad and complex. Different cell types can have receptors for the same effector but may respond differently. For example, adrenalin targets cells of the liver and blood vessels among others, with different effects in each. As it happens, adrenalin is also a neurotransmitter. Apparently, as organisms evolved, they responded to environmental imperatives, adapting by co-opting already existing signaling systems in the service of new pathways. Just as the same signal transduction event can lead to different pathways of response in different cells, evolution has allowed different signal transduction pathways to engage in ***crosstalk***. This occurs when two different signal transduction pathways intersect in the same cells. In one example, the cAMP produced at the front end of the PKA signaling pathway can activate (or under the right circumstances, inhibit) enzymes in the MAP kinase pathway. These effects result in changes in the levels of active or inactive transcription factors and can therefore modulate the expression of a gene using two (or more) signals. We are only beginning to understand what looks less like a linear pathway and more like a web of signal transduction.

Some iText & VOP Key Words and Terms

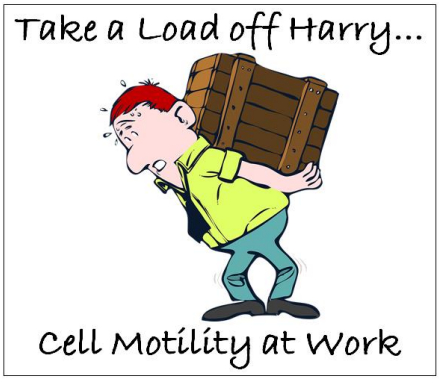
action potential	**fight-or-flight**	**peroxisomes**
active transport	**flaccid**	**phagocytosis**
adaptin	**free energy**	**phospholipase C**
adenylate cyclase	**G protein subunits**	**phosphorylase kinase**
adherens junctions	**gap junctions**	**pinocytosis**

adrenaline	gluconeogenesis	PKA
allosteric change regulates transport	GLUT1	PKC
antiport	glycolysis	plasmodesmata
aquaporins	*good* cholesterol	plasmolysis
bad cholesterol	G-Protein-linked receptors	poikilothermic organisms
basal lamina	Heat shock protein	potential difference
belt desmosomes	HSP70 protein	Protein kinase A
Ca++ ions	hydrophilic corridor	protein kinase C
cadherin	hypertonic	protein packaging
cargo receptor	hypotonic	protein phosphorylation
carrier proteins	IgG light chain	proton gate
cell adhesion molecules	inositol triphosphate	proton pump
cell-cell attachment	integrin	receptor-mediated endocytosis
cell-cell recognition	ion channels	RER membrane
cell-free translation	ion flow	resting potential
channel proteins	ion pumps	secondary active transporters
chaperone proteins	IP_3	serine-threonine kinases
cholesterol effects in membranes	isotonic	signal peptide
clathrin	LDL (low density lipoprotein)	signal recognition particle
coated pits	ligand (chemically) gated channels	signal sequence
coated vesicle	lysosome	signal transduction
connexins	MAP kinase	Smooth endoplasmic reticulum
contact inhibition	mechanically gated channels	sodium-potassium pump
contractile vacuole	membrane depolarization	solute concentration gradients
COP	membrane hyperpolarization	solute transport
cotransport	membrane invagination	sorting vesicle
coupled transport	membrane potential	spot desmosomes
cytoskeleton	microbodies	stop-transfer sequence
DAG	mitochondrial membrane contact proteins	symport

diffusion kinetics	**mitogenic effects**	**tight junction membrane proteins**
early endosome	**nerve growth factor**	**tight junctions**
ECM	**neurotransmitters**	**TJMPs**
effector molecules	**NGF**	**tonoplast**
EGF	**nuclear envelope**	**T-SNARE**
endocytosis	**nuclear pore fibrils**	**turgid**
endomembrane system	**nuclear transport receptor**	**turgor pressure**

Chapter 18: The Cytoskeleton & Cell Motility

Microfilaments, intermediate filaments and microtubules – roles in cell structure, secretion, cell migration, organelle and muscle movements



18.1 Introduction

The cell as it appears in a microscope was long thought to be a bag of liquid surrounded by a membrane. The electron microscope revealed a ***cytoskeleton*** composed of thin and thick rods, tubes and filaments that organize and maintain cell shape and that are responsible for cell motility. We will see that intracellular structures and organelles are enmeshed in these ***microfilaments***, ***intermediate filaments*** and ***microtubules***. To start this chapter, we revisit these structures and more closely examine how they work to support cell shape, motility and overall organization. Remember that cell motility includes the movement of cells and organisms, as well as the movements of organelles (e.g., vesicles) and other structures inside the cell. Of course, these movements are not random…, and they require chemical energy. After a close look at movements in and of cells, we'll look at the interaction of ***actin*** and ***myosin*** in ***skeletal muscle contraction***. We'll look a famous ***paradox*** arising from early studies showing that that ATP was required for muscle contraction *but also* for relaxation. Then we look at experiments that resolve the paradox. Animals control skeletal muscle contraction, but some muscles contract rhythmically or with little or no control on the part of the animal - think cardiac muscles of the heart, or smooth muscles like those in the digestive and circulatory systems. We will focus here on the regulation of skeletal muscle contraction by calcium ions and regulatory proteins in the response of so-called voluntary (i.e., skeletal) muscles to neural commands. Finally, we'll look at skeletal muscle ***elasticity*** and how it contributes to their function.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast roles of cytoskeletal structures in different kinds of *cell motility*.
2. distinguish between the roles of microfilaments, microtubules and intermediate filaments in the *maintenance and alteration of cell shape* and structure.
3. suggest how ciliary and spindle fiber microtubules can maintain their length.
4. explain how spindle fiber microtubules can change their length.
5. propose an experiment to show which part of a *motor protein* has *ATPase activity*.
6. Describe the key experiments that led to the actin-myosin *contraction paradox.*
7. outline the steps of the *micro-contraction cycle* involving myosin and actin.
8. compare and contrast muscle and flagellar structure and function.
9. explain why *smooth muscles* do not show striations in the light microscope.
10. outline the structure of a skeletal muscle, from a whole muscle down to a sarcomere.
11. propose alternate hypotheses to explain *hereditary muscle weakness* involving specific proteins/genes and suggest how you might test for one of them in a patient with muscle weakness symptoms.

18.2 Overview of Cytoskeletal Filaments and Tubules

In a light microscope, typical eukaryotic cells look like a membrane-bound sac of cytoplasm containing a nucleus and assorted organelles. But by the late 19th century, microscopists had described the appearance of fibers that accompanied a dramatic change the structure of dividing cells. No doubt you recognize this as ***mitosis*** in which duplicated chromosomes (***chromatids***) materialize just as the nuclear membrane dissolves and ***spindle fibers*** form. Over time the fibers seem to pull the chromatids apart to opposite poles of the cell. Spindle fibers turn out to be bundles of ***microtubules***, each of which is a polymer of ***tubulin*** proteins. Let's look again at a fluorescence micrograph of a mitosing ***metaphase*** cell (Fig. 18.1); most of the cell other than what is fluorescing is not visible in the micrograph.

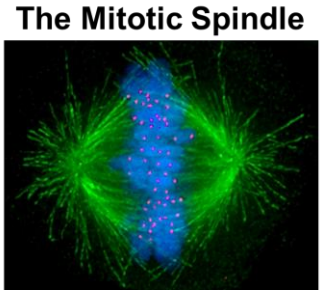


Fig. 18.1: Fluorescence micrograph of the mitotic spindle treated with antibodies to chromosomal proteins (blue) and spindle fiber proteins (green).

To get this image, *antibodies* were made against purified microtubule, kinetochore and chromosomal proteins (or DNA), and then linked to different ***fluorophores*** (organic molecular fluorescent tags). When the tagged antibodies were added to dividing cells in metaphase, they bound to their respective fibers. In a fluorescence microscope, the *fluorophores* emit different colors of visible light. Microtubules are green, metaphase chromosomes are blue, and kinetochores are red in the micrograph.

Both mitosis and meiosis are very visible examples of movements *within* cells. As for movement in whole organisms, early-to-mid 20th century studies asked what the striations (stripes) seen in skeletal muscle by light microscopy might have to do with muscle contraction. The striations turned out to be composed of an isolable protein complex that investigators called ***actomyosin*** (*acto* for active; *myosin* for muscle). Electron microscopy later revealed that actomyosin (or ***actinomyosin***) is composed of thin filaments (***actin***) and thick filaments (***myosin***) that slide past one another during muscle contraction.

Electron microscopy also hinted at a more complex cytoplasmic structure of cells in general. The ***cytoskeleton*** consists of fine rods and tubes in more or less organized states that permeate the cell. As noted, the most abundant of these are ***microfilaments***, ***microtubules*** and ***intermediate filaments***. Though myosin is less abundant, it is nonetheless present in non-muscle cells. Microtubules account for chromosome movements of mitosis and meiosis and together with microfilaments (i.e., actin), they enable organelle movement inside cells (you may have seen ***cytoplasmic streaming*** of *Elodea* chloroplasts in a biology lab exercise). Microtubules also underlie the movements of the ***cilia*** and ***flagella*** that power the movement of whole cells like paramecium, amoeba, phagocytes, etc. Actin and myosin enable muscle contraction and thus, higher animal movement. Finally, the cytoskeleton is a dynamic structure. Its fibers not only account for the movements of cell division, but they also give cells their ***shape*** and ***mechanical strength***. All of the fibers can disassemble, reassemble and rearrange, allowing cells to change shape. These changes range from the *pseudopods* extended by amoeboid cells to the spindle fibers that stretch cells in mitosis and meiosis to constriction of a dividing cell that eventually pinches off daughter cells…, and more! In this chapter we look in some detail at the roles of these tubules and filaments in cell structure and different forms of cell motility

18.3 The Molecular Structure and Organization of Cytoskeletal Components

Of the three main cytoskeletal fibers, intermediate filaments serve a mainly structural role in cells. Microtubules and microfilaments have dual functions, dynamically maintaining cell shape and enabling cell motility. For example, when attached to the plasma

membrane, microfilaments maintain cell shape. And when they interact with ***motor proteins*** (e.g., *myosin*), they can pull or push against a muscle cell membrane, changing the shape of the cell. Likewise, motor proteins such as *dynein* and *kinesin* can move *cargo* back and forth along microtubule tracks from one point to another in the cell. We will look at how motor proteins interact with microtubules and microfilaments shortly.

Let's take another look at the drawings and micrographs of the three main cytoskeletal filaments of eukaryotic cells that we saw earlier in the text (Fig. 18.2, below).

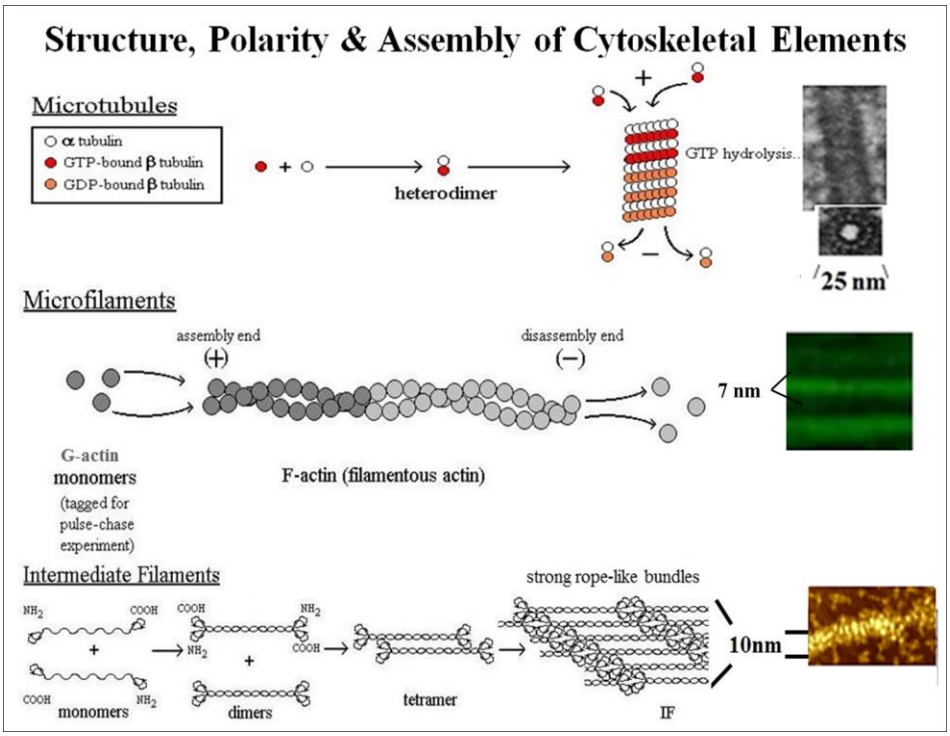


Fig. 18.2: Illustration of assembly, structure and polarity of microtubules, microfilaments and intermediate filaments alongside fluorescence micrographs made using fluorescent antibodies against isolated microtubule, microfilament and intermediate filament proteins (TOP, MIDDLE and BOTTOM, respectively).

321-2 Introduction to the Cytoskeleton

The location and general functions of microtubules, microfilaments and intermediate filaments was demonstrated by immunofluorescence microscopy. Fluorescence micrographs like those in Fig. 18.3 (below) show the different locations of fibers in cells.

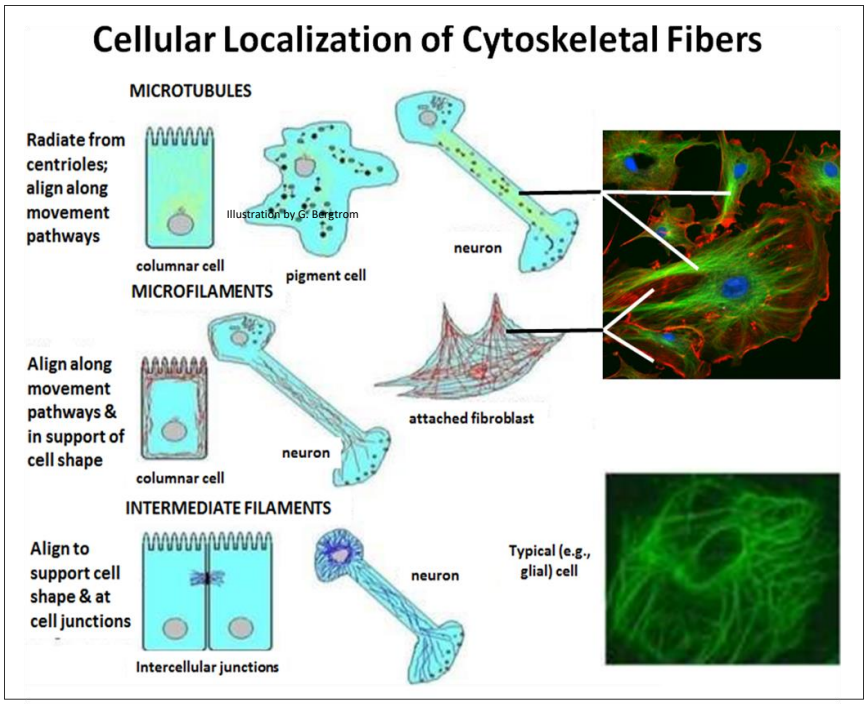


Fig. 18.3: Illustration and immunofluorescence microscope localization of microtubules, microfilaments and intermediate filaments in cells.

These localizations are consistent with known functions of the major cytoskeletal component filaments in cell structure and motility. Despite the small size of prokaryotic cells, they too were recently found have previously unsuspected cytoplasmic structures that could serve as a cytoskeleton (NCBI-A Prokaryotic Cytoskeleton?). So perhaps ***all*** (not just eukaryotic) cells are more than an unorganized bag of fluid sap! Next, we consider specific roles of microtubules, microfilaments, intermediate filaments and related proteins in the eukaryotic cytoskeleton.

322-2 Microtubules, Microfilaments and Intermediate Filaments in Cells

18.4 *Microtubules* are Dynamic Structures Composed of Tubulin Monomers

Microtubules assemble from dimers of ***α-tubulin*** and ***β-tubulin*** monomers. After their formation, ***α/β-tubulin dimers*** add to a growing, or *plus end* (***+end***), fueled by ***GTP*** hydrolysis (see Fig. 18.2). Disassembly at the ***-end*** of microtubules powers changing the shape of cells or the separation and movement of chromatids to opposite poles of cells during mitosis or meiosis. Isolated single microtubules were shown to grow by addition to one end and to disassemble at the opposite end, thus distinguishing the *+ends* and *-ends*. Find a summary of evidence for microtubule ***polarity*** in the link below.

323-2 Demonstration of the Polarity & Dynamics of Microtubules

Microtubules in most cells (other than when they are dividing) can seem disordered. In non-dividing (*interphase*) animal cells they tend to radiate from ***centrioles*** without forming discrete structures. But as cell division approaches, microtubules reorganize to form spindle fibers. The reorganization is *nucleated* from *centrioles* in animal cells and from a more amorphous ***microtubule organizing center*** (***MTOC***) in plant cells. A typical centriole (or ***basal body***) in has a '*9 triplet*' microtubule array as seen in electron micrograph cross sections (Fig. 18.4).

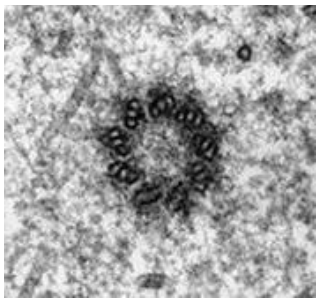


Fig. 18.4. Transmission electron micrograph of the characteristic *9 triplet* microtubule array of *centrioles* and *basal bodies*.

18.4.1 The Two Kinds of Microtubules in Spindle Fibers

The spindle fibers of mitosis and meiosis are made up of ***kinetochore microtubules*** and ***polar microtubules***. The former use chemical energy to pull duplicated chromatids apart; the latter use chemical energy to separate daughter cells during ***cytokinesis***.

18.4.1.a Kinetochore Microtubules

Duplicated chromosomes condense in prophase of mitosis and meiosis, forming visible paired ***chromatids*** attached at their ***centromeres***. Specific proteins associate with centromeres to make a *kinetochore* during condensation. As the spindle apparatus forms, some spindle fibers attach to the kinetochore; these are the *kinetochore microtubules*. By *metaphase*, bundles of kinetochore microtubules stretch from the kinetochores at the cell center to centrioles at opposite poles of a dividing animal cell, as drawn in Fig.18.5.

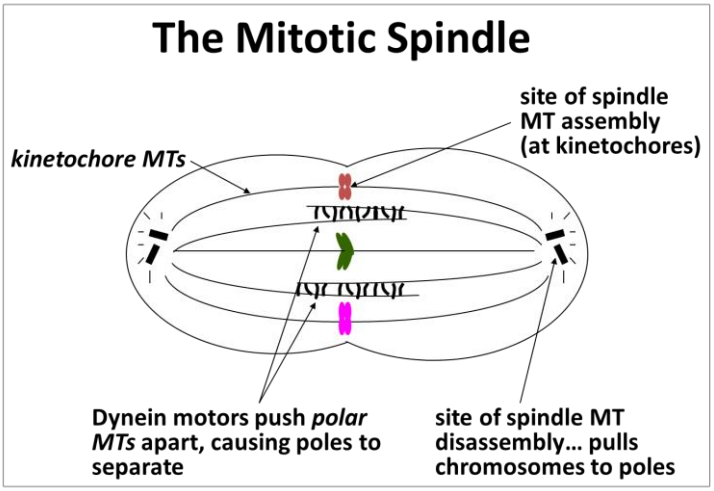


Fig. 18.5: Starting mitotic anaphase, spindle fiber microtubules exert forces that separate and pull chromatids apart and also push the poles of the cell apart (see text for details).

We now know that the ***+ends*** of kinetochore microtubules are in fact at the kinetochores, where these fibers assemble! During **anaphase**, kinetochore microtubules disassemble and shorten at their *–ends* (at the centrioles in animal, or MTOCs in plant cells). The forces generated separate the chromatids and draw daughter chromosomes to the opposite poles of the dividing cell.

The role of kinetochore microtubule disassembly at the centrioles (i.e., at their ***-ends***) was shown in a clever experiment in which a tiny laser beam was aimed into a cell at spindle fibers attached to the kinetochore of a pair of chromatids (see Fig. 18.6 below, and an animation of the events at the link below).

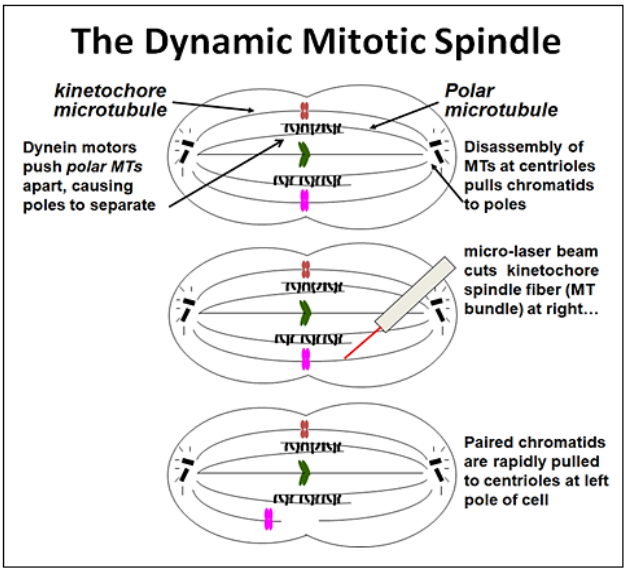


Fig. 18.6: Disrupting a kinetochore spindle fiber demonstrates a strong force from microtubule disassembly that quickly pulls a pair of chromatids towards centrioles.

324 Spindle Fiber Microtubules Generate Force on Chromatids

18.4.1.b Polar Microtubules

Spindle fiber ***polar microtubules*** extend from centrioles/MTOCs at opposite poles towards the center of dividing cells, but instead of binding to kinetochores, they overlap at the center of the dividing cells. While the kinetochore microtubules are tugging apart the paired chromatids at the venter of the cell, the *polar microtubules* are sliding past one another in opposite directions, pushing apart the poles of the cell. In this case, ***dynein motor proteins*** attached to microtubules (illustrated in Figs. 18.5 1nd 18.6 above) hydrolyze ATP to power microtubule sliding. Dynein motors on the microtubules from one pole of the cell in effect 'walk' along overlapping microtubules extending from the opposite pole.

18.4.2 Microtubules in Cilia and Flagella

The microtubules of cilia or flagella emerge from a ***basal body***, shown in the electron micrograph at the left in Fig.18.7, below.

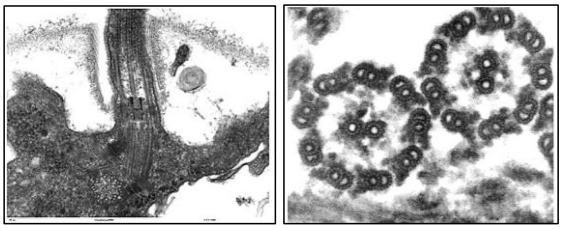


Fig. 18.7: Transmission electron micrographs of a basal body (LEFT) and cross-sections of a pair of 9+2 microtubule arrays (RIGHT) as they would emerge from a basal body.

Basal bodies are structurally similar to centrioles, organized as a ***9-triplet*** ring of microtubules. The formation of cilia and flagella begins at basal bodies but soon show a typical ***9+2 arrangement*** (9 outer doublets plus 2 central microtubules) in cross-section (above right in the micrograph).

It turns out that eukaryotic flagella and cilia can be stripped from their cells in a very high-speed blender (not the kind you may have in your kitchen!). Treatment with detergents dissolves their membranes, leaving behind ***axonemes*** with a 9+2 array of microtubules. Cross sections of axonemes are shown in Fig. 18.8 below.

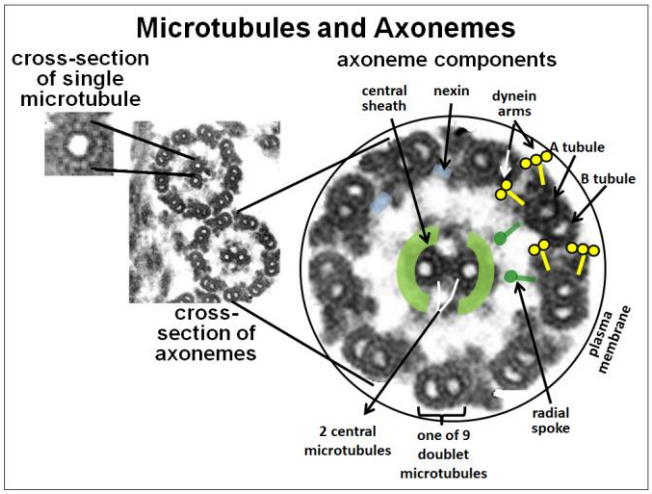


Fig. 18.8: Transmission electron micrographs of *axonemes* show that microtubules are made up of a ring of 13 tubulins (LEFT). Microtubules in cilia or flagella are arranged in typical 9+2 arrays (MIDDLE). The enlarged cross-section illustrates specific microtubule-associated proteins (*MAPs*) that maintain the 9+2 structure, including dynein motors that powers motility.

Axonemes from cilia and flagella are virtually identical. It is also possible to see the tubulin subunits that make up a microtubule polymer in cross section (micrograph at the upper left). Each tubule is made up of a ring of 13 tubulin subunits. Microtubules in the 'doublets' share tubulins but are also composed of 13 tubulins. When fully formed, the 25 nm diameter microtubules appear to be a hollow cylinder. When isolated, they typically come along with the dynein motor proteins and other *Microtubule-Associated Proteins* (***MAPs***) indicated in the micrograph at the right. These proteins hold microtubules together in an axoneme and play a role in motility.

18.4.3 Microtubule Motor Proteins Move Cargo from Place to Place in Cells

Motor proteins such as dynein and kinesin, are *ATPases*; they use the free energy of ATP hydrolysis to power intracellular motility. Let's take a closer look at how these two major motor proteins carry ***cargo*** from place to place inside of cells, activities summarized below in Fig. 18.9.

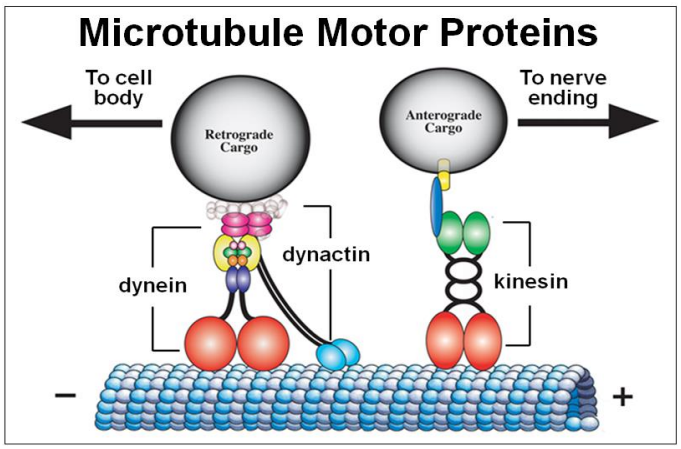


Fig. 18.9: Roles of *dynein* and *kinesin* in retrograde (backwards) and anterograde (forward) movement of cargo vesicles along microtubules (see text for details).

Organelles are a typical *cargo*. Examples include ***vesicles*** formed at the *trans Golgi face* containing secretory proteins, pigments or neurotransmitters. ***Secretory vesicles*** move along microtubule tracks to the plasma membrane for *exocytosis*. Vesicles containing *neurotransmitters* move from the cell body of neurons along microtubule tracks in the axons, reaching the nerve ending where they become ***synaptic vesicles***. In a chameleon, ***pigment vesicles*** in skin cells disperse or aggregate along microtubule tracks to change skin color to match the background.

Vesicle transport in neurons is well-understood. Neurotransmitter vesicles arise from the endomembrane system in neuron ***cell bodies***. ATP-dependent ***kinesin*** motor proteins power ***anterograde*** movement of the vesicles from the cell body to nerve endings). In contrast, ATP-dependent ***dynein*** motors (part of a ***dynactin*** complex) power ***retrograde*** movement of empty vesicles back to the cell body.

325-2 Microtubule Motor Proteins

A fanciful (and *not too inaccurate*!) animation of a motor protein in action on an axonal microtubule is at this link: Kinesin 'walking' an organelle along a microtubule. Next we'll let's look at some elegant studies of isolated axonemes and what they tell us about microtubule-based cell motility.

18.4.4 Demonstrating Sliding Microtubules

Experiments on *axonemes* isolated from demembranated cila or flagella confirm the sliding microtubule mechanism of ciliary and flagellar motility. An axoneme isolation protocol is illustrated in Fig. 18.10 below.

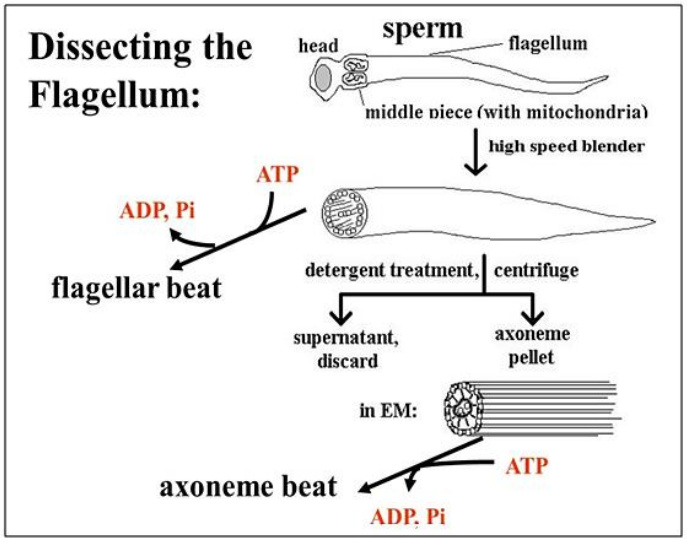


Fig. 18.10: Removing the membrane from isolated cilia or flagella leaves behind the *axoneme*; when provided ATP, both the isolated structures and de-membranated axoneme will beat as the ATP hydrolyzed.

Adding ATP added to detached cilia or flagella makes them beat in much the same way as they do when attached to their cells. The phenomenon is easily seen in a light microscope. But isolated axonemes (with their original 9+2 microtubule arrangement) also 'beat' (after a fashion!) in the presence of ATP!

326-2 9+2 Microtubule Array in Axonemes that Beat

Selective addition of different detergents removes ***radial spokes***, ***nexin*** and other proteins from the axoneme, causing the microtubules to separate. Dissociated microtubule doublets and central 'singlets' can then be observed in the electron microscope. When such separated microtubules are dialyzed to remove the detergents, doublet microtubules re-associate to form sheets as shown in Fig. 18.11.

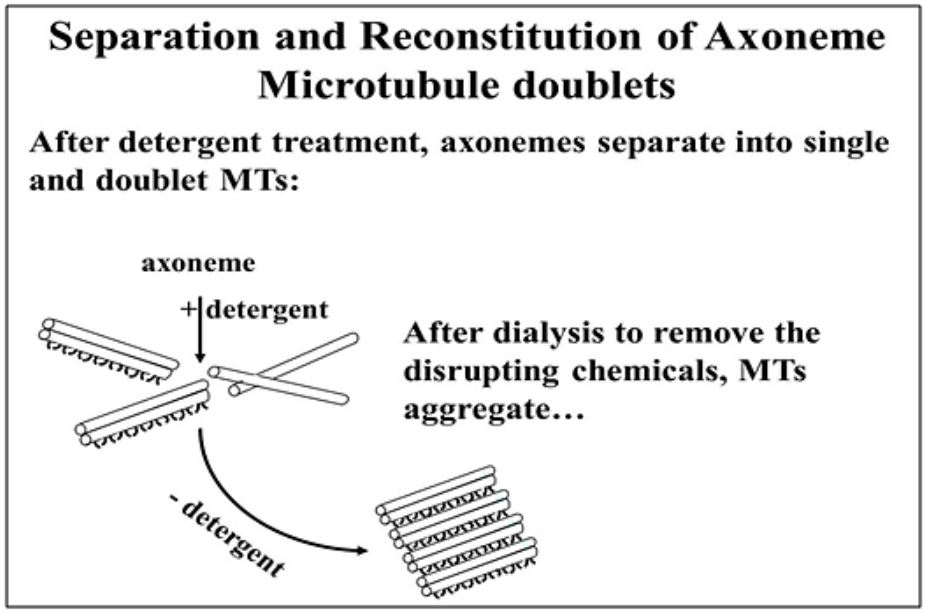


Fig. 18.11: Detergents can dissociate axonemes into individual and doublet microtubules. Dialysis of separated microtubules to remove the detergent cause the microtubules to re-associate (or *reconstitute*) into a sheet with connections resembling those seen in intact axonemes.

ATP added to *reconstituted* microtubule doublets causes the microtubules to separate as the ATP is hydrolyzed. When such preparations are fixed for electron microscopy

immediately after adding the ATP, they are caught in the act of sliding. See this animated in the first link below (#327-2).

327-2 Proof of Sliding Microtubules in Flagella and Cilia

328 Bacterial Flagella are Powered by a Proton Gradient

329 The Effects of Different Drugs on Microtubules... and Cancer

18.4.5 The Motor Protein Dynein Enables Axonemes to Bend

Take another look at the cross-section of axonemes in Fig. 18.9. In the 9+2 *axoneme* of cilia and flagella, dynein arms attached to the ***A tubules*** of the outer doublets walk along the ***B tubules*** of the adjacent doublet. If only the doublets on one side of an axoneme take a walk while those on the other side hold still, the microtubules will slide past one another and the axoneme (and therefore a cilium or flagellum) will bend. This microtubule sliding is constrained by flexible ***nexin*** and ***radial spoke*** attachments. Fig. 18.12 compares the movements of cilia and flagella.

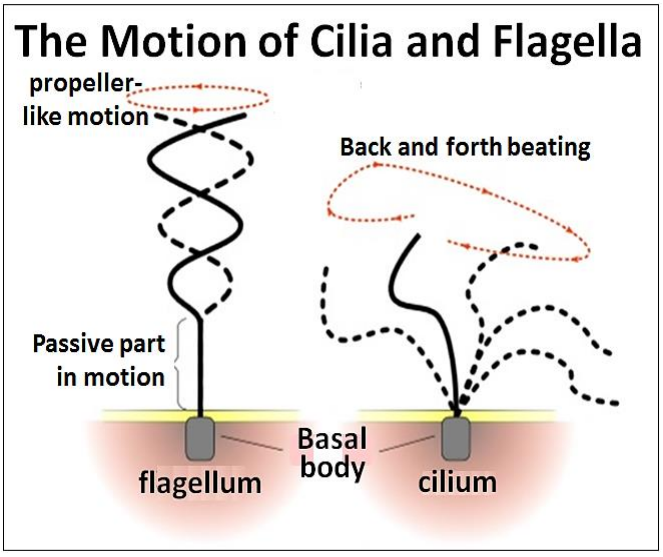


Fig. 18.12: Comparison of ciliary beat with propeller like generation of flagellar wave motion (see text for details).

The differences in flagellar motion (wave-like propeller) and ciliary motion (back and forth beat in a single plane) result in part from which microtubules are sliding at a given moment and the nature of their restraint by axoneme proteins.

18.5 Microfilaments – Structure and Role in Muscle Contraction

At 7 nm in diameter, ***microfilaments*** (actin filaments) are the thinnest cytoskeletal component. Globular actin (***G-actin***) monomers polymerize to form linear ***F-actin*** polymers. Two polymers then combine to form a twin-helical actin microfilament. As with microtubules, microfilaments have a **+end** to which new actin monomers are added to assemble *F-actin*, and a **–end** at which they disassemble when they are in a dynamic state, such as when a cell is changing shape. When one end of a microfilament is anchored to a cellular structure, for example to ***plaques*** in the cell membrane, motor proteins like myosin can use ATP to generate a *force* that deforms the plasma membrane and thus, the shape of the cell. One of the best-studied examples of myosin/actin interaction is in skeletal muscle where the sliding of highly organized thick myosin rods and the thin actin microfilaments results in muscle contraction.

18.5.1 The Thin (Micro-) Filaments and Thick Filaments of Skeletal Muscle

Bundles of parallel muscle cells make up a skeletal muscle. Thin sections of skeletal muscle cells (***myocytes***) appear ***striated*** in the light microscope (Fig. 18.13).

Fig. 18.13: Light micrograph of skeletal muscle stained to showing characteristic striations.

The dark purplish structures surrounding the myocyte are *mitochondria*, which will provide the ATP to fuel contraction. Skeletal muscle is made up of 'aligned', bundled myocytes. The bundled myocytes (also called *myofibers*) are further organized into fascicles that are finally bundled into a muscle.

The blowout illustration in Fig. 18.14 (below) shows the anatomical organization and fine structure of a muscle (left panel).

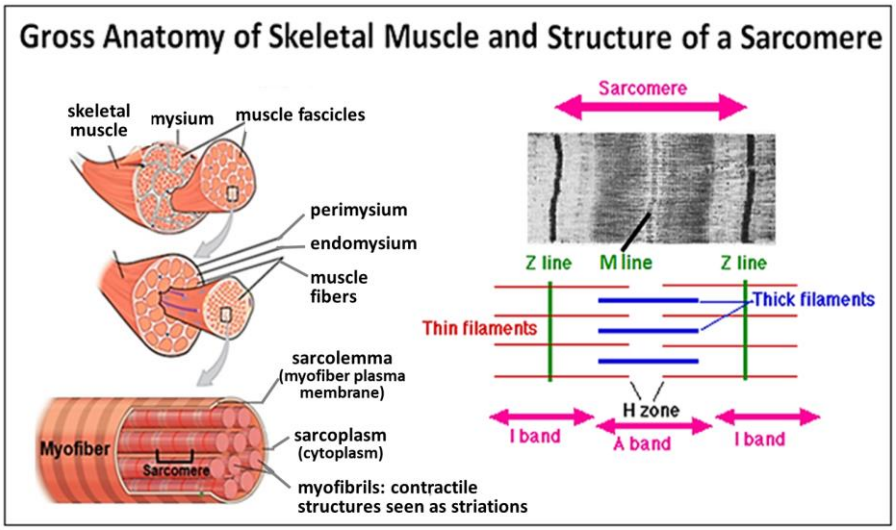


Fig. 18.14: Skeletal muscle organization and the anatomy of a muscle cell *sarcomere* (see text for details).

Light passing through the more or less ordered regions of the sarcomere will bend to different degrees because the regions each have a different ***refractive index***. Polarizing light microscopy detects these differences, enhancing the contrast between the regions of the sarcomere, defining them as either ***isotropic*** (i.e., with a low refractive index) or ***anisotropic*** (with a high refractive index). High-resolution electron microscopy from the 1940s revealed the fine structure of skeletal muscle (right panel). A pair of dark vertical ***Z-lines*** defines a ***sarcomere***. In the light micrograph of myocytes, the *Z lines* (shown in grey) aligned in register across sarcomeres of fascicles myofibrils. Based on light and electron microscopy, we can define the regions of the sarcomere:

- The ***A*** or ***Anisotropic band*** of overlapping, aligned actin and myosin filaments in the middle of the sarcomere is more ordered than the I band and so, has a higher refractive index.
- The ***I band*** or ***isotropic band,*** has a low refractive index compared to the *A band*. It is largely made up of thin (actin) microfilaments.
- A pair of ***Z lines*** demarcates the *sarcomere* (Z for *zwischen*, German for *between*).

- The ***H zone*** is a region where myosin does not overlap actin filaments.
- An ***M line*** lies at the center of the H zone.

The multiple repeating sarcomeres of myocytes *aligned in register* in the fascicles are what give the appearance of striations in whole muscles.

18.5.2 The Sliding Filament Model of Skeletal Muscle Contraction

Electron microscopy of relaxed and contracted muscle is consistent with the sliding of thick and thin filaments during contraction (Fig 18.15).

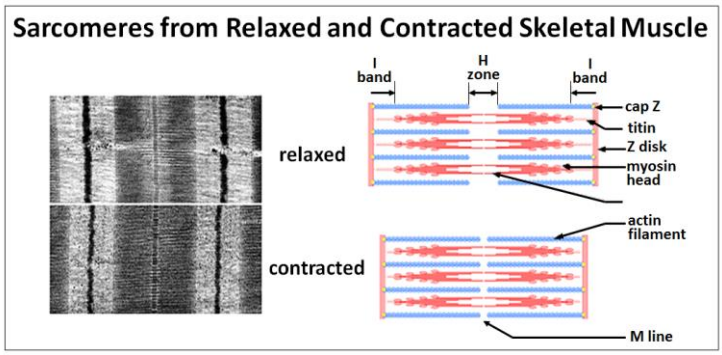


Fig. 18.15: Transmission **e**lectron micrograph and a corresponding illustration showing sarcomere shortening, consistent with the sliding of filaments during skeletal muscle contraction.

Additional key structures of the sarcomere can be seen in the drawing at the right. Note that, in the sarcomeres of a contracted muscle cell, the H zone has almost disappeared. While the width of the A band has not changed after contraction, the width of the I bands has decreased and the Z-lines are closer. The best explanation here was the ***Sliding Filament Hypothesis*** (***model***) of skeletal muscle contraction.

330-2 The Sliding Filament Model of Skeletal Muscle Contraction

18.5.3 The Contraction Paradox: Contraction and Relaxation Require ATP

The role of ATP in fueling the movement of sliding filaments during skeletal muscle contraction was based in part on experiments with ***glycerinated fibers***. These are muscle fibers that were soaked in glycerin to permeabilize the plasma membrane.

The soluble cytoplasmic components normally in myocytes leak out of these fibers but leave sarcomere structures intact as seen in electron micrographs. Investigators found that if ATP and calcium were added back to glycerinated fibers, the ATP was hydrolyzed and the fibers could still contract… and even lift a weight! The contraction of a glycerinated muscle fiber in the presence of ATP is illustrated in Fig. 18.16.

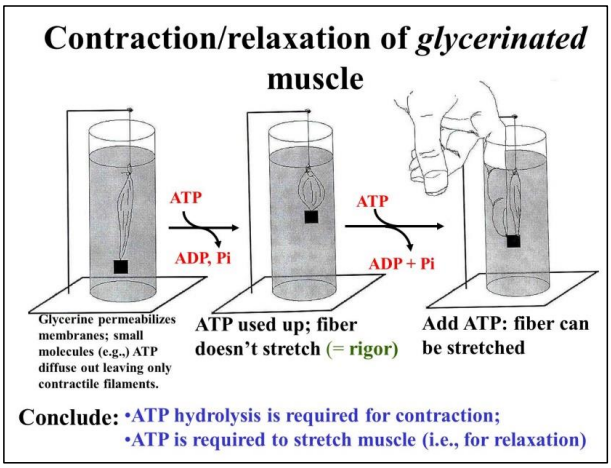


Fig. 18.16: The skeletal muscle *Contraction Paradox*: Given ATP, *glycerinated* muscle fibers contract and pull a weight. When all the ATP is hydrolyzed the fiber can't stretch unless more ATP is added.

When assays showed that all of the added ATP had been hydrolyzed, the muscle remained contracted. It would not relax, even with the weight it had lifted still attached. Attempts to manually force the muscle back to its relaxed position didn't work. But the fiber could be stretched when fresh ATP was added to the preparation. Moreover, if the experimenter let go immediately after stretching the fiber, it would again contract and lift the weight. A cycle of forced stretching and contraction could be repeated until all of the added ATP was hydrolyzed. At that point, the fiber would again no longer contract…, or if contracted, could no longer be stretched.

The contraction paradox then, was that ATP hydrolysis is required for muscle contraction *as well as* for relaxation (stretching). The paradox was resolved when the functions of the molecular actors in contraction were finally understood. Here we review some of the classic experiments that led to this understanding.

331 The Contraction Paradox

18.6 Actin-Myosin Interactions *in vitro*: Dissections and Reconstitutions

Several experiments hinted at the interaction of actin and myosin in contraction. For example, ***actomyosin*** was first observed as the main component of viscous skeletal muscle homogenates. Under appropriate conditions, adding ATP to such *actomyosin* preparations caused a decrease in viscosity. However, after the added ATP was hydrolyzed, the mixture became viscous again. Extraction of the non-viscous preparation (before it re-congealed and before the ATP was consumed) led to the biochemical separation of the two main substances we now recognize as the ***actin*** and ***myosin*** (*thin* and *thick*) filaments of contraction. What's more, adding these components back together reconstituted the viscous *actomyosin* (now renamed ***actinomyosin***). Adding ATP once again to the reconstituted solution eliminated its viscosity. The ATP-dependent viscosity changes of actinomyosin solutions were consistent with an ATP-dependent separation of thick and thin filaments. Do actin and myosin also separate in glycerinated muscles exposed to ATP, allowing them to stretch and relax? This question was answered with the advent of electron microscopy. The purification of skeletal muscle myosin from actin (still attached to Z Lines) is cartooned in Fig. 18.17, showing what the separated components looked like in the electron microscope.

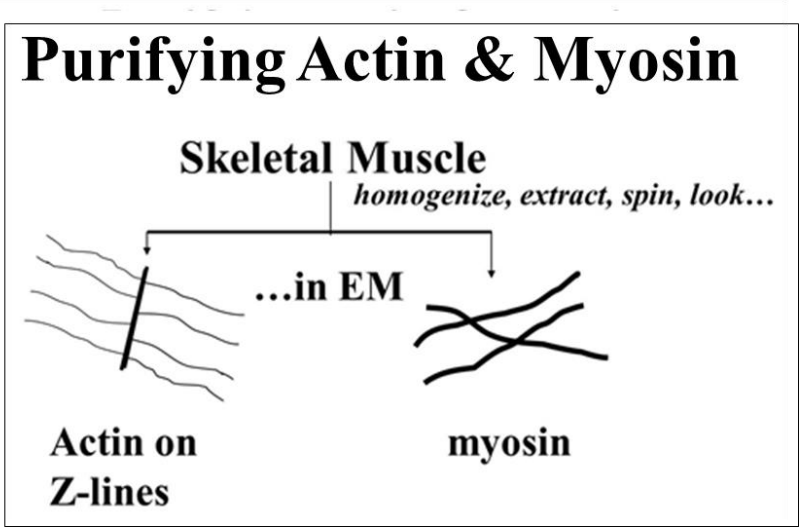


Fig. 18.17: Overview of the isolation of actin thin filaments (still on Z-Lines) from myosin thick filaments.

Next, when actin-Z Line and myosin fractions were mixed, electron microscopy of the resulting viscous material revealed thin filaments interdigitating with thick filaments. Fig. 18.18 shows the result of this reconstitution experiment.

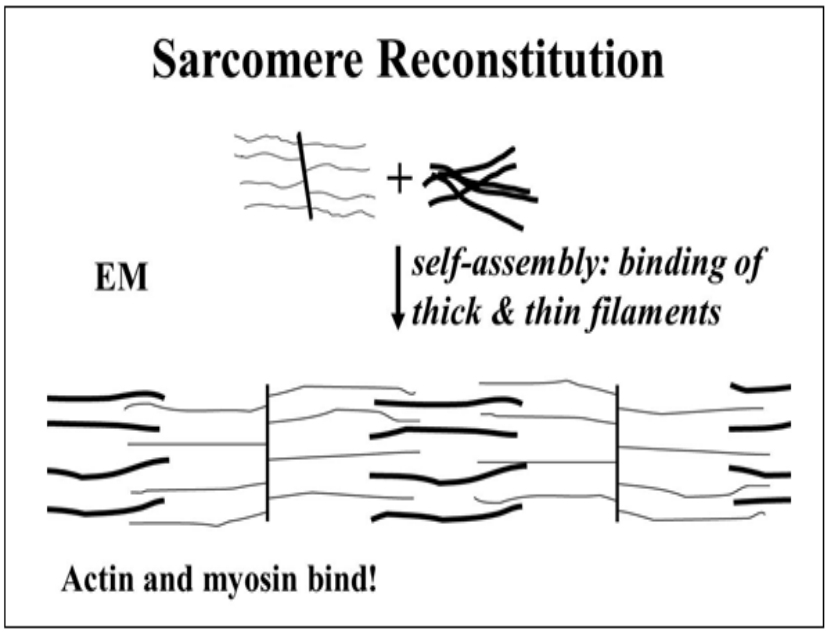


Fig. 18.18: Reconstitution of actin thin filaments (on Z-Lines) with myosin filaments.

As expected, when ATP was added to these extracts, the solution viscosity dropped and electron microscopy the revealed that the myosin and actin filaments had again separated. The two components could again be isolated and separated by centrifugation.

In yet further experiments, actinomyosin preparations could be spread on over an aqueous surface, producing a film on the surface of the water. When ATP was added to the water, the film visibly "contracted", pulling away from the edges of the vessel, reducing its surface area! Electron microscopy of the film revealed shortened sarcomere-like structures with closely spaced Z lines and short I bands…, further confirming the sliding filament model of muscle contraction.

332 *In Vitro* & Electron Microscope Evidence for a Sliding Filament Model

When actin and myosin were further purified from isolated actinomyosin, the thick myosin rods could be dissociated into large myosin monomers. In fact, at ~599Kd, myosin monomers are among the largest known proteins. Thus, thick filaments are massive polymers of huge myosin monomers! The molecular structure of myosin thick filaments is shown in Fig. 18.19.

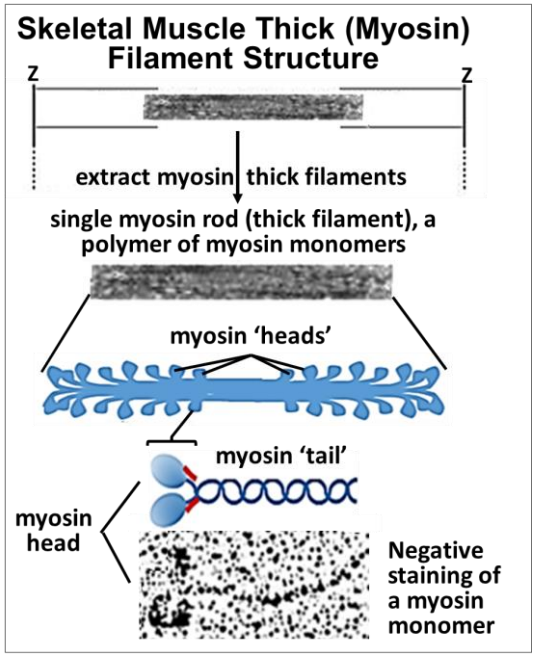


Fig. 18.19: Structure of a skeletal muscle myosin filament and the myosin monomer. Shown is ***myosin II***, the thick filament that spans both sides of the H zone in a sarcomere (upper). The head-&-tail structure of a myosin monomer is shown in the high magnification electron micrograph and is illustrated in the cartoon (lower). The myosin monomer is itself a polymer of four polypeptides.

An early observation of isolated mammalian actin filaments was that they had no ATPase activity. On the other hand, while isolated myosin preparations did have an ATPase activity, they would only catalyze ATP hydrolysis very slowly compared to intact muscle fibers. Faster ATP hydrolysis occurred only if myosin filaments were mixed with microfilaments (either on or detached from Z-lines). In the electron microscope, isolated myosin protein monomers appeared to have a double-head and single tail regions. Biochemical analysis showed that the monomers themselves were composed of the two heavy chain and two pairs of light chain polypeptides shown in the illustration above.

In Fig 18.20 a high magnification/high resolution electron micrograph simulation and corresponding drawings illustrate a myosin monomer and its component structures.

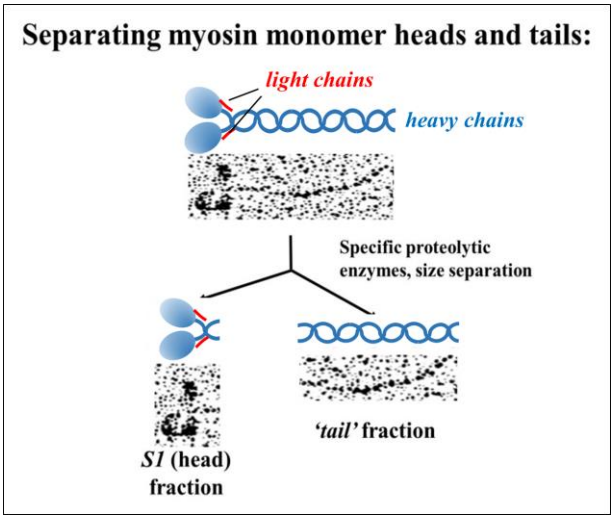


Fig. 18.20: Digestion of purified myosin monomers with enzymes that hydrolyze peptide bonds between specific amino acids produces an *S1 head* and a *tail* fraction with different properties.

Proteolytic enzymes that only hydrolyze peptide linkages between specific amino acids can 'cut' the myosin monomers into ***S1*** (head) and ***tail*** fragments. Electron micrographs of enzymatic digest fractions after separation by ultracentrifugation are shown above. The tail fragments turn out to be remnants of the two myosin heavy chain polypeptides. The S1 fragments consist of a pair of light chains and the rest of the heavy chains. Upon further analysis, the fraction containing the S1 myosin heads had a slow ATPase activity, while the tails had none. The slow activity was not an artifact of isolation; mixing the S1 fraction with isolated actin filaments resulted in a higher rate of ATP hydrolysis. Thus the myosin heads must be ATPases that bind and interact with actin microfilaments.

333-2 Thick Filament & Myosin Monomer Structure

In fact, S1 myosin heads bind directly to actin, ***decorating*** the actin with "***arrowheads***" visible in the electron microscope (https://www.flickr.com/photos/actin_arrowheads). Even intact myosin monomers could decorate muscle actin. These results are consistent with the requirement that myosin must bind to actin to achieve a maximum rate of ATPase activity during contraction.

The arrowheads on ***decorated actin*** still attached to Z lines are illustrated below in Fig. 18.21.

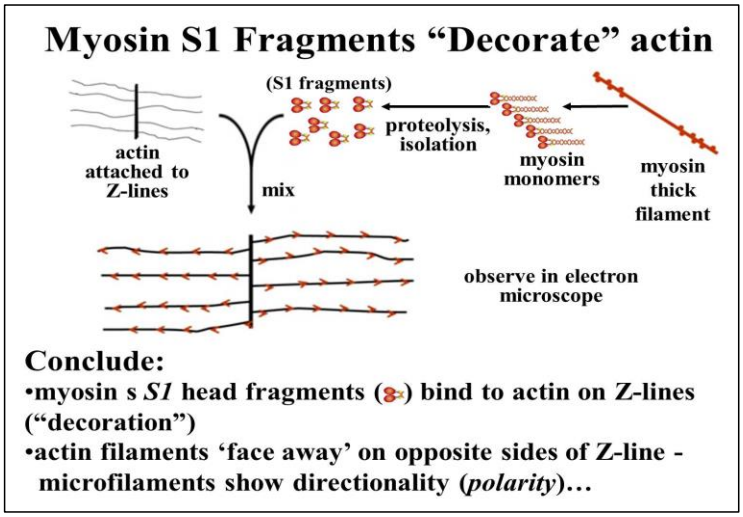


Fig. 18.21: Illustration of myosin decoration by myosin monomer S1 fragments, showing opposing polarity of actin filaments on opposite sides of the Z-line.

Note that the 'arrowheads' always face in opposite directions on either side of the Z line. These opposing arrowheads suggest that the actin filaments attached to the two Z-Lines of a sarcomere are drawn towards each other along the opposite sides of bipolar myosin rods. This is consistent with sliding filaments that draw Z-lines closer together during skeletal muscle contraction, shortening the sarcomeres. For a more dramatic look at decorated actin, Check out the 'arrowheads' in the electron micrograph at https://www.slideserve.com/brinda/muscle-structure-physiology-i-rosenbluth-0409

334-2 Myosin Monomers & S1 Heads Decorate Actin

18.7 Allosteric Change and the Micro-Contraction Cycle

Whereas dynein and kinesin are *motor proteins* that 'walk' along microtubules, myosins are motor proteins that walk along microfilaments. All of these motor proteins are ATPases that use the free energy of ATP hydrolysis to effect conformational changes that result in the walking, i.e., motility. In skeletal muscle, allosteric changes in myosin

heads enable the myosin rods to do the walking along F-actin filaments. When placed in sequence, the different myosin head conformations illustrated in Fig. 18.22 (below) are the likely changes that would occur during a ***micro-contraction cycle***.

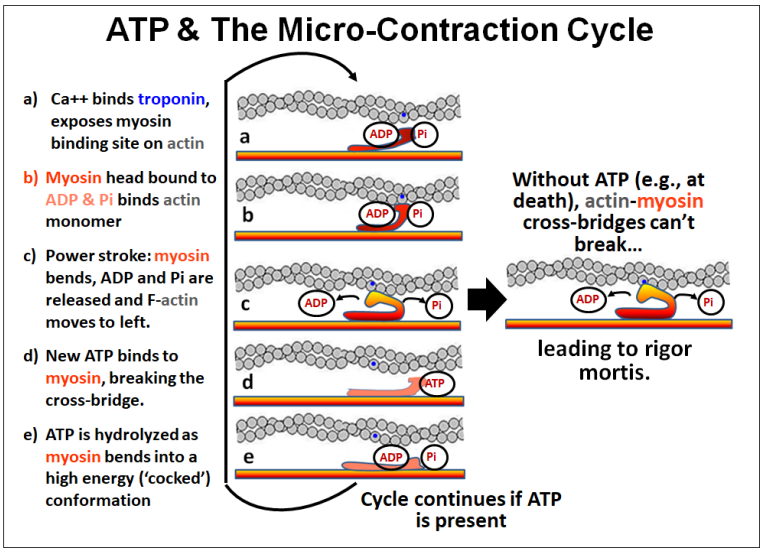


Fig. 18.22: Steps in the *Microcontraction Cycle* explain the muscle contraction paradox (see text for details).

To help you follow the sequence, find and follow the small (red) dot on a single monomer in the actin filament. Here are the steps:

a. Calcium is required for contraction; in the presence of Ca++ ions, myosin binding sites on actin are open (Ca^{++}-regulation of muscle contraction is discussed in detail below).
b. Myosin heads with attached ADP and Pi bind to open sites on actin filaments.
c. The result of actin-myosin binding is an allosteric change in the myosin head that bends a ***hinge region*** to pull the attached microfilament (note the dot - it has moved to the left!). This ***micro-sliding*** of actin along myosin is the ***power stroke***. The myosin head remains bound to an actin monomer in the F-actin
d. ATP displaces ADP and Pi on myosin heads in this bent conformation. The resulting allosteric change in the head breaks the cross-bridge between it and the actin.
e. Once dissociated from actin, myosin heads catalyze ATP hydrolysis, resulting in yet another conformational change. The head, still bound to ADP and Pi, has bent at its hinge, taking on a high-energy conformation that stores the energy of ATP hydrolysis.

Micro-contraction cycles of actin sliding along myosin continue as long as ATP and Ca++ are available. During repetitive *micro-contraction cycles*, myosin heads on the thick filaments pull actin filaments attached to Z-lines as stored free energy is released during *power strokes*, bringing the Z lines closer to each other. The result is the shortening of the sarcomere and ultimately of muscle cells and of course, the entire muscle. When the muscle no longer needs to contract, the Ca++ required for contraction is withdrawn, micro-contraction cycles cease, the myosin heads remain in the high-energy conformation of step ***e***, and the muscle can relax (stretch). Neural stimulation can then cause another release of Ca++ to signal contraction again.

The *micro-contraction cycle* also stops when ATP is gone, usually a more permanent state brought about by death! But this time, myosin heads remain bound to the actin filaments in the state of muscle contraction at the time of death. This is ***rigor mortis*** at the molecular level (see the illustration above). At the level of whole muscle, ***rigor mortis*** results in the inability to stretch or otherwise move body parts when ATP has once and for all departed.

18.8 The Micro-contraction Cycle Resolves the Contraction Paradox

The micro-contraction cycle resolves the contraction paradox:

- ***ATP is necessary for muscle contraction***: In step ***e*** in the illustration above, as ATP on myosin heads is hydrolyzed, the heads change from a low-energy to a high-energy conformation. The myosin heads can now bind to actin monomers (step ***b*** in the micro-contraction cycle). This results in the ***power stroke*** (step ***c***). Free energy released by an allosteric change in myosin pulls the actin along the myosin, in effect causing a micro-shortening of the sarcomere, in other words, contraction!

- ***ATP is necessary for muscle relaxation***: At the end of step ***c***, myosin remains bound to actin until ATP can again bind to the myosin head. Binding of ATP in step ***d*** displaces ADP and inorganic phosphate (Pi)… and breaks actin-myosin cross-bridges. A removal of Ca^{++} from sarcomeres at the end of a contraction event blocks myosin binding sites on actin, while the rapid breakage of actin-myosin cross-bridges by ATP-myosin binding allows muscle relaxation and the sliding apart of the actin and myosin filaments (i.e., stretching). This leaves the myosin heads in the 'cocked' (high-energy) conformation, ready for the next round of contraction.

No more paradox! The displacement of ADP and Pi by ***ATP binding*** to myosin heads breaks actin-myosin cross-bridges, allowing for relaxation (stretching). The ***hydrolysis of ATP*** bound to myosin cocks the myosin head in a high free-conformation that is released during the microcontraction power stroke.

Electron microscopic examination of myosin monomer heads at different ionic strengths provides visual evidence that myosin heads are flexible and can take on alternate stable conformations, as would be expected during the micro-contraction cycle (Fig 18.23).

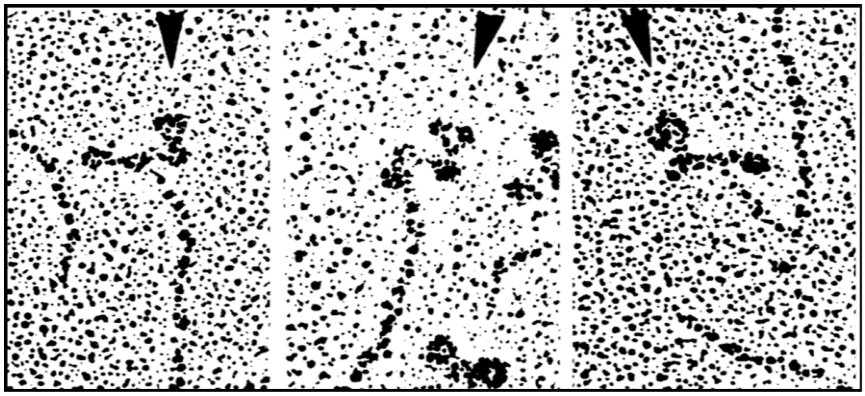


Fig. 18.23: The ability of flexible myosin heads to bend and change conformation is consistent with their proposed activity during the micro-contraction cycle.

The arrows point to myosin heads in different conformations. This visualization was made possible by treatment of the preparations with anti-myosin antibodies (immunoglobulins). See a cool video of conformational change in myosin monomers at Myosin heads in Action.

- **335-2 Actin-Myosin Contraction Cycle Resolves the Contraction Paradox**

336-2 ATP Binding & Hydrolysis Changes Myosin Head Conformation

18.9 Ca^{++} Ions Regulate Skeletal Muscle Contraction

Typically, the neurotransmitter acetylcholine released by a motor neuron binds to receptors on muscle cells to initiate contraction. Early experiments had already revealed that Ca^{++} was required along with ATP to get glycerinated skeletal muscle to contract. It was later demonstrated that Ca^{++} ions were stored in the ***sarcoplasmic reticulum*** (the smooth endoplasmic reticulum) of intact muscle cells. As we have seen, an action potential generated in the cell body of a neuron propagates along an axon to the nerve terminal, or synapse. The action potential at a neuromuscular junction that initiates contraction is summarized in the illustration in Fig. 18.24 (below).

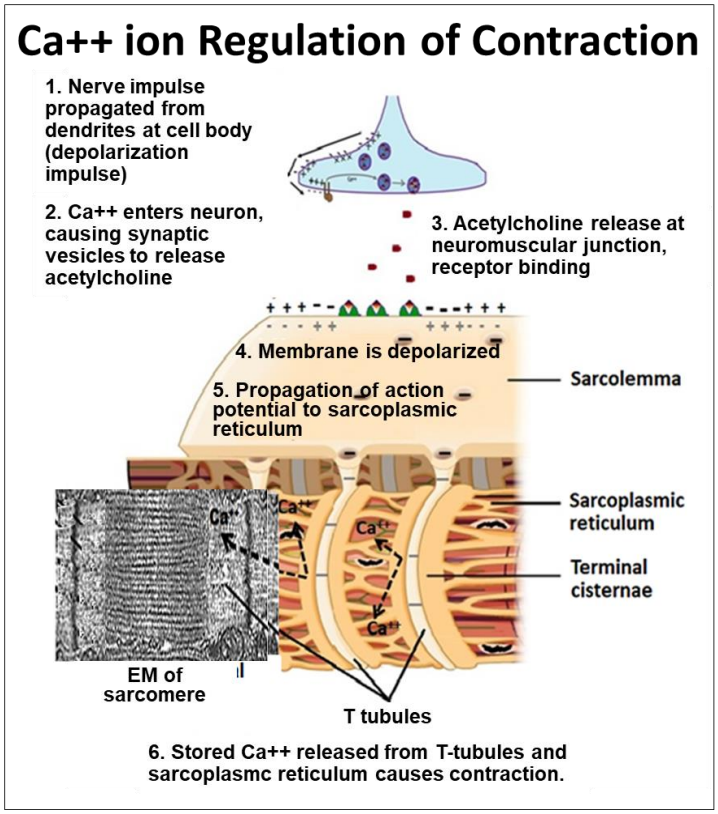


Fig. 18.24: Innervation leads to Ca++ release from *sarcoplasmic reticulum* to regulate contraction (see text for details).

In a similar fashion, an action potential generated at a neuromuscular junction travels along the ***sarcolemma*** (the muscle plasma membrane) to points where it is continuous with ***transverse tubules*** (***T-tubules***). The action potential then moves along the T-tubules and then along the membranes of the sarcoplasmic reticulum. This propagation of an action potential opens Ca^{++} channels in the sarcoplasmic reticulum, releasing Ca^{++} to bathe myofibril sarcomeres where they bind to one of three ***troponin*** molecules to allow skeletal muscle contraction, i.e., to allow filaments to slide. The ***three troponins*** and a ***tropomyosin*** molecule are bound to actin filaments. Experiments using a*nti-troponin* and *anti-tropomyosin* antibodies localized the three proteins in electron micrographs at regularly spaced intervals along actin filaments, as modeled in Fig. 18.25 below.

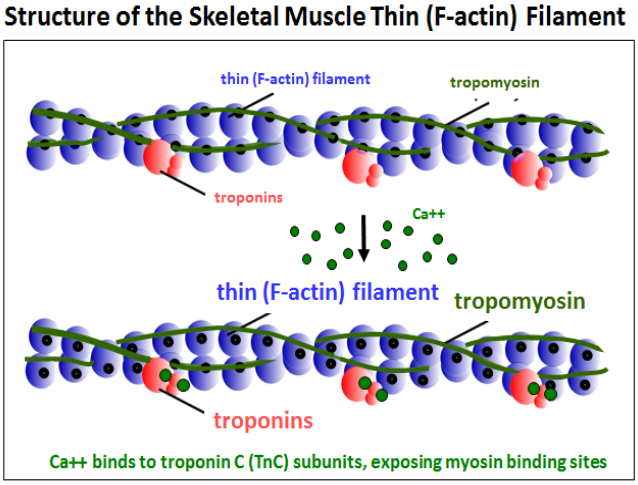


Fig. 18.25: Skeletal muscle thin filaments consist of actin associated with troponins and tropomyosin. These actin-associated proteins participate in the response to Ca++ ions to regulate interactions with myosin.

In resting muscle, tropomyosin (a fibrous protein) lies along the actin filament where it covers up the myosin binding sites of seven G-actin monomer subunits in the microfilament. Click https://www.e-sciencecentral.org/articles/Figure.php?xn=am/am-47-226&id=f1-am-47-226 to see an electron micrograph of negatively stained actin bound to the regularly spaced tropomyosin molecules shown in the illustration above.

The 'cross-section drawn in Fig. 18.26 (below) illustrates how ***troponin T*** (*tropomyosin-binding troponin*) and ***troponin I*** (*inhibitory troponin*) hold the tropomyosin in place, and how the binding of Ca^{++} ions to troponin C regulates contraction.

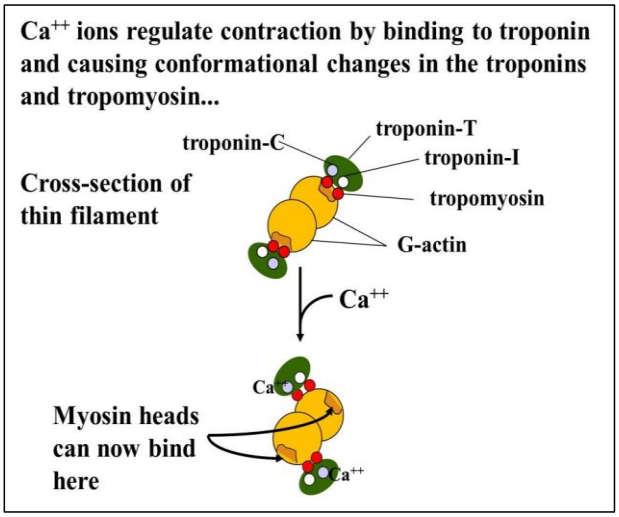


Fig. 18.26: Ca++ ions initiate uncovering myosin binding sites on actin by binding to *troponin-C*, resulting in allosteric changes in the troponins and ultimately tropomyosin.

A chain reaction of conformational changes begins when Ca^{++} ions bind to ***troponin-C***. The result is that the three-subunit troponin complexes bound to tropomyosin shift position along the filament to expose the myosin-binding sites on the G-actin subunits. Only after this shift can ATP-bound myosin in turn bind to actin and initiate the *micro-contraction cycle* discussed earlier. The regulation of contraction by Ca++ is animated in the link below.

337 Regulation of Skeletal Muscle Contraction by Calcium

18.9.1 Muscle Contraction Generates Force

Contraction by ATP-powered sliding of thin along thick filaments generates force on the *Z-lines*. In three dimensions, the Z-lines are actually ***Z-disks*** to which the actin thin filaments are attached. The protein ***α-actinin*** in the Z-disks anchors the ends of the actin filaments to the disks so that when the filaments slide, the Z-disks are drawn closer, shortening the sarcomeres. Another Z-disk protein, ***desmin***, is an intermediate filament organized around the periphery of Z-disks. Desmin connects multiple Z-disks in a myofibril. By keeping the Z-Disks in register, muscle cell, and ultimately whole muscle contraction is coordinated. Finally, actin filaments at the ends of the muscle

cell must be connected to the plasma membrane for a muscle cell to shorten during myofibril contraction. Several proteins, including ***syntrophins*** and ***dystrophin*** (another intermediate filament protein) anchor the free ends of microfilaments coming from Z-disks to the cell membrane.

Still other proteins anchor the cell membrane in this region to the extracellular matrix (tendons) that are in turn, attached to bones! Force generated by myosin hydrolysis of ATP and the sliding of filaments in individual sarcomeres are thus transmitted to the ends of muscles to effect movement. If the name *dystrophin* sounds familiar, it should! The gene and its protein were named for a mutation that causes muscular dystrophy, resulting in a progressive muscle weakening.

338 Contraction Generates Force Against Z Disks and Cell Membranes

18.9.2 The Elastic Sarcomere: Do Myosin Rods Just Float in the Sarcomere?

In fact, myosin rods do not "float" in sarcomeres, but are anchored to proteins in the ***Z discs*** and ***M-lines***. In 1954, R. Natori realized that when contracted muscle relaxes, it lengthens beyond its resting state, then shortening again to its resting length. He proposed that this elasticity must be due to a fiber in the sarcomere. Twenty-five years later, the elastic structure was identified as ***titin***, a protein that holds several molecular records! The gene for titin contains the largest number of *exons* (363) of known proteins. After actin and myosin, titin is also the most abundant protein in muscle cells. At almost 4 x 10^6 Da, the aptly named *titin* is the also the largest known polypeptide, much larger than even myosin! Extending from the Z discs to the M line of sarcomeres, titin coils around thick filaments along the way. *Titin* is anchored at Z-disks by ***α-actinin*** and ***telethonin*** proteins. At the *M-line*, titin binds to a ***myosin-binding protein C*** (***MYBPC3***) and to ***calmodulin***, among others (*myomesin*, *obscurin*, *skelamin*…). Some if not all of these proteins must participate in keeping the myosin thick filaments positioned and in register in the sarcomere. This is similar to how desmin binds Z-disks to each other to keep sarcomeres in register. The location of titin and several other sarcomere proteins is illustrated in Fig. 18.27 (below).

Coiled titin molecules (red in the illustration) extend from the Z to M lines. The colorized electron micrograph of one extended titin molecule in the middle of the illustration above should convince you of the length (35,213 amino acids!) of this huge polypeptide. Titin's elastic features are largely in a region labeled P in the electron micrograph that lies between Z discs and the ends of myosin rods. The many domains of this *P* region are shown expanded at the bottom of Fig. 18.27.

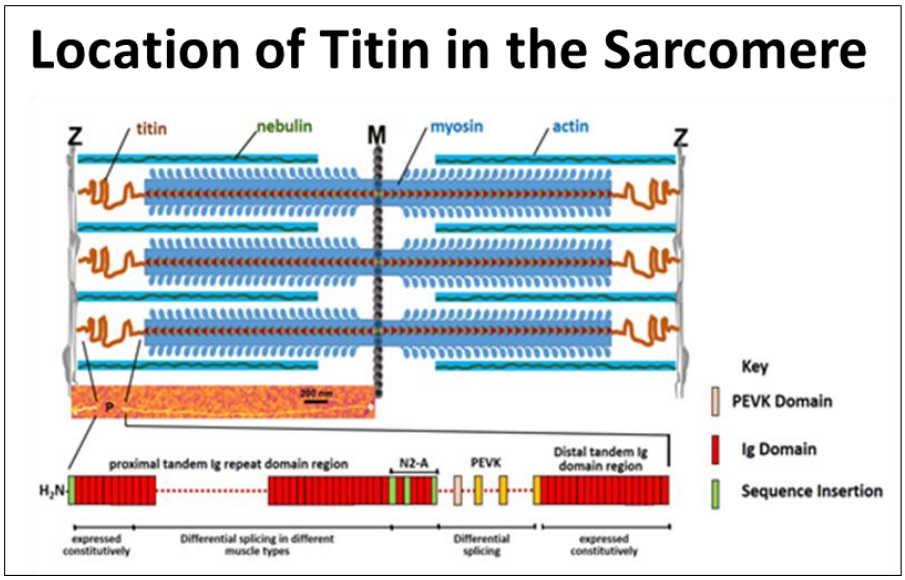


Fig. 18.27: Structure of *titin* and its location in the sarcomere (see text for details).

With all the binding (and other) functions, you might expect that titin has many domains. It does! They include ***Ig*** (immunoglobulin) domains, ***fibronectin*** domains (not shown here) and ***PEVK*** and ***N2A*** domains that help bind titin to *α-actinin* in Z-disks. Which and how many Ig and/or PEVK domains are present in a particular muscle depends on which alternative splicing pathway is used to form a titin mRNA. Over a micron long, titin functions as a molecular spring, as Natori predicted. Its coiled domains compress during contraction, passively storing some of the energy of contraction.

When a skeletal muscle relaxes, Ca^{++} is withdrawn from the sarcomere. ATP can still displace ADP from myosin heads, breaking actin-myosin cross-bridges. When actin and myosin dissociate in the absence of Ca^{++}, the troponins and tropomyosin reverse their conformational changes, once again covering myosin head binding sites on F-actin. The muscle then stretches, typically under the influence of gravity or an opposing set of muscles.

But during contraction, 244 individually folded titin domains were compressed. During relaxation, these domains de-compress and the stored energy of compression also helps to power relaxation. At the same time, titin connections limit the stretch so that

a potentially overstretched muscle can 'bounce' back to its normal relaxed length. In a particularly elegant experiment, R. Linke et al. provided a visual demonstration of myofiber ***elasticity*** consistent with the coiled spring model of titin structure. They made antibodies to peptide domains on either side of the *PEVK* domain of *titin* (*N2A* and *I20-I22*) and attached them to ***nanogold particles***. The gold particles are electron dense and will appear as black granules in transmission electron microscopy. Here is the experiment: Individual *myofibers* were stretched to different lengths, fixed for electron microscopy and treated with the *nanogold*-linked antibodies. The *antibodies* localize to and define the boundaries of the titin *PEVK* domains in myofibers stretched to different lengths (Fig. 18.28, below, showing electron micrographs with simulated localization of nanogold particles that reflect the actual results).

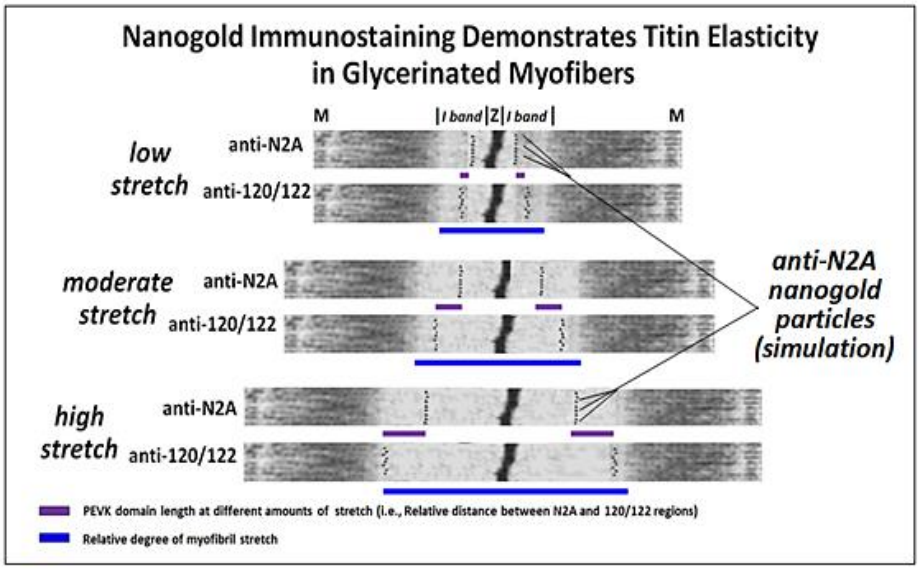


Fig. 18.28: Elasticity of skeletal muscle shown by the location of anti-N2A-linked and anti-120/122-linked nanogold particles in sarcomeres. Note the increased separation of the PEVK domains targeted by the antibodies as a muscle fiber is increasingly stretched. This titiin elasticity facilitates skeletal muscle relaxation.

In the experiment, increased stretch lengthened the I bands on either side of Z lines of sarcomeres (blue bars). Likewise, the titin PEVK domains also lengthened as is evident from the increased distance between the nanogold-linked N2A and 120/122 antibodies that bind on either side PEVK domains. This demonstration of titin (and

therefore sarcomere) elasticity is consistent with the storage of some of the free energy of contraction when the molecule is compressed, and the passive release of that energy during relaxation. Since titin tethers thick filaments to Z-disks and M-lines, it also limits the amount of sarcomere stretch during relaxation. An animation from Linke's lab is at http://www.titin.info/.

18.10 Actin Microfilaments in Non-Muscle Cells

Microscopy revealed that thin (~10 nm) filaments permeate the cytoskeleton of eukaryotic cells (see Fig. 18.3). These were suspected to be actin microfilaments. Microfilaments typically lie in the cortex of cells, just under the plasma membrane, where they support cell shape. These same microfilaments can also re-organize dynamically, allowing cells to change shape. A dramatic example of this occurs during cytokinesis in dividing cells, when the dividing cell forms a *cleavage furrow* in the middle of the cell (discussed further in another chapter). The cortical microfilaments slide past each other with the help of *non-muscle myosin*, progressively pinching the cell until it divides into two new cells. To test whether these 10 nm 'microfilaments' were in fact actin, myosin monomers or S1 myosin head fragments were placed atop actins isolated from many different cell types. When viewed in the electron microscope, such preparations always revealed that the 10 nm microfilaments were decorated with *arrowheads*, just like S1 fragment decorated muscle cell actin or Z line-bound actin. Clearly, the cytoplasmic microfilaments are a form of F-actin. Check out the arrowheads on *cortical ring* actin in cells in cytokinesis after treatment with S1 fragments at https://www.flickr.com/photos/actin_arrowheads**.** Also, see the role of cortical filaments in cell division at Cortical Actin Filament Action in Cytokinesis. Of course, actin microfilaments are involved in all manner of cell motility in addition to their role in cell division. They enable cell movement and cytoplasmic streaming inside cells. And while they give intestinal microvilli strength, they even enable them to move independent of the passive pressures of *peristalsis*.

Microfilaments are found throughout the eukarya, and they are readily identified since most if not all of them can be decorated my S1 fragments or intact myosin monomers. The S1 myosin-decorated actin filaments you see at the link above were in sea urchin embryos! Other examples of microfilaments in cell motility include the ability of *amoeba* and other *phagocytic cells* to extend *pseudopodia* to engulf food or foreign particles (e.g., bacteria), respectively.

A well-studied example of microfilament-powered cell movement is the spread of fibroblast cells along surfaces. These migrating cells extend thin *filipodia* into the direction of movement by assembling actin bundles along the axis of cell movement. In Fig. 18.29 (below), actin *stress fibers* that maintain cell shape fluoresce green in the immunofluorescence micrograph (left). The dual roles of actin in fibroblast shape and movement are also illustrated at the right.

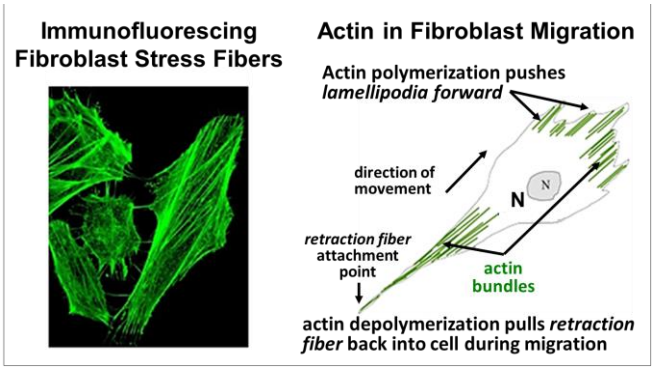


Fig. 18.29: In the anti-actin immunofluorescence micrograph of fibroblasts, actin localizes with stress fibers which help maintain cell shape (LEFT). Actin also localizes in lamellipodia and retraction fibers in migrating fibroblasts (RIGHT), orienting in the direction of movement.

The extension of filipodia at the moving front of a fibroblast is mainly based on actin assembly and disassembly (not unlike motility based on microtubules). A ***retraction fiber*** forms at the hind-end of the cell as the fibroblast moves forward. The retraction fiber remains attached to the surface (*substratum*) on which it is migrating until actin-myosin interactions (in fact, sliding) causes *retraction* of most of this 'fiber' back into the body of the cell. Studies of non-muscle cell motility suggest the structure and interacting molecular components of stress fibers modeled in Fig. 18.30 (below).

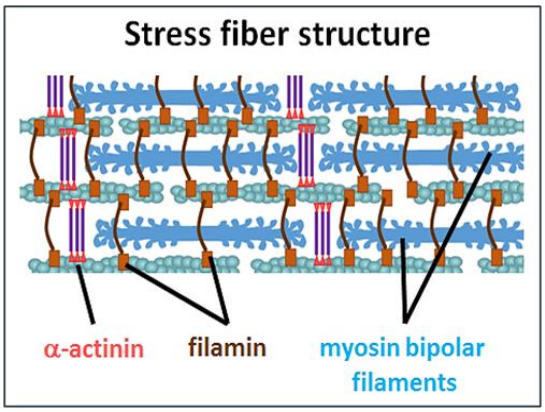


Fig. 18.30: The molecular structure of stress fibers; myosin as well as other actin-binding proteins interact with actin in non-muscle cell motility.

The illustration suggests roles for actin-binding proteins in the sliding of overlapping myosin and actin filaments during movement. This model may also explain the cytoplasmic streaming that distributes cellular components and nutrients throughout a cell. In fact, the movements of both involve actin-myosin interactions. ***Filamin*** in this drawing is shown holding actin filaments together at an angle, while ***α-actinin*** also helps to bundle the actin filaments. ***Titin*** (not shown) also seems to be associated with stress fibers. Unlike highly organized skeletal muscle sarcomeres, the proteins and filaments in stress fibers are not part of Z- or M-line superstructures.

Could something like these less-organized non-muscle stress fiber filament bundles be evolutionary ancestors of muscle cell sarcomeres?

18.11 Both Actins and Myosins are Encoded by Large Gene Families

Actins may be the most abundant protein in cells! At least six different actin ***isoforms*** encoded by a large actin ***gene family*** have nearly identical amino acid sequences, all of which are involved in cytoskeletal function. The β-actin isoform predominates. Genes for some isoforms are expressed in a cell-specific manner.

Are all actin isoforms functionally significant? Myosin monomers (or S1 heads) decorate virtually all actins. This makes one wonder if any one actin is an adaptation (however subtle) such that the absence of one isoform would pose a significant threat to the survival of an organism. Since amino acid sequence differences between actins would not predict dramatically different protein function, could they underlie some as yet unknown physiological advantage to different cells?

In mice, the loss of a γ-actin gene has little effect on the organism, while loss of the β-actin gene in mice is lethal at embryonic stages. So what is the function of γ-actin? Does it even have one? Or is it a *vestigial* protein that like our appendix once served a now long-lost function? In contrast, studies in humans show that a non-lethal β-actin mutation correlates with delayed development and later neurological problems (e.g., epilepsy), kidney and heart abnormalities. Nevertheless, people with such mutations can lead nearly normal, healthy lives (Beta–Actin Gene Mutations and Disease).

Like the actins, myosin genes encoding variant isoforms comprise a large eukaryotic gene family. All isoforms have ATPase activity, and some (if not all) are clearly involved in cell motility. Unique functions are not yet known for other isoforms, but different myosin monomers can decorate actin, and myosins from one species can decorate actin filaments of other species, even across wide phylogenetic distances. Several studies ask why there are so many isoforms of actin and myosin. Check out the questions and research in challenge box below.

18.12 Intermediate Filaments

These 10 nm filaments are proteins with extended secondary structure that don't readily fold into tertiary structures, and they have no enzymatic activity. Recall their intercellular location in desmosomes where they firmly bind cells together to confer tensile strength to tissues. Within cells, intermediate filaments permeate the cells where they participate in regulating and maintaining cell shape. Recall their role in anchoring actin to either Z-disks or plasma membrane *plaques* in muscle cells, transmitting the forces of contraction to the shortening of the sarcomeres and then to the actual shortening of a muscle. The extracellular ***keratins*** that make up fur, hair, fingernails and toenails, are proteins related to intermediate filaments. Keratins are bundles of rigid, insoluble extracellular proteins that align and bind to form stable, unchanging secondary structures. Finally, intracellular intermediate filaments are not confined to the cytoplasm; ***lamins*** are intermediate filaments that make up structural elements of the *nuclear lamina*, a kind of ***nucleoskeleton***.

As we saw earlier, intermediate filament subunits have a common structure consisting of a pair of monomers. The monomers are non-polar; i.e., unlike microtubules and actin filaments, they do not have 'plus' and 'minus' ends. Globular domains at the C- and N-terminal ends of monomer filaments are separated by coiled rod regions. The basic unit of intermediate filament structure is a dimer of monomers. Dimers further aggregate to form tetramers and larger filament bundles. The structural features of intermediate filaments are illustrated in the cartoon in Fig. 18.31 below.

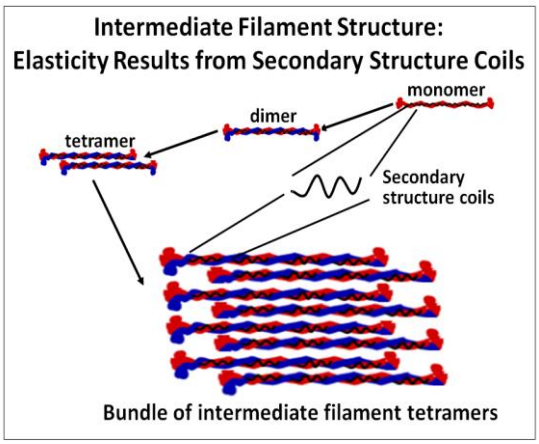


Fig. 18.31: Coiled secondary structure of the proteins permits elasticity of intermediate filament bundles that contribute to the viscosity of cytoplasm.

Like microtubules and actin filaments, intermediate filament bundles can disassemble and reassemble as needed when cells change shape. Unlike microtubules and actin, intermediate filaments can stretch, a property conferred by the coiled rod regions of the filaments. This should be reminiscent of titin molecules!

In the bundled intermediate filaments that permeate the cytoplasm of cells, the ability to stretch contributes to the viscosity of cytoplasm and is even called ***viscoelasticity***. This elastic property is thought to allow actins and microtubules some freedom of movement of and within cells. Like the actins, there are many isoforms of intermediate filaments.

Some iText & VOP Key Words and Terms

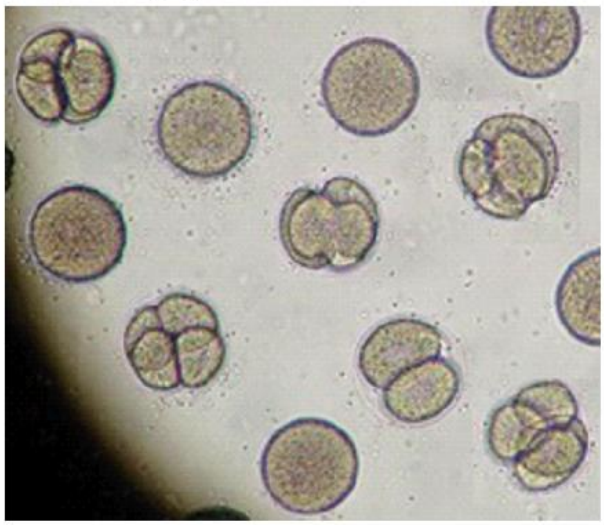
"9+2"	F-actin	myosin ATPase
α-tubulin	F-actin polarity	myosin
A-band	flagella	myosin "heads"
acetylcholine	fluorescence microscopy	neuromuscular junction
acidic keratin	force transduction	nuclear lamina
actin	G-actin	plus and minus ends
actin-binding proteins	hair, horn	protofilaments
actin-myosin interactions	I-band	pseudopodia
actin-myosin paradox	intermediate filaments	sarcomere
action potential	intestinal microvilli	sarcoplasmic reticulum
amoeboid movement	keratin	sarcolemma
ATPase	keratin isoforms	scales, feathers, fingernails
axoneme	lamins	secretion vesicle transport
β-tubulin	membrane depolarization	skeletal muscle contraction
basal body	microfilaments	skeletal muscle relaxation
basic keratin	microtubule assembly end	sliding filament model
Ca++ regulation of contraction	microtubule disassembly end	syncytium
Ca++ release v. active transport	microtubule doublets	thick and thin filaments
cell motility	microtubule organizing center	titin
centriole	microtubule polarity	transverse (T) tubules
cilia	microtubule-associated proteins	tread-milling

contraction regulation	microtubules	tropomyosin
cortical cellular microfilaments	mitotic, meiotic spindle fibers	troponin I
creatine phosphate	M-line	troponin T
cross-bridges	motor proteins	troponins
cytoplasmic streaming	MTOC	troponin C
cytoskeleton	muscle cell	tubulin heterodimer
desmosomes	muscle fiber	tubulins
dynein	myocyte	viscoelasticity
evolution of actin genes	myofiber	Z-disks
evolution of myosin genes	myofibril	Z-line

Chapter 19: Cell Division and the Cell Cycle

Separation of replication from cell division in eukaryotes; cell cycle checkpoints, cyclins and MPF, apoptosis, cancer, radiation, chemotherapy, immunotherapy

Cleavage: the first mitoses of fertilized sea urchin eggs will form an embryo…



19.1 Introduction

Bacteria divide by binary fission, duplicating their nearly 'naked' circular chromosomes as the cell enlarges and divides into two daughter cells. Under favorable conditions, bacteria (*E. coli* for example) can divide every 20-40 minutes. Many eukaryotic cells stop dividing when they reach maturity (their *terminally differentiated state*) while those that do divide may have varying life spans between divisions, ranging from 15-24 hours. Cell division begins with ***mitosis***, the condensation of chromosomes from chromatin. ***Cytokinesis*** is the process near the end of mitosis that actually divides one cell into two new cells after duplicated chromosomes are safely on opposite sides of the cell. Although mitosis lasts only about an hour in the life of the typical eukaryotic cell, it has been parsed into 4 or 5 phases (depending on whose text you are reading!); it is the last of these phases that overlaps cytokinesis. Usually, mitosis and cytokinesis together take about 1.5 hours.

By far the longest period between successive cell divisions or the ***cell cycle*** is ***interphase***, so-called because 19th century microscopists who described mitosis saw nothing happening in cells when they were not actually dividing. But by the 1970s, experiments had shown that interphase itself was divisible into discrete phases of cellular activity called ***G_1***, ***S*** and ***G_2*** (occurring in that order). It turns out that ***kinases*** regulate progress through the cell cycle, catalyzing timely *protein phosphorylations*. The first of these kinases to be discovered was *mitosis-promoting factor* (***MPF***). Kinase-regulated events are ***checkpoints*** a cell must pass through in order to enter the next phase of the cell cycle. As you might guess, the failure of a checkpoint can have serious consequences. One consequence is ***carcinogenesis***, the runaway proliferation of cancer cells. We begin this chapter with a brief description of binary fission in *E. coli*, followed by a closer look at the eukaryotic cell cycle. We close with a look at alternate fates of eukaryotic cells and at details of cellular end-of-life events, including ***apoptosis,*** or ***programed cell death***).

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. describe the *phases of the cell cycle* and what occurs in each.
2. interpret experiments leading to our understanding of the *separation* of chromosomal events from duplication of the DNA contained in those chromosomes.
3. describe the role of *cyclin* and *cdk* (cyclin-dependent kinase) in MPF.
4. compare the roles of different cyclins and cdks in regulating the cell cycle.
5. define cell-cycle checkpoints and how/why they monitor progress through the cell cycle, including their roles in detecting DNA damage and pathways to *apoptosis*.
6. state an hypothesis to explain how cell cycling errors can *transform* normal cells into *cancer* cells.
7. list some examples of apoptosis in humans and other organisms.
8. compare and contrast examples of apoptosis and *necrosis.*
9. formulate an hypothesis to account for the degradation of cyclin after mitosis.
10. research and explain how different chemotherapeutic agents work and the biochemical or molecular basis of their side effects.

19.2 Cell Division in a Prokaryote

The life of actively growing bacteria is not separated into discrete times for duplicating genes (i.e., DNA synthesis) and then ***binary fission*** (dividing and partitioning the duplicated DNA into new cells). Instead, the single circular chromosmome of a typical bacterium is replicating even before fission is complete, so that the new daughter cells already contained partially duplicated chromosomes. Cell growth, replication and fission as it might be seen in *E. coli* are illustrated below in Fig. 19.1.

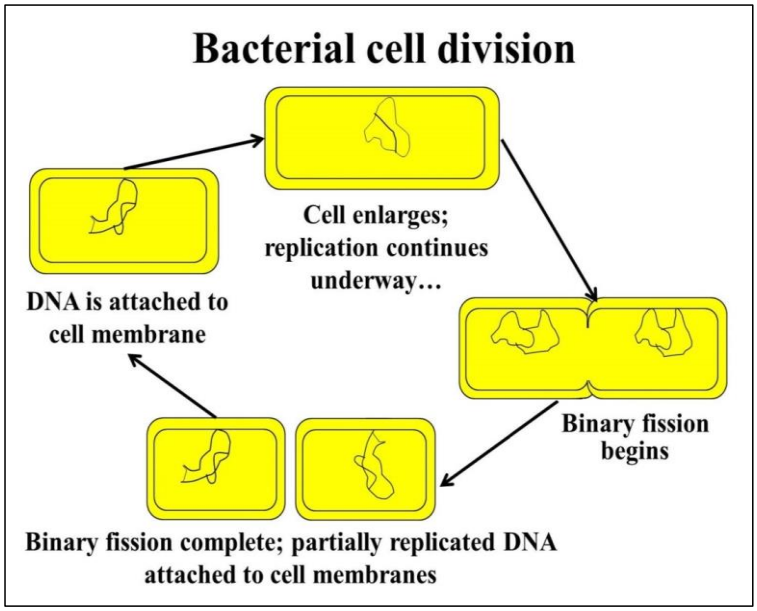


Fig. 19.1: Bacteria divide by binary fission. When growing in culture, they divide continually, partially replicating their circular DNA molecules during one division in preparation for the next.

339 Binary Fission

In marked contrast to to bacteria, the life cycle of a typical eukaryotic cell is divided into sepatrate events. Next we will see how the timing of separated events of the cell cycle was discovered.

19.3 Cell Division in Eukaryotes

Depending on cell type, typical eukaryotic cells have a roughly 16-24 hour cell cycle divided into ***phases***. One of these, ***mitosis***, was discovered In the late 1800s using light microscopy (you may have seen mitosing onion root tip cells in a biology class laboratory). In any cluster of cells some were seen to lose their nuclei and to form ***chromosomes*** (from *chroma*, colored; *soma*, bodies). In *mitosis*, paired chromosomes (**chromatids**) are attached at their centromeres. The chromatids were seen to separate and to be pulled apart by ***spindle fibers***. Once separated and on their way to opposite poles of dividing cells they are once again called chromosomes. Thus homologous chromosomes were equally partioned to the daughter cells at the end of cell division.

Because the same chromosomal behavior was observed in mitosis in diverse organisms, chromosomes were soon recognized as the stuff of inheritance, the carrier of genes! The events of mitosis, lasting about an hour, were seen as occurring in 4 phases over that short time (Fig. 19.2).

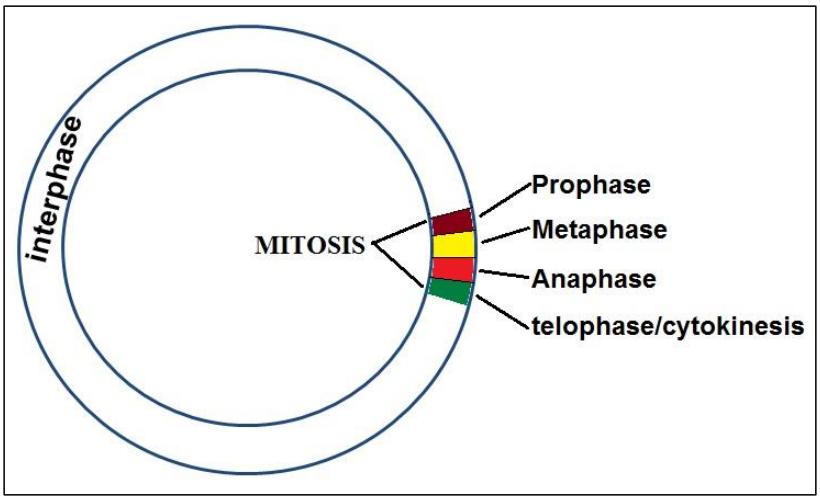


Fig. 19.2: Mitosis and cytokinesis in eukaryotic cells are separated in time, and mitosis is further divisible into 5 phases.

To the early microscopists, this short period of intense mitotic activity was in stark contrast to a much longer '*quiet'* time in the life of the cell. For lack of a more descriptive term, they called this period ***interphase***. Also, depending on whom you ask, ***cytokinesis*** (the cell movements that actually divide a cell in two) is *not* part of mitosis. In that sense, we can think of three stages in the life of a cell: interphase, mitosis and cytokinesis. Of course, it turned out that interphase is not cellular 'quiet time' at all!

19.3.1 Defining the Phases of the Eukaryotic Cell Cycle

Correlation of the inheritance of specific traits with the inheritance of chromosomes was shown early in the early 20th century, most elegantly in genetic studies of the fruit fly, *Drosophila melanogaster*. At that time, chromosomes were assumed to contain the genetic material and that both were duplicated during mitosis. The first clue that this was not so came only after the discovery that DNA was in fact the chemical *stuff of genes*. The experiment outlined in Fig. 19.3 (below) distinguished the time of chromosome formation from the time of DNA duplication.

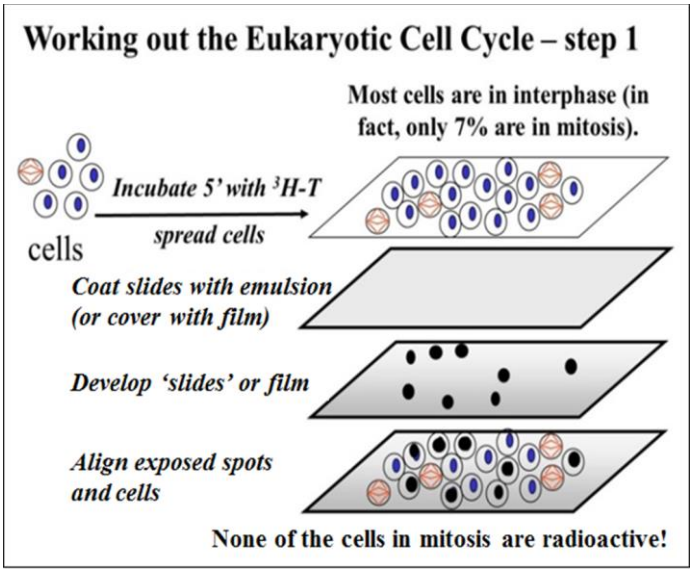


Fig. 19.3: First experiment demonstrating that replication and cell division in eukaryotes are separate events. (see text for details

Here are the details of the experiment:

1. Cultured cells were incubated with ***^{3}H-thymine***, the radioactive base that cells will incorporate into deoxythymidine triphosphate (dTTP, one of the four nucleotide precursors to DNA synthesis).
2. After a short period of culture, unincorporated ^{3}H-thymine was washed away and the cells were fixed and spread on a glass slide.
3. Slides were dipped in a light-sensitive emulsion, containing chemicals similar to the light sensitive chemicals found in the emulsion-side of film (do you remember photographic film!!?).
4. After sufficient time to allow the radioactivity on the slide to '*expose'* the emulsion, the slides were developed (in much the same way as developing photographic film).
5. The resulting ***autoradiographs*** in the microscope revealed images in the form of dark spots created by exposure to ***hot*** (i.e., radioactive) DNA.

If DNA replicates in chromosomes undergoing mitosis, then when the developed film is placed back over the slide, any dark spots should lie over the cells in mitosis, and not over cells that are not actively dividing. But observation of the autoradiographs showed that none of the cells in mitosis were radioactively labeled. On the other

hand, some of the cells in interphase were! Therefore, DNA synthesis must take place sometime in interphase, before mitosis and cytokinesis, as illustrated in Fig. 19.4, below.

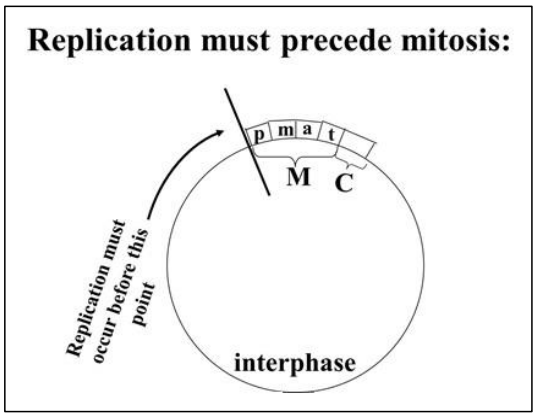


Fig. 19.4: The autoradiographic data from the experiment outlined in Fig. 19.3 demonstrates that DNA synthesis begins and ends some time before the beginning of mitosis.

340-2 Experiments Revealing Replication in Cell Cycle Interphase

As we will see, the synthesis of nuclear DNA in eukaryotic cells (replication) occurs many hours before the start of mitosis, and in fact lasts a good chunk of time of the typical cell cycle.

Next a series of ***pulse-chase*** experiments were done to determine when in the cell cycle DNA synthesis actually takes place. Cultured cells given a short *pulse* (exposure) to ^{3}H-thymine and then allowed to grow in non-radioactive medium for different times (the *chase*). At the end of each chase time, cells were spread on a glass slide and again prepared for autoradiography. Analysis of the autoradiographs identified distinct periods of activity within interphase: $\boldsymbol{G_1}$ (*Gap1*), ***S*** (a time of DNA *synthesis*) and $\boldsymbol{G_2}$ (*Gap 2*). Here are the details of these very creative experiments, performed before it became possible to synchronize cells in culture so that they would all be growing and dividing at the same time.

1. Cells were exposed to ^{3}H-thymine for just 5 minutes (the ***pulse***) and then centrifuged. The radioactive supernatant was then discarded
2. The cells were rinsed and spun again to remove as much labeled precursor as possible.

3. The cells were re-suspended in fresh medium containing unlabeled (i.e., non-radioactive) thymine and further incubated for different times (the ***chase*** periods).
4. At each chase time, cells washed free of radioactive precursor and spread on glass slides.

After dipping the slides in light-sensitive emulsion, exposing and developing the film, the autoradiographs were examined, with the results shown in Fig 19.5, below.

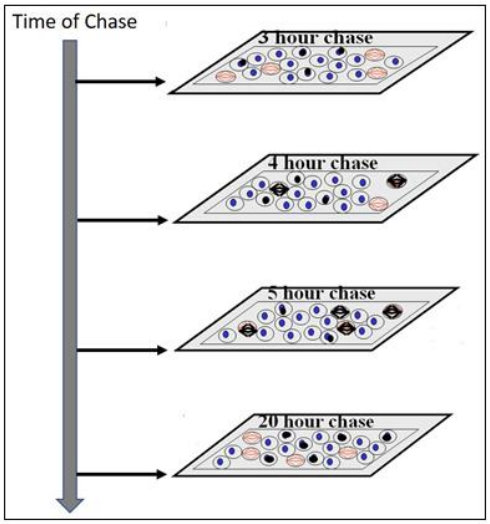


Fig. 19.5: These autoradiographs from a *pulse-chase* labeling experiment led to the identifying the phases of the eukaryotic cell cycle (see text for explanation).

Here is a description of the results:

- After a 3-hour (or shorter) chase period, the slides looked just as they would immediately after the pulse. That is, while 7% of the cells were in mitosis, none are radioactively labeled. In contrast, many *interphase* cells showed labeled nuclei.
- After 4 hours of chase, a few of the 7% of the cells that were in mitosis were labeled, along with others in interphase.
- After a 5-hour chase, most cells in mitosis (still about 7% of cells on the slide) were labeled; many fewer cells in interphase were labeled.
- After a 20-hour chase, none of the 7% of cells that were in mitosis is labeled. Instead, all of the labeled cells are in interphase.

The graph in Fig.19.6 plots a count of *radiolabeled mitotic cells* against *chase times*.

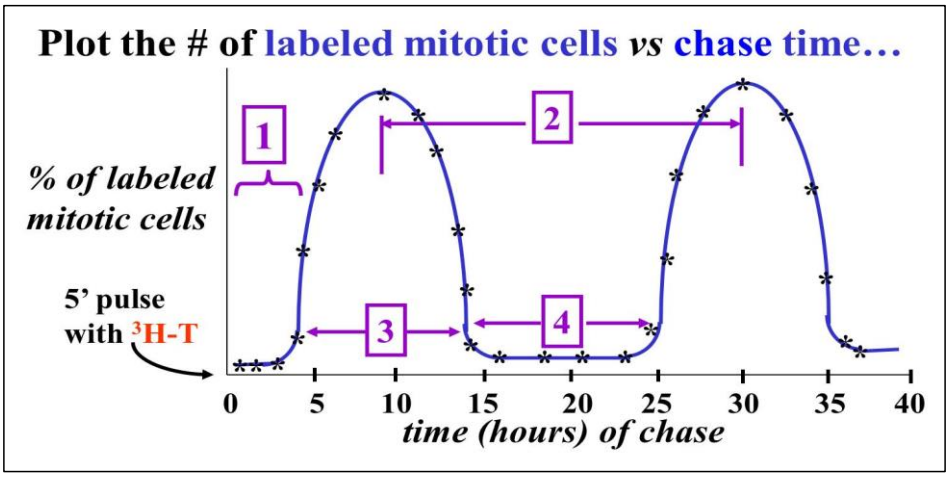


Fig. 19.6: Graph plotting the number of radioactive cells in mitosis over time of chase in the pulse - chase experiment described in Fig. 19.5 (see text for explanation).

The plot defines the duration of events (phases) of the cell cycle as follows:

1. The first phase (interval ***#1*** on the graph) must be the time between the end of DNA synthesis and the start of mitosis, defined as ***Gap 2*** (***G_2***).
2. Cell doubling times are easily measured. Assume that the cells in this experiment doubled every 20 hours. This would be consistent with the time interval of 20 hours between successive peaks in the number of radiolabeled mitotic cells after the pulse (interval ***#2***).
3. Interval ***#3*** is easy enough to define. It is the time when DNA is synthesized, from start to finish; this is the ***synthesis***, or ***S*** phase.
4. One period of the cell cycle remains to be defined, but it is not on the graph! That would be the time between the end cell division (i.e., mitosis and cytokinesis) and the beginning of DNA synthesis (*replication*). That interval can be calculated from the graph as the time of the cell cycle (~20 hours) minus the sum of the other defined periods of the cycle. This phase is defined as the ***Gap 1*** (***G_1***) phase of the cycle.

Fig. 19.7 is a typical eukaryotic cell cycle, summarizing events in each phase.

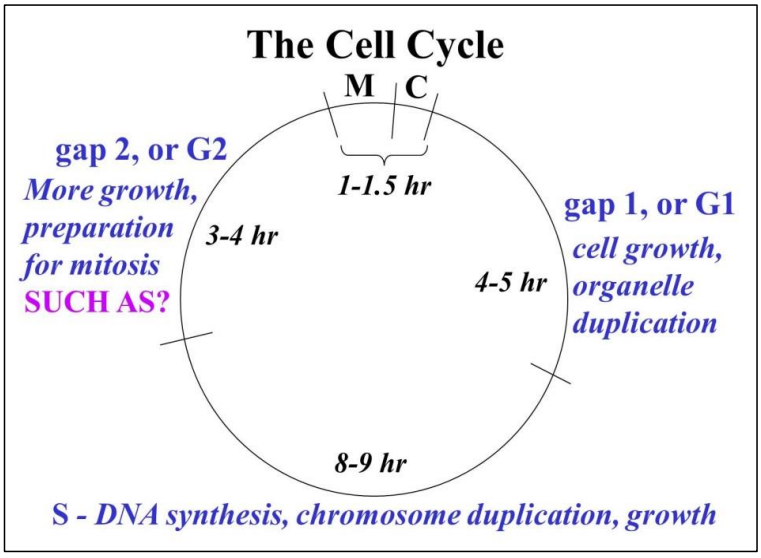


Fig. 19.7: A typical eukaryotic cell cycle: phases G1, S and G2 follow cytokinesis; G2 immediately precedes prophase of mitosis. The length of time for each phase differs for different cell types.

During ***G₁, S*** and ***G₂*** of interphase, the cell grows in size, preparing for the next cell division. As you might guess, ***G₁*** includes synthesis of enzymes and other proteins needed next for replication.

DNA replicates in the S phase, along with the synthesis of new *histone* and other proteins that will be needed to assemble new *chromatin*. ***G₂*** is the shortest time of interphase and is largely devoted to preparing the cell for the next round of mitosis and cytokinesis. Among the proteins whose synthesis increases in this time are the *tubulins* and proteins responsible for condensing chromatin into paired chromatids representing the duplicated chromosomes. ***Cohesin*** is a more recently discovered protein made in the run-up to mitosis. It holds centromeres of chromatids together until they are ready to separate.

341-2 Events in the Phases of the Cell Cycle

We noted that typical dividing cells have generation times ranging from 16 to 24 hours. Atypical cells, like newly fertilized eggs, might divide every hour or so! In these cells, events that normally take many hours must be completed in just fractions of an hour.

19.3.2 When Cells Stop Dividing…

Terminally differentiated cells are those that spend the rest of their lives performing a specific function. These cells no longer cycle. Instead, shortly after entering G_1 they are diverted into a phase called ***Go***, as shown in Fig. 19.8.

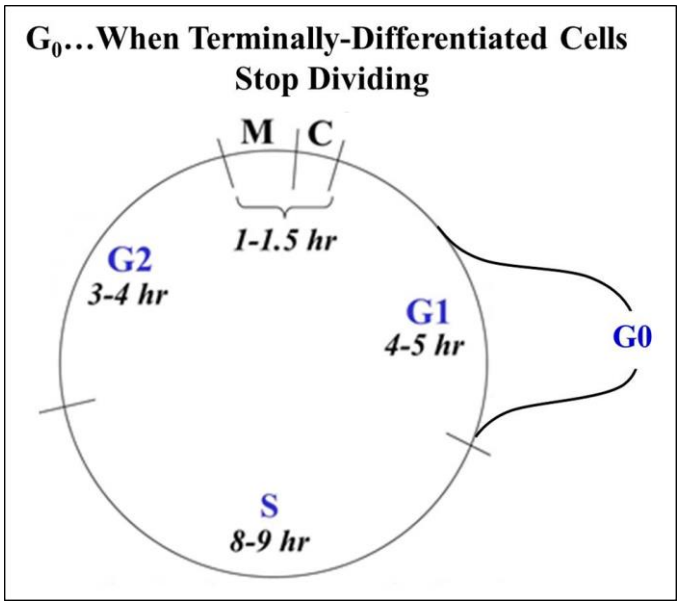


Fig. 19.8: Terminally differentiated cells no longer divide, entering the Go state. While they can sometimes resume cycling, such cells more typically experience cell death and replacement by stem cells.

Referred to as ***terminally differentiated***, these cells normally never divide again. With a few exceptions (e.g., many neurons), most terminally differentiated cells have a finite lifespan, and must be replaced by stem cells. A well-known example is the erythrocyte, or red blood cell. With a half-life of about 60 days, these cells are regularly replaced by precursor reticulocytes produced in bone marrow.

19.4 Regulation of the Cell Cycle

Progress through the cell cycle is regulated. The cycle can be controlled or put on 'pause' at any one of several phase transitions. Such ***checkpoints*** monitor whether the cell is on track to complete a successful cell division event. Superimposed on these controls are

signals that promote cell differentiation. Embryonic cells *differentiate* as the embryo develops. Even after *terminal differentiation* of cells that form all adult tissues and organs, *adult stem cells* will divide and differentiate to replace worn out cells. Once differentiated, cells are typically signaled in G_1 to enter ***Go*** and stop cycling. In some circumstances cells in ***Go*** are recruited to resume cycling. However, if this resumption should occur by mistake, the cells may be *transformed* to cancer cells. Here we consider how the normal transition between phases of the cell cycle is controlled.

19.4.1 Discovery and Characterization of *Maturation Promoting Factor (MPF)*

To monitor their progress through the cell cycle, cells produce internal chemical signals that tell them when it's time to begin replication or mitosis, or even when to enter into ***Go*** when they reach their terminally differentiated state. The experiment that first demonstrated a chemical regulator of the cell cycle involved fusing very large frog's eggs! The experiment is described in Fig. 19.9.

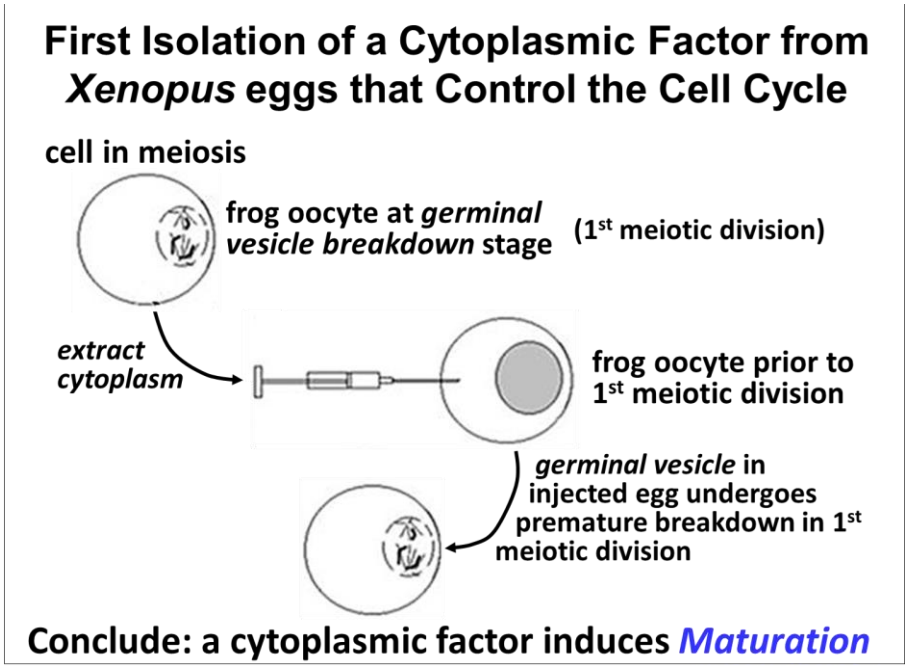


Fig. 19.9: Experiment leading to the discovery of *meiosis-promoting factor* (*MPF*), the first known chemical regulator of cell division.

The hypothesis tested here was that frog oocyte cytoplasm from ***germinal vesicle stage*** oocytes (i.e., oocytes in mid-meiosis) contain a chemical that causes the cell to lose its nuclear membrane, condense its chromatin into chromosomes and enter meiosis. The test was to withdraw cytoplasm from one of these large, mid-meiotic oocytes with a fine hypodermic needle and inject it into a pre-meiotic oocyte. The result of the experiment was that the mid-meiotic oocyte cytoplasm induced premature meiosis in the immature oocyte. Subsequently a ***maturation promoting factor*** (***MPF***) was isolated from the mid-meiotic cells that when injected into pre-meiotic cells, caused them to enter meiosis. ***MPF*** turns out to be a protein kinase made up of two polypeptide subunits (Fig. 19.10).

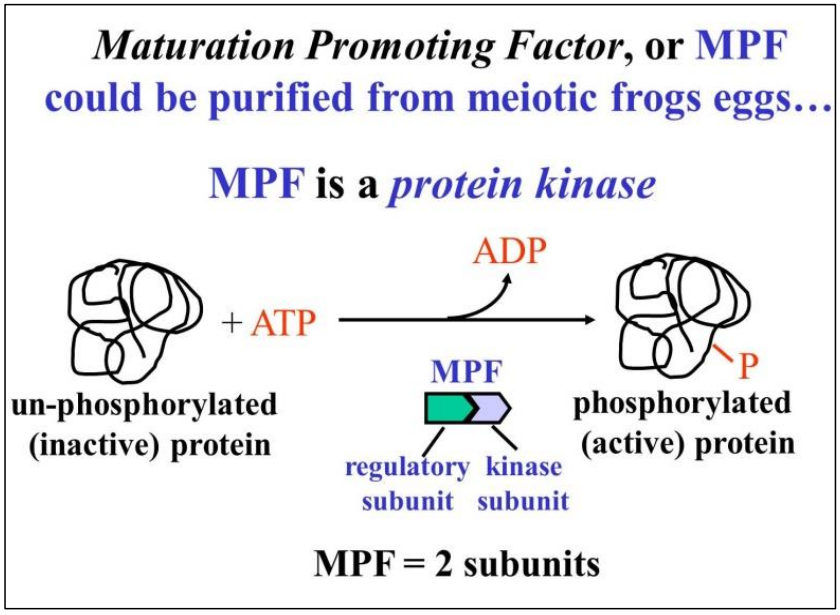


Fig. 19.10: MPF was shown to be a two-subunit *protein kinase* that transfers phosphates from ATP to several different proteins.

MPF was then also shown to stimulate *somatic cells* in G_2 to enter premature mitosis. So (conveniently) *MPF* can also be ***Mitosis Promoting Factor***! Hereafter we will discuss the effects of *MPF* as being equivalent in mitosis and meiosis. When active, *MPF* targets many cellular proteins.

342 Discovery of MPF Kinase and Its Role in Meiosis and Mitosis

Assays of *MPF* activity as well as the actual levels of the two subunits over time during the cell cycle are graphed in Fig. 19.11.

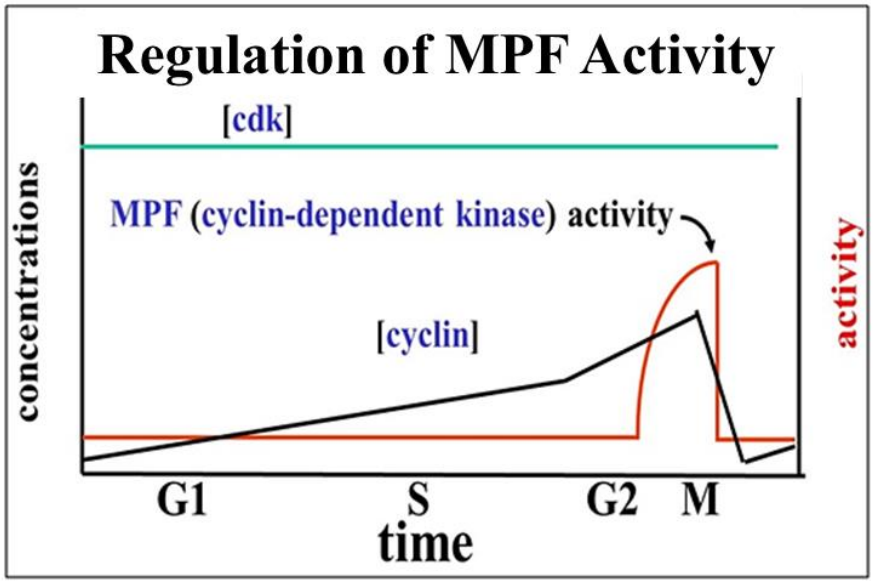


Fig. 19.11: Graph comparing plots of cellular *cdk* and *cyclin* levels over time with one of *MPF* activity (see text for details).

One subunit of *MPF* is ***cyclin***. The other subunit, ***cyclin-dependent kinase*** (***cdk***), contains the kinase enzyme *active site*. Both subunits must be bound to make an active kinase. Cyclin was so-named because its levels rise gradually after cytokinesis, peak at the next mitosis, and then fall. Levels of the cdk subunit do not change significantly during the life of the cell. Because the kinase activity of MPF requires *cyclin*, it tracks the rise in cyclin near the end of the G_2, and its fall after mitosis. Cyclin begins to accumulate in G_1, rising gradually and binding to more and more *cdk* subunits. *MPF* reaches a threshold concentration in G_2 that triggers entry into mitosis. For their discovery of these central molecules Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse won the 2001 Nobel Prize in Physiology or Medicine.

19.4.2 Other Cyclins, CDKs and Cell Cycle Checkpoints

Other chemical signals accumulate at different points in the cell cycle. For example, when cells in ***S*** are fused with cells in G_1, the G_1 cells begin synthesizing DNA (visualized as ^{3}H-thymine incorporation).

Fig 19.12 describes an experiment showing control of progress to different phases of the cell cycle.

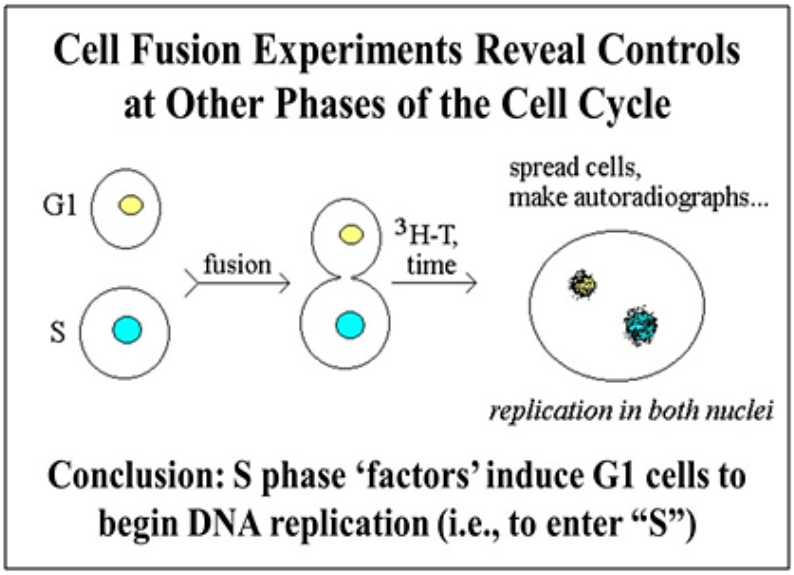


Fig. 19.12: Cell fusion experiments revealed additional chemical regulators of the cell cycle activity (see text for details).

An ***S*** phase factor could be isolated from the ***S*** phase cells. This factor also turns out to be a two-subunit protein kinase, albeit a *different* one from MPF. Just as MPF signals cells in G_2 to begin mitosis, the ***S*** phase kinase signals cells in G_1 to enter the ***S*** phase of the cell cycle and to start replicating DNA. *MPF* and the ***S*** phase kinase govern activities at two of several cell cycle ***checkpoints***. In each case, the activity of the kinases is governed by prior progress through the cell cycle. In other words, if the cell is not ready to begin mitosis, active *MPF* production is delayed until it is. Likewise, the ***S*** phase kinase will not be activated until the cell is ready to begin DNA synthesis.

343 Cell Cycle Control at Check Points and the Go "Phase"

The sequence of signals that control progress through the cell cycle is probably more intricate and extensive than we currently know, but the best-described checkpoints are in G_1, G_2 and ***M*** (Fig. 19.13, below).

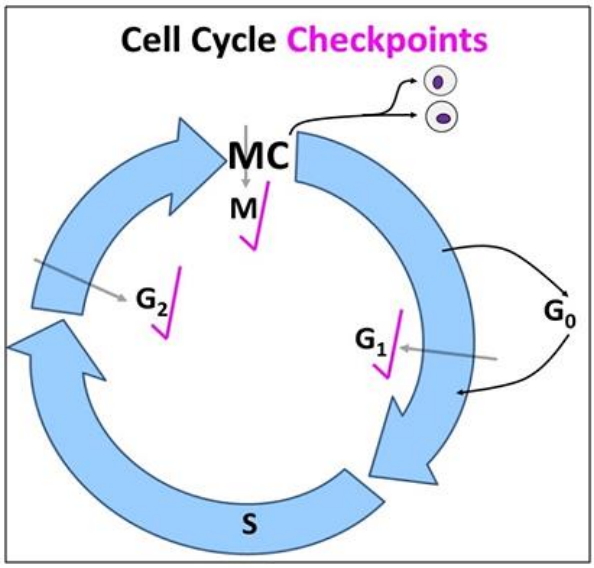


Fig. 19.13: Simplified diagram of cell cycle checkpoints at which progress through the cycle is assessed. If progress through a phase is incomplete, cell cycle regulators (kinases) delay onset of the next phase. (see text for details).

We generally envision checkpoints as monitoring and blocking progress until essential events of a current phase of the cell cycle phase are completed. These kinases are part of molecular sensing mechanisms that act by phosphorylating cytoplasmic and/or nuclear proteins required by upcoming phases of the cycle. Let's take a look at some events that are monitored at these *checkpoints* in more detail.

19.4.2.a The G1 Checkpoint

The ***G_1 checkpoint*** controls the transition from the *G_1* to the S phase of the cell cycle. If actively dividing cells (e.g., stem cells) in G_1 fail to complete their preparation for replication, the S-phase kinase won't be produced and the cells won't proceed the S phase until the preparatory biochemistry catches up with the rest of the cycle. To enter S, a cell must be ready to make proteins of replication, like DNA polymerases, helicases, primases…, among others. Only when these molecules have accumulated to (or become active at) appropriate levels, is it "safe" to enter S and begin replicating DNA. Now, what about those cells that are fully differentiated? *Terminally* differentiated cells stop producing the active ***G_1*** *checkpoint* kinase and stop dividing. Thus they are arrested in ***Go*** (see below).

19.4.2.b The G_2 Checkpoint

Passage through the ***G₂*** checkpoint is only possible if DNA made in the prior ***S*** phase is not damaged. Or if it was, that the damage has been (or can be) repaired (review the proofreading functions of DNA polymerase and the various DNA repair pathways). Cells that do successfully complete replication and pass the ***G₂*** checkpoint must prepare to make the proteins necessary for the upcoming mitotic phase. These include nuclear proteins necessary to *condense* chromatin into chromosomes, *tubulins* for making *microtubules*, etc. Only when levels of these and other required proteins reach a threshold can the cell begin mitosis. Consider the following two tasks required of the ***G₂*** checkpoint (in fact, any checkpoint):

- sensing whether prior phase activities have been successfully completed.
- delaying transition to the next phase if those activities are unfinished.

But what if sensing is imperfect and a checkpoint is leaky? A recent study suggests that either the ***G₂*** checkpoint ***is*** leaky, or at least, that incomplete activities in the ***S*** phase are tolerated, and that some DNA repair is not resolved until mitosis is underway in M***!*** Check it out at DNA repair and replication during mitosis.

19.4.2.c The M Checkpoint

The ***M*** checkpoint is monitored by the original *MPF*-catalyzed phosphorylation of proteins that

a) bind to chromatin causing it to condense and form chromatids,
b) lead to the breakdown of the nuclear envelope, and
c) enable spindle fiber formation.

We have seen that the tension in the spindle apparatus at metaphase tugs at the kinetochores holding the duplicated chromatids together. When this tension reaches a threshold, MPF peaks and an activated ***separase*** enzyme causes the chromatids to separate at their centromeres. Beginning in *anaphase*, continuing tension in the spindle apparatus draws the new chromosomes to opposite poles of the cell. Near the end of mitosis and cytokinesis, proteins phosphorylated by MPF initiate the breakdown of cyclin in the cell. Passing the ***M*** checkpoint means that the cell will complete mitosis and cytokinesis, and that each daughter cell will enter a new ***G₁*** phase.

Dividing yeast cells seem to have the three checkpoints discussed here. More complex eukaryotes use more *cyclins* and *cdk*s to control the cell cycle at more checkpoints. Different *cyclins* show cyclic patterns of synthesis, while their *cdks* remain at constant levels throughout the cell cycle (as in *MPF*). Different gene

families encode evolutionarily conserved *cdks* or *cyclins*. But each *cyclin*/*cdk* pair has been coopted in evolution to monitor different cell cycle events and to catalyze phosphorylation of phase-specific proteins. To learn more, see Elledge S.J. (1996) *Cell Cycle Checkpoints: Preventing an Identity Crisis.* Science 274:1664-1672.

344-2 Cyclin/cdk Checkpoints for Cell Cycle Phases

19.4.2.d The Go State

Go is not really a phase of the cell cycle, since cells in *Go* have reached a terminally differentiated state and have stopped dividing. Terminally differentiated cells in tissues and organs no longer divide. But even these cells have finite half-lives (recall our red blood cells that must be replaced every 60 days or so). Because cells in many tissues are in *Go* and can't divide, they must be replaced by stem cells, which can divide and differentiate. Some cells live so long in ***Go*** that they are nearly never replaced (muscle cells, neurons). Other cells live short lives in ***Go*** (e.g., stem cells, some embryonic cells). For example, a ***lymphocyte*** is a differentiated *immune system* white blood cell type.

However, exposure of lymphocytes to foreign chemicals or pathogens activates ***mitogens*** that cause them to re-enter the cell cycle from ***Go***. The newly divided cells then make the antibodies that neutralize toxic chemicals and fight off the pathogens. The ***retinoblastoma*** (***Rb***) protein is such a mitogen, a transcription factor that turns on genes that lead to cell proliferation.

What if cells cycle when they shouldn't? What if they are inappropriately signaled to exit ***Go***? Such cells are in trouble! Having escaped normal controls on cell division, they can become a focal point of cancer cell growth. You can guess from its name that the retinoblastoma gene was discovered as a mutation that causes retinal cancer. For more about the normal function of the Rb protein and its interaction with a ***G1*** *cdk*, check out the link below.

345-2 Rb Gene Encodes Transcription Factor Regulatory Subunit

19.5 When Cells Die

As noted, few cell types live forever; most are destined to turn over (another euphemism for dying), mediated by ***programmed cell death***, or ***apoptosis***. Apoptosis occurs in normal development when cells are only temporarily required for a maturation process

(e.g., embryonic development, metamorphosis). When older cells are no longer necessary or when cells are genetically damaged, they too are detected and signaled to undergo *apoptosis*. Frog metamorphosis is a well-known example of Apoptosis. Signaled by *thyroid hormone*, tadpoles digest their tail cells, reabsorbing and recycling the digestion products. These in turn serve as nutrients to grow adult frog structures. Normal and apoptotic cells are compared in micrographs and drawings in Figure 19.14 (below).

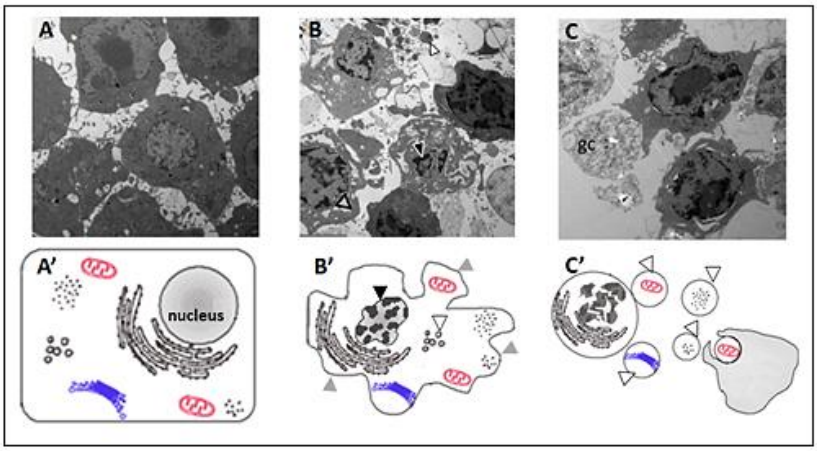


Fig. 19.14: Apoptotic Cells: In the 3 electron micrographs and corresponding illustrations, normal cells (**A** & **A'**) are followed by apoptotic cells [**B** & **B'**] and [**C** & **C'**]. In [**B** & **B'**], a black arrowhead indicates nuclear condensation (*pyknosos*), membrane blebbing (grey arrowheads) and apoptotic bodies (white arrowhead). In [**C** & **C'**] the nucleus has broken down (*karyorrhexis*) and the cell itself cell has fragmented into apoptotic bodies (white arrowheads). In **C'**, a phagocyte is engulfing one of them.

Apoptosis is first associated with nuclear fragmentations and cell shrinkage ([B, B'] and [C, C'] images). Apoptotic bodies in the cell leave the cell in or as 'blebs' (evaginations) where they may be engulfed by phagocytes.

Click http://www.cellimagelibrary.org/images/43705 to see a time lapse movie of live prostate cancer cells that were chemically induced to undergo apoptosis. Look for the formation of numerous apoptotic bodies; you might also see some non-apoptotic cells undergoing cytokinesis!

We now understand the basic metabolic pathway leading to apoptosis. During development, apoptotic cell death starts with an external signal programmed to appear at a specific time in development. Similar signals appear in response to cell obsolescence or genetic damage. The biochemical pathway to apoptosis is illustrated in Fig. 19.15 (below).

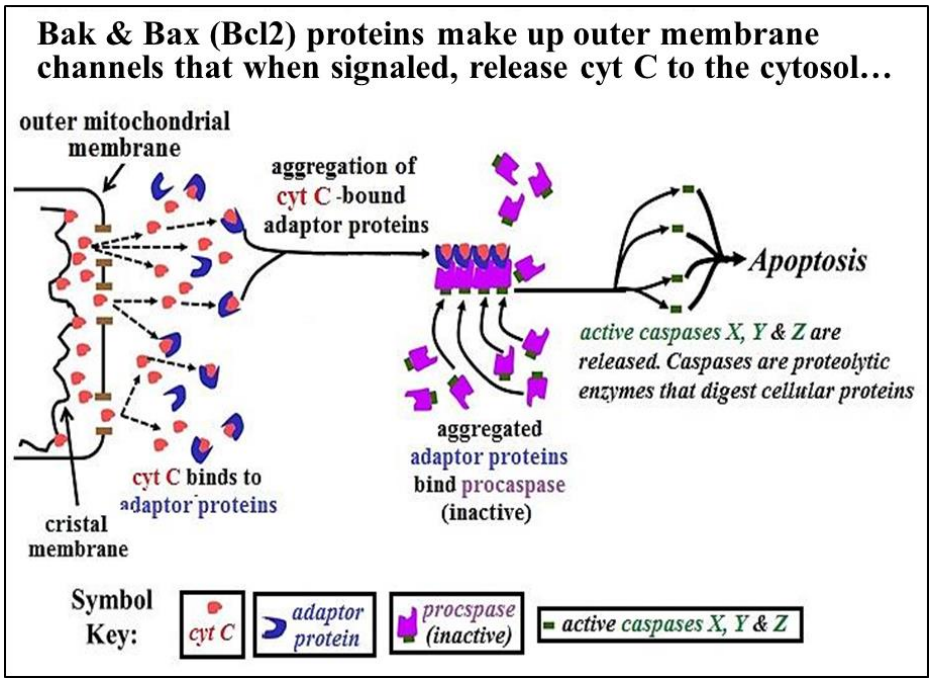


Fig. 19.15: Biochemical steps of *apoptosis*, or *programmed cell death* (see text for details).

The signal molecule acts on target cells to induce transcription of ***Bcl2*** genes. *Bcl2* proteins ***Bak*** and ***Bax*** are outer mitochondrial membrane channel components that allow the release of *cytochrome C into the cytoplasm.* This sets off molecular events leading to *apoptosis*.

Mitochondrial exit of cytochrome C is possible because it is a *peripheral* membrane protein, only loosely bound to the cristal membrane. It exists in equilibrium between membrane-bound and unbound states. As some cytochrome C molecules exit the intermembrane space, others detach from the cristal membrane and follow. In the cytosol, cytochrome C binds to ***adaptor*** proteins that then aggregate. The cytochrome c-adaptor complex has a high affinity for a biologically ***inactive procaspase***. Binding of p*rocaspase* to the *cytochrome C-adaptor complex* causes an allosteric change in the *procaspase,* releasing an active ***caspase***. *Caspases* are *proteolytic enzymes* that start the auto-digestion of the cell. For their work in identifying apoptosis genes, Sydney Brenner, H. Robert Horvitz and John E. Sulston shared the 2002 Nobel Prize in Physiology or Medicine.

In contrast to apoptosis, cells that die unexpectedly from external injury undergo ***necrosis***, an *accidental* rather than a programmed death. Differences in the apoptotic and necrotic pathways are compared in the illustration in Fig. 19.16 (below).

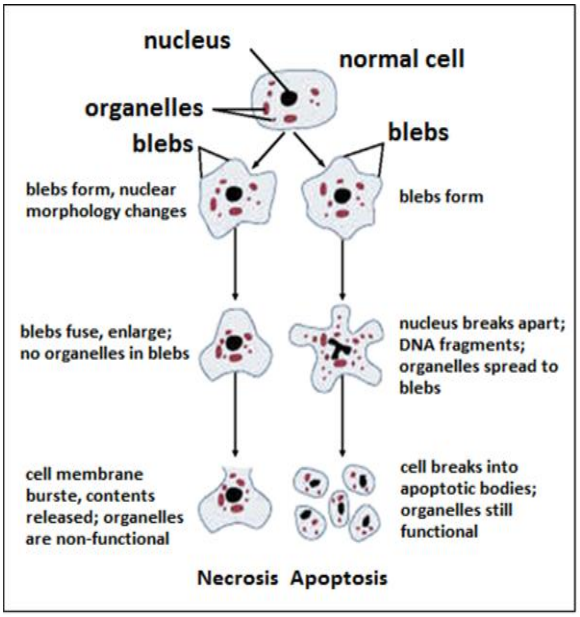


Fig. 19.16: Comparison of the cellular events of apoptosis and necrosis.

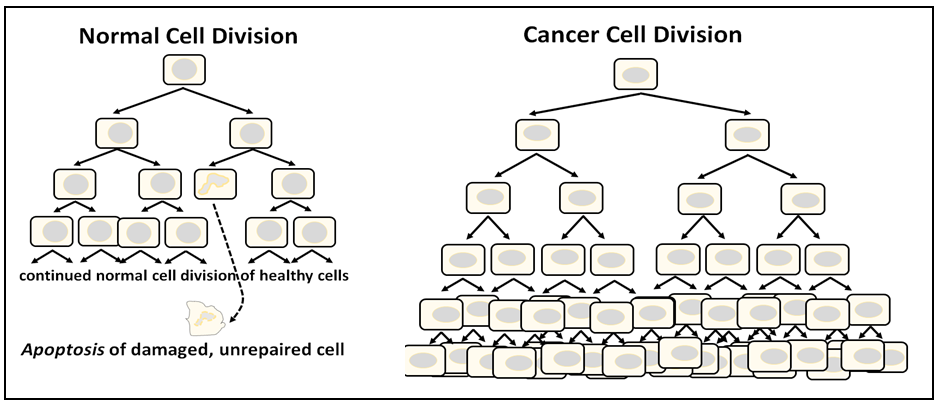
Key differences between the two kinds of cell death are:

- in necrotic cells the nucleus ***does not shrink or fragment***, blebs do form but eventually fuse, the cell membrane eventually bursts and ***cell contents are released*** into the extracellular environment.
- In apoptotic cells, the nucleus ***does fragment***, blebs surround cell contents (including organelles), the ***blebs disrupt the cell into apoptotic bodies***, and ***cell contents are not released*** into the extracellular environment.

19.6 Disruption of the Cell Cycle Checkpoints Can Cause Cancer

As we've noted, cycling cells continue to divide until they attain ***Go*** in the terminally differentiated state. Most terminally differentiated cells are cleared by *apoptosis* when they reach the end of their effective lives, to be replaced by stem cells. We also noted

that accidental signaling can bring cells out of ***Go***, leading to renewed cell proliferation. While these cells are obviously abnormal, they may not be detected by apoptotic defense mechanisms. As a result, they undergo uncontrolled cell divisions, becoming cancer cells. Likewise, If a checkpoint fails or if a cell suffers physical damage to chromosomes during cell division, or if it suffers a debilitating somatic mutation in a prior ***S*** phase, it may self-destruct in response to a consequent biochemical anomaly. This is another example of ***apoptosis.*** But if a physically damaged or mutated cell should escape apoptotic clearance, it too may begin proliferating, producing cancer cells. Apoptotic clearance and uncontrolled cancer cell proliferation are compared in Fig. 19.17.



19.17: Results of non-cancer cell divisions in which abnormal cells are targeted for apoptosis, and cancer cell division, in which damaged or altered cells escape apoptotic controls.

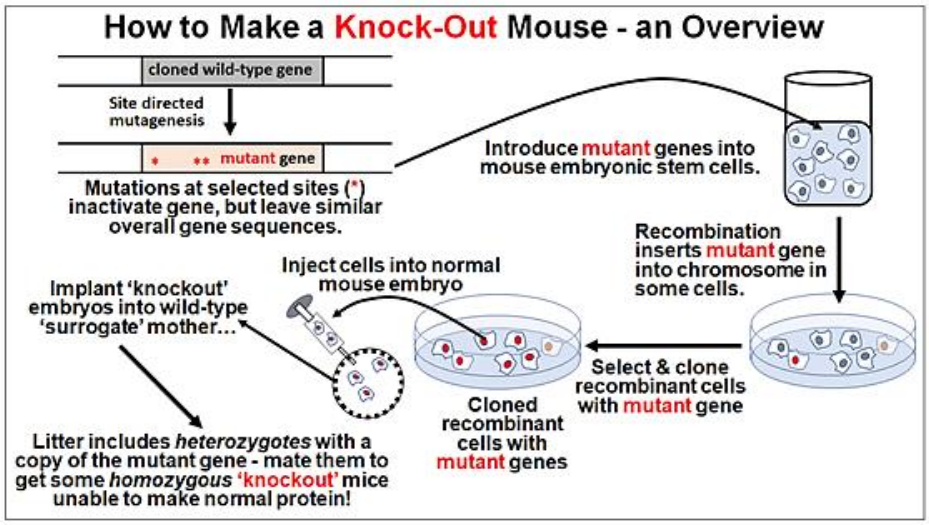
346-2 Regulating Cell Death - Apoptosis *vs.* Necrosis

19.7 p53 Protein Mediates Normal Cell Cycle Control

Cancerous growth may result if a normal dividing cell suffers a somatic mutation that disrupts normal cell cycle control. Think over-expression of *cdk* for example, or imagine *cyclin* levels in daughter cells that never drop; such cells would never stop cycling.

Other possibilities include a cell in ***Go*** that is stimulated to begin cycling again by an inappropriate encounter with a hormone or other signal. If undetected, these anomalies can transform normal cells to cancer cells. This is apparently the case for a mutation in the human ***p53*** gene that causes *Li-Fraumeni syndrome*. The symptoms include a high

incidence of early-age bone, blood and breast cancers (see Li-Fraumeni syndrome for more details). The role of ***p53*** protein in regulating cancer cell formation was shown using ***knockout mice***. We noted earlier the use of ***knockout mutations*** in studies of specific gene function. Fig. 19.18 is a protocol for making knockout mutations in mice.



Fig, 19.18: Making knockout mutant mice. Specific nucleotides in a cloned gene of interest are altered by ***site-directed mutagenesis***. The altered gene is inserted into embryonic stem cells where it recombines with and replaces the homologous gene already in the chromosome. Recombinant clones are selected re-injected into embryos that are then incubated in the uteri of 'foster mother' mice. Knockout mice in the newborn litter can be selected and studied for the effects of removing a gene.

For their demonstration how knockout organisms could be engineered using molecular technologies, Mario R. Capecchi, Martin J. Evans and Oliver Smithies shared the 2007 Nobel Prize in Physiology or Medicine (read all about it at The Knockout Nobel Prize).

The role of ***p53*** protein in regulating cancer cell formation in mice was shown in a knockout experiment when the normal mouse *p53 genes* were replaced with a pair of mutant *p53* genes unable to make functional **p53** ***protein***. The resulting ***knock-out mice*** developed tumors and cancers at a much higher rate than normal mice. In general, many ***knock-out*** organisms have been created to study the effects of ***loss-of-function*** mutations *in vivo* (i.e., in whole animals). Using similar strategies, ***knock-in*** protocols can insert normal genes into an organism lacking the genes, allowing study of the effects of ***gain-of-function*** mutations.

19.7.1 p53 is a DNA-Binding Protein

If a cell in ***Go*** is stimulated to begin cycling again by an inappropriate encounter with a hormone or other signal, it may be transformed into a cancer cell. It is the job of the **p53 *protein*** to detect these anomalies and enable dividing cells to repair the damage before proceeding through cell cycle check points. Failing that, the p5 protein will signal apoptosis of the cell. p53 is a DNA-binding, gene-regulatory protein. Fig. 19.19 (below) shows the structure of the P53 protein and how it binds to the DNA double helix.

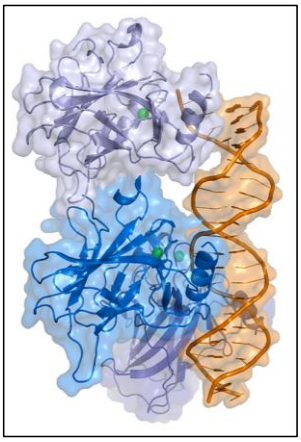
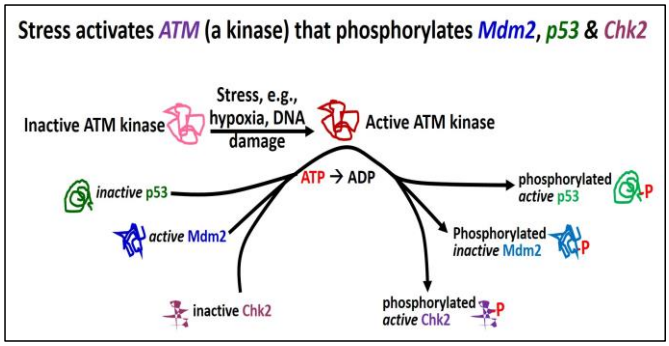


Fig. 19.19: Structure of the *P53* gene-regulatory protein bound to DNA. P53 was originally called a *tumor suppressor protein* because when mutated, tumors arose.

Mutations in the gene for the *P53 protein* in humans (where it is called ***TP53***) are associated with many human cancers. Cultured cells mutagenized p53 genes also exhibit key characteristics of cancer cells, including unregulated cell proliferation and suppression of apoptosis.

19.7.2 How p53 Works to Salvage Cells

The *p53 protein* is normally bound to an active ***Mdm2*** protein. To enable cell cycle checkpoints, *p53-Mdm2* must separate and be kept separate to allow p53 time to act. In dividing cells, physical stress or chemical stress such as DNA damage during cell growth can activate an ***ATM kinase***. ATM kinase in turn, phosphorylates *Mdm2,* causing it to dissociate from p53. The same kinase also phosphorylates another kinase, ***Chk2***, as well as the now 'free' ***p53.*** ATM kinase-initiated events are further detailed in Fig. 19.20 (below).

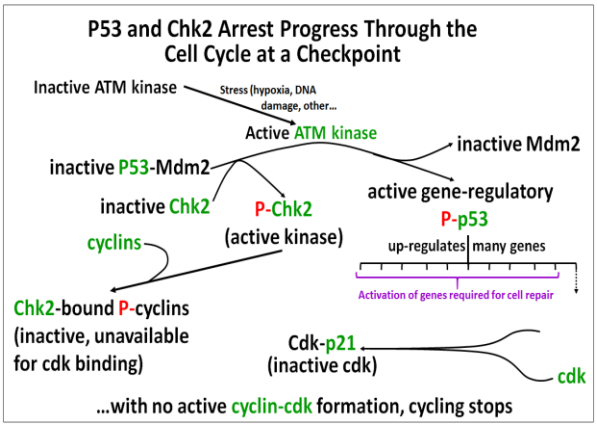


19.20: Normal function of the *P53* gene-regulatory protein (see text for details).

Each of the proteins and enzymes phosphorylated by the ATM kinase has a role in cell cycle checkpoint function and cell cycle arrest while errors are corrected:

1. Now separated from Mdm2, ***Phospho-p53*** actively *up-regulates* several genes, including the ***p21*** gene.
2. The ***P21*** protein binds to ***cdks***; ***cyclins*** can't bind ***P21-cdks***.
3. Active ***Phospho-Chk2*** catalyzes cyclin phosphorylation; ***phospho-cyclins*** can't bind to ***p21-cdks***.
4. The inability of *cyclins* to bind **cdks** specifically blocks the cell cycle between the ***G_1*** and ***S***, and the ***G_2***-to-***M*** phases.

Fig. 19.21 (below) illustrates the kinase-mediated events at cell cycle checkpoints.



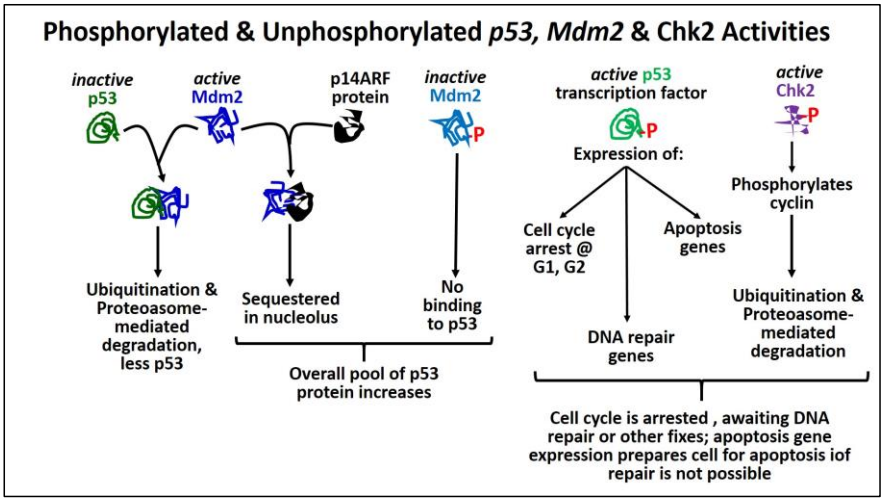
19.21: Role of the *P53* in decision-making at cell cycle checkpoints. (see text for details).

The cell cycle will remain arrested while the cell attempts to finish essential biochemical activities necessary to correct stress-induced or other physical or chemical aberrations before moving on to the next phase of the cycle. If DNA repairs or other corrections are successful, the cell can progress to the next phase. But what happens if repairs are unsuccessful?

19.7.3 How p53 Works When Cells Can't Be Saved

If DNA repair or other corrections fail, the damaged cell is routed to apoptosis. In this case, ***proteasomes*** target the ***Chk2-cyclin*** complex for degradation. Likewise, any ***P53*** remaining bound to unphosphorylated ***Mdm2*** is also targeted for proteasome destruction. The result is that any cell unable to correct effects of stress or chemical damage, or to repair DNA damage, is target for ***apoptosis***.

Levels and activity of ***p53*** (as well as the other proteins discussed above) control both the amount of ***p53*** protein available to respond to cell cycling anomalies and the responses themselves. While phosphorylation (activation) of ***p53*** leads to a rapid arrest of the cell cycle, it also results in the activation of genes for proteins required for DNA repair *and* for proteins required for apoptosis, just in case repair efforts fail! The interactions of ***p53*** with different proteins leading to alternate cell fates are summarized in Fig. 19.22, below.



19.22: Summary of different roles of p53 protein in protein degradation and apoptosis, cell cycle progress and DNA repair (see text for details).

To sum up, ***p53*** suppresses malignant tumor growth either by

- allowing DNA or other cellular repair before resumption of normal cell cycling, preventing unregulated cell divisions; after repair, ***p53*** and other proteins are inactivated and/or destroyed, the cell cycle can resume.
- The inability to repair/correct cell cycling problems sets in motion events leading to apoptosis, thereby also blocking tumorigenesis by killing off damaged cells.

It should be clear now why a mutant ***p53*** that reduces or eliminates ***p21*** protein production or blocks essential DNA repair protein production, will instead allow damaged cells to enter ***S***, transforming them into cancer cells.

In an interesting twist, it seems that compared to humans, few whales or elephants die from cancer, despite having thousands of times more cells than humans. The reason seems to be that, at least for elephants, they have as many as 20 copies (40 alleles) of their p53 genes! Thus, a mutation in one p53 allele may have little effect, while the tumor-repressing effects of the remaining p53 genes prevail. Read about this recent research at Whales and Elephants Don't Get Cancer!

19.8 The Centrality of p53 Action in Cell Cycle Regulation

Because of its multiple roles in regulating and promoting DNA repair, and in controlling cell cycle checkpoints, p53 has been called "*the Guardian of the Genome*"! Here is further evidence of this central role.

19.8.1 'Oncogenic Viruses'

Cancer causing viruses include Human Papilloma Virus (**HPV**), Epstein Barr Virus (**EBV**), human immunodeficiency virus (**HIV**), Hepatitis B and C viruses (**HBV**, **HCV**), Human herpes virus 8 (**HHV-8**) and simian virus 40 (**SV40**). The SV40 virus was discovered as contaminant of polio vaccines that were used in the 1960s.

While an association of SV40 and cancer in humans is as yet unproven, there is a demonstrated link between SV40, p53 and cancer in other mammals. After infection of cells by SV40, viral DNA enters the nucleus where it can integrate into the host cell genome. Such SV40 infections are usually latent, (i.e., they cause no harm) but when activated, they can lead to cellular transformation and the growth of malignant sarcomas in muscles as well as tumors in other organs. Upon activation, RNA polymerase II in the infected cells transcribes the SV40 genes to produce enzymes that replicate viral DNA and encapsulate the DNA in a membrane envelope to make new viral particles. Since the relatively small SV40 genome does not encode all of the enzymes and factors need for viral DNA replication, the infected cells also provide

these factors, producing them only during the ***S*** phase. At that time, the SV40 ***large T antigen*** (already made soon after infection) enters the host cell nucleus where it regulates transcription of genes essential to viral replication and viral particle formation. While that may be OK for the SV40 virus, the kicker is that the *large T antigen* also binds to ***p53***, interfering with transcription of proteins whose genes are regulated by ***p53***.

Unable to exercise checkpoint functions, the host cell starts dividing uncontrollably, forming cancerous tumors. Deregulation of the cell cycle by *large T antigen* ensures progress to the S phase and unregulated co-replication of viral and host cell DNA.

19.8.2 p53 and Signal Transduction

Stress can activate signal transduction pathways. For example, mutations affecting the ***MAPK*** (MAP kinase) signaling pathway can lead to tumorigenesis. This can be explained by the observation that when activated, the MAPK pathway leads to amplified production of a kinase that phosphorylates ***p53***. Active ***phospho-p53*** in turn augments activation of the MAPK signal transduction pathway. You may recall that MAPK signal transduction typically ends with a mitogenic response.

Another example of p53 interaction is with ***FAK*** (***focal adhesion kinase***) proteins. *FAK* activity is increased by *integrin*-mediated signal transduction. Recall that membrane integrins bind *fibronectin*, contributing to formation of the extracellular matrix, or *ECM*. Elevated *FAK* activity participates in the regulation of cell-cell and cell-ECM adhesion at ***focal adhesion points***. Another role for FAK is to bind directly to inactive p53 and increase p53-Mdm2 binding. As we have just seen, persistent p53-Mdm2 is targeted for ubiquitination… and ultimate destruction! In fact, abnormally high levels of FAK are associated with many different tumor cell lines (colon, breast, thyroid, ovarian, melanoma, sarcoma…). These result when p53 is unable properly to activate cell cycle checkpoints. While the interactions implied here are complex and under active study, these ***p53*** activities certainly confirm its central role as both ***guardian of the genome*** and as ***guardian of cell division***.

19.9 Cancer Cell Growth and Behavior; Cancer Treatment Strategies

Different cancer cell types have different growth and other behavioral properties. You may have heard of ***slow-growing*** and ***fast-growing*** cancers. *Colon* cancers are typically slow growing. Periodic *colonoscopies* that detect and remove colorectal tumors in middle-age or older people can prevent the disease (although the risks of disease and the procedure itself must be balanced). *Pancreatic* cancers are fast growing and usually go

undetected until they reach an advanced stage. The twin goals of medical research are to detect the different cancers early enough for successful intervention, and of course, to find effective treatments.

19.9.1 Cancer Cell Origins, Growth and Behavior

A single mutated cell in a tissue can become the growth point of a ***tumor***, essentially a mass of cells cloned from the original mutated one. ***Benign tumors*** or growths (for example breast and uterine *fibroids* in women, or common moles in any of us) usually stop growing and are not life threatening. They are often surgically removed for the comfort of the patient (or because cells in some otherwise benign tumors may have a potential to become cancerous). ***Malignant tumors*** (also called *malignant neoplasms*) are cancerous and can grow beyond the boundaries of the tumor itself. When tumor cells are shed they may enter the bloodstream and travel to other parts of the body, the phenomenon called ***metastasis***. Cancer cells that metastasize can become the focal point of new tumor formation in many different tissues. Because cancer cells continue to cycle and replicate their DNA, they can undergo yet more somatic mutations. These further changes can facilitate metastasis and cancer cell growth in different locations in the body.

19.9.2 Cancer Treatment Strategies

There are many different kinds of cancers originating in different tissues of the body. They all share the property of uncontrolled cell division, albeit for different molecular (and not always well-understood) reasons. The two major cancer treatment strategies developed in the 20th century all aim at disrupting replication in some way.

- ***Radiation therapy*** relies on the fact that most cells in our bodies do not divide. They aim mutagenic radiation at tumors in the hope that replicating DNA will be mutated at so many sites (i.e., genes) that the tumor cells can no longer survive or replicate properly.
- ***Chemotherapy*** is used to attack tumors that do not respond well to radiation or that are not easily be reached by radiation technologies, and to fight cancers that do not even form focused tumors (such as lymphomas and leukemias involving lymph and blood cells). These *chemotherapies* also aim to derange replication or mitotic activities. For example, recall ***cordycepin*** (dideoxyadenosine triphosphate, or ddATP). When present during replication, ddATP is incorporated into a growing DNA chain, after which no additional nucleotides can be added to the DNA strand. That makes ddATP a potent chemotherapeutic disruptor of replication. ***Taxol*** is another chemo drug. In this case, it prevents the depolymerization of spindle fiber microtubules, thus blocking mitotic anaphase and telophase in the latter part of the

M and C phases of the cycle. ***Colchicine*** (a plant alkaloid) attacks cancer (and other dividing) cells by blocking microtubule polymerization in the first place, thus preventing spindle fiber formation in mitotic prophase.

These therapies are not effective against all cancers and of course, they don't target specific kinds of cancer cells. Their success relies simply on the fact that cancer cells proliferate rapidly and constantly while other cell types do not. Many if not all of the side effects of radiation and chemotherapies result from the damage done to normal dividing cells (e.g., hair follicle cells accounting for hair loss among many cancer patients, depletion of blood cells that fail to be replaced by stem cells in bone marrow).

Much research now is focused on mobilizing the body's own immune system to create more specific, targeted cancer treatments. In a fascinating bit of history, more than 100 years ago, Dr. William B. Coley injected a terminal cancer patient with streptococcal bacteria. Remarkably, this and many subsequent patients emerged tumor-free upon his recovery from the infection (for details, check out The Earliest Cancer Immunotherapy Trials). The phenomenon of "Dr. Coley's Toxins" was initially thought to be an anti-tumor effect of the bacteria. But by 1948 it was widely attributed to the immune response activated by the infection. In the 1990s, scientists revisited the immune response to cancer, and by the turn of the 21st century, studies of cancer immunotherapy picked up steam (and more substantial research funding!).

Recent animal immunotherapy experiments and human clinical trials are promising. A few immunotherapies have already been approved by the U.S. FDA (Food and Drug Administration). Our immune system can recognize cancer cell surface molecules as foreign, but only mounts a weak immune defense that can't clear those cells. Those that survive continue to proliferate, causing cancer.

There are different, sometimes overlapping approaches to cancer immunotherapy. All are based on the fact that cancer cells that have mutated in some way and are producing aberrant proteins that the immune system can see as foreign enough to elicit an immune response, however slight. The goal now is to create conditions for a strong immune response to the cancer cell antigens. Some immunotherapies seek to boost that innate immune response. Others seek isolate or synthesize unique cancer cell antigens *in vitro* that upon injection will generate an immune response in the patient strong enough to kill the cancer cells.

Some immunotherapies are summarized in Table 19.1. As you can see from the table, immuno-targeting cancer cells has already proven to be highly effective. In some cases, the therapy is an example of *personalized medicine*, in which treatments are uniquely tailored to you as a patient.

Known Issues with immunotherapies are that

- they are time and labor intensive…, and costly to produce.
- while they may 'cure' you, they likely won't not work on someone else.
- like radiation and chemotherapy, immunotherapies come with their own unpleasant and sometimes severe side effects.

Summary of Cancer Immunotherapies in Use or Under Study

Type	Monoclonal antibodies	Immune checkpoint inhibitors	Cancer vaccines	Non-Specific immunotherapies	Chimeric Antigen Receptor CAR T-Cell Therapies
Brief Description	Monoclonal antibodies (mAbs) are typically prepared from isolated, cloned immune cells B-lymphocytes (or B-cell) that secrete blood-borne antibodies, or IgGs. IgGs from such cloned B-cells, chosen because they produce IgGs to cancer cell surface proteins (antigens) can thus attack a very specific part of the cancer cell, leading to its destruction.	Immune checkpoints protect normal cells from immune attack. The PD-1 protein on T-cell surfaces normally binds to PD-L1 receptors on normal cell, cloaking them against T-cell attack. PD-L1 proteins on cancer cells also bind PD-1. Thus protected from immune response, they proliferate. The disruption the interaction of PD-1 and PD-L1 (and others), checkpoint inhibitors 'un-cloaks' cancer cells, allowing T-cells to attack and destroy them.	These are made using whole tumor cells or purified proteins (or bits there) encoded by mutant genes active in cancer cells. They are often injected with adjuvants (chemicals that boost an immune response). When recognized as 'foreign' by a patient's immune system, cancer cells are attacked. Some vaccines are made sourcing the patient's own cancer cells for antigens. Some effective vaccines can also be prepared against a cancer from any patient's tumor.	Tumor shrinkage in response to bacterial infection can be mimicked by signal molecules such as Cytokines that can enhance an otherwise weak immune cell response to cancer cells. Anti-cancer cytokines include interferons, interleukins, and even thalidomide! Examples of this activity include the isolation of tumor infiltrating lymphocytes (TILs) found in tumors. Their anti-cancer activity is low, but can be boosted by cytokine treatment to levels where they may shrink the tumor.	This is similar to cytokine-stimulation of tumor-infiltrating cells proliferation to attack a tumor. But in this case, T-lymphocytes isolated from a cancer patient's blood are genetically engineered to contain and express genes for receptors specific for antigens (proteins) on the surface of cancer cells (the CARS). Once multiplied by growth in culture. these cells with their engineered chimeric antigen receptors, are injected back into the patient, where they can target and kill the cancer cells.
Possible Susceptible Cancers	pancreatic cancer, brain tumors, breast cancer, cervical cancer, prostate cancer, lymphoma, colorectal cancer, kidney cancer, lung cancer, melanoma	melanoma, non-small cell lung cancer, Hodgkin lymphoma, bladder cancer…, and potentially, many others!	pancreatic cancer, brain tumors, breast cancer, cervical cancer, metastatic prostate cancer, lymphoma, colorectal cancer, kidney cancer, lung cancer, melanoma	metastatic melanomas, cervical squamoous cell carciniima, cholangio carcinoma	pancreatic cancer, brain tumors, breast cancer, neuroblastoma, acute myeloid leukemia, multiple myeloma, non-Hodgkin's lymphoma

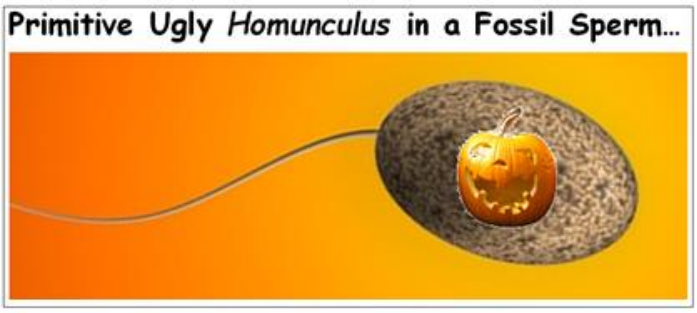
Note that in the table, the term *checkpoint inhibitor* in the context of immunotherapies is different than the term *checkpoints* describing portals to progress through the eukaryotic cell cycle. A more detailed discussion of cancer immunotherapies is on the *cancer.gov* website at Cancer Treatment Immunotherapy.

Some iText & VOP Key Words and Terms

anaphase	G_2 phase	mTOR signaling
apoptosis	*Guardian of the Genome*	necrosis
ATM kinase	immunotherapy	oncogenic viruses
benign tumors	integrin	p14ARF
cancer cells	interphase	p21
CDKs	invasive tumors	P53
cell cycle	LFS	PD-1 checkpoint protein
cell cycle checkpoints	Li-Fraumeni Syndrome	PD-L1
chemotherapy	M checkpoint	programmed cell death
Chk2	M phase of the cell cycle	prophase
colchicine	malignant tumors	protein phosphorylation
cyclin	MAPK	proteasome
cyclin level in cell cycle	maturation	radiation therapy
cytokinesis	maturation promoting	S phase
dideoxyNTP	Mdm2	signal transduction
elephant p53 genes	metaphase	SV40
FAK	metastasis	T antigens
Go of the cell cycle	mitosis	taxol
G_1 checkpoint	mitosis promoting factor	telophase
G_1 phase	mitotic phases	tumor suppressor protein
G_2 checkpoint	MPF	ubiquitination

Chapter 20: Origins of Life

A short history; Origins under reductive vs. non-reductive conditions; Prebiotic chemical and metabolic evolution; Origins of communication (catalysis, autocatalysis, co-catalysis, biochemical pathways; Transition from the RNA World



20.1 Introduction

It is nearly universally accepted that there was a time, however brief or long, when the earth was a lifeless planet. Given that the cell is the basic unit of life, and that to be alive is to possess all of the ***properties of life***, any cell biology textbook would be remiss without addressing the questions of ***when*** and ***how*** the first cells appeared on our planet. ***Abiogenesis*** is the origin of life from non-living matter. Of course, any observation of abiogenesis in nature is no longer possible! But experiment and educated guesswork makes it possible to construct reasonable (if sometimes conflicting) scenarios to explain the ***origins of life***, and hence our very existence.

In this chapter, we will see that different scenarios require consistent assumptions about climatic, geologic, thermodynamic and chemical conditions that favored abiogenesis. The right conditions would lead to a prebiotic accumulation of organic molecules, chemical reactions and proto-structures that could support the formation of a cell. One might reasonably speculate such a ***prebiotic laboratory*** would have led to experiments in ***chemical evolution***, which best survived and interacted in a hostile pre-biotic environment, even before the origin of the first cell… or cells. Hence the chapter title "Origins of Life"!

While many consider the creation of the first cell to be a singular, one-off event, it has been argued that multiple independent origins were not only possible under these conditions, but also probable! According to Jeremy England, of MIT, the laws of thermodynamics dictate that "... when a group of atoms is driven by an external source of

energy (like the sun or chemical fuel) and surrounded by a heat bath (like the ocean or atmosphere), matter inexorably acquires the key physical attribute(s) associated with life" (Statistical Physics of Self Replication). Here is a reminder of those key attributes, or properties of life:

Evolution:	**long-term *adaptation/speciation***
Cell-based:	**the cell is the fundamental *unit of life***
Complexity:	***dynamic order*; allows physical/biochemical change**
Homoeostasis:	**living things maintain *balance* between change and order**
Requires Energy:	**needed to do *work*, i.e., all cellular functions**
Irritability:	**immediate sensitivity and *response to stimuli***
Reproduction:	**sort of self-explanatory, yes?!**
Development:	***programmed change*; most obvious in multicellular organisms, but found in some form in all organisms.**

Remember that to be alive is to possess not just some, but all of these properties! If entities with all of the properties of life (i.e., cells) did originate independently, they would have reproduced to form separate populations of cells, each of which would embark on an independent evolutionary pathway. In this scenario, less successful populations would go extinct as successful ones become dominant. Successful organisms would have spread, spawning populations, evolving and generating new species. This could go on until all other independent lineages of life were extinct. The ***take-home message*** here is that if conditions on a prebiotic earth favored the formation of the 'first cell', then why not the formation of two or dozens or even hundreds of 'first cells'? We may never know, because there are no survivors of such lineages. If they ever existed, they are now extinct! We will see the evidence that there is only one successful population of cells from which the common ancestor of all known living things.

As to the question of ***when*** life began, we'll look at geological and geochemical evidence suggesting the presence of life on earth as early as 4.1 billion years ago. As for ***how*** life began, this remains the subject of ongoing speculation. *All* of the scenarios described below attempt to understand the physical, chemical and energetic conditions that might have been the ideal laboratory for prebiotic "*chemistry experiments*". What *all* Origins of Life scenarios *must* share are the following:

- ***prebiotic synthesis* of organic molecules & polymers**
- **origins of *catalysis* & *replicative chemistry***
- ***sources of free energy* to sustain prebiotic chemistry**
- **beginnings of *metabolism* sufficient for life**
- **origins of *molecular communication* (information storage and retrieval)**
- ***enclosure* of life's chemistry behind a semipermeable boundary**

Let's consider some tricky definitions. If one believes the origin of life was so unlikely that it could only have happened once (still a common view), then the very first cell, the ***progenote***, is the progenitor of us all, the common ancestor of all living things. The evolutionary tree in Fig. 20.1 (below) puts the progenote at the root of the tree.

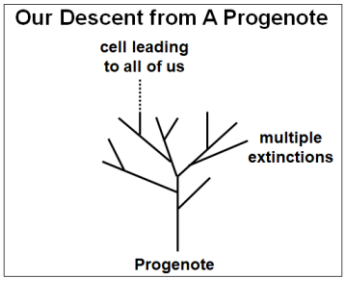


Fig.20.1: Descent (evolution and speciation) from a single progenitor cell formed by abiogenesis on a prebiotic earth.

The progenote, that first and only cell, would have given rise to multiple lineages, i.e., as it reproduced, some cells would have diverged into separate and multiple species. In other words, the descendants of the progenote would have evolved from the get-go. Like any populations of cells and organisms, whether from of a single or many 'progenotes', descendant populations would have competed for resources (and ultimately survival) on an early earth. Thus one of these lineages (the dashed line in the illustration) would have survived as others fell to extinction, a process that continues today as the fossil record attests. Now let's imagine multiple independent 'first cells'. How would that change our view of life's descent? Figure 20.2 (below) illustrates a likely scenario.

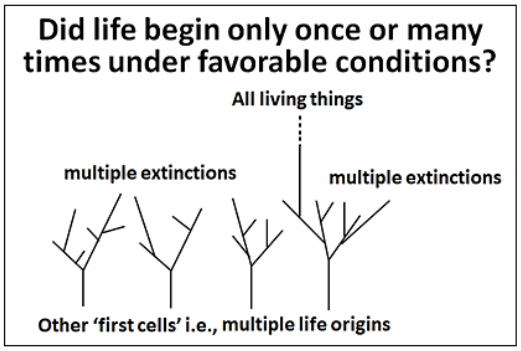


Fig.20.2: Descent (evolution and speciation) from multiple 'first' cells.

Based on this illustration, we can surmise that:

1. Independent descent from independent 'first cells' may have occurred, but without evidence we must assume that the evolution of all but one of them led to extinction of their lineage, leaving only one evolutionary path to life on earth.

2. The descendants of a 'first cell' that survived (illustrated on the right) include many lineages that went extinct, while a surviving lineage that includes the ancestor to us, along with the extinctions that continue to this day.

In other words, whether or not life had one or many origins in a permissive prebiotic environment, we can anchor our assumptions the evolution of life from a single origin. We'll see clear evidence to support that all organisms alive today are descended from a single cell. In a scenario where only one cell population survives the competition with other populations, its evolved cells would have been the source of our ***Last Universal Common Ancestor***, or ***LUCA***. As illustrated below in Fig. 20.3, the *LUCA* must be that highly evolved cell whose genome, biochemistry and basic metabolic infrastructure is shared among all things alive today.

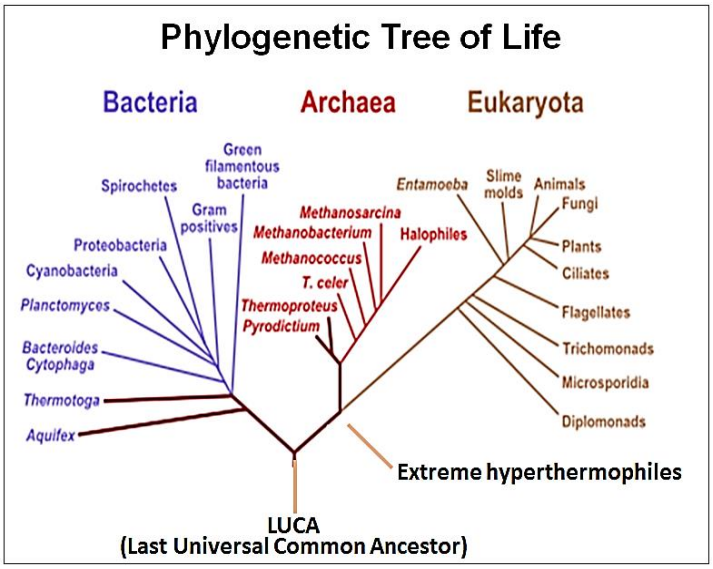


Fig. 20.3: Evolutionary (i.e., phylogenetic) tree of all living organisms, showing descent from the ***Last Universal Common Ancestor*** (***LUCA***) of the 3 domains of life (Bacteria, Archea and Eukaryota).

This and similar evolutionary trees for known living things on earth are based on studies of nucleotide sequences of many genes in existing organisms. Whatever the prebiotic pathway (or pathways) to the first living cells on earth, computers can be used to trace differences in gene nucleotide sequences back to changes that would have begun in a single common ancestor that we have defined as our ***LUCA***.

Let's think about the basis for our conclusion that descendants of multiple 'first cells' are no longer with us. If there were multiple origins, then each of these first cells may have survived long enough to produce its own lineage of evolved cell populations. But cells in each of these independent lineages would have evolved biochemistries and cellular structures that that differ from one another. Perhaps it will be easiest to imagine that they evolved very different genetic codes, even if they used RNA and DNA as their genetic material, and even if the best solution to storing and retrieving genetic information was the 3 base codon for their amino acids. But which codons encode which amino acids was a fortuitous choice in evolution, made by LUCA's ancestors. So if life originated multiple times and any descendants survived to this day, we would expect them to have a different genetic code, if not some very unusual way to store genetic information. Since there is no evidence for different genetic codes among organisms alive today, we conclude either that all but one of the multiple progenote lineages went extinct, or that they never happened. Either way, among the descendants of *our* progenote is a lineage, a population that includes the first cell that must have had a DNA genome, with the same genetic code used by virtually all organisms alive today. This LUCA, *our* LUCA, and its descendants display the high level of conservation of gene and protein sequences that we find today.

We'll look at more supporting evidence that this is so as we move through this chapter; this evidence is perhaps the strongest support for realized expectations. We'll begin by looking at the following basic requirements of any life-origins scenario:

- reduction of inorganic molecules to form organic molecules
- a source of free energy to fuel the formation of organic molecules
- a scheme for catalytic acceleration of biochemical reactions
- separation of early biochemical 'experiments' by a semipermeable boundary.

Then, we'll consider some proposed scenarios for the creation of organic molecules:

- import of organic molecules (or even life itself) from ***extraterrestrial*** sources.
- organic molecule synthesis on an earth with a ***reducing atmosphere.***
- organic molecule synthesis on an earth with a ***non-reducing atmosphere***.

We'll explore alternate free-energy sources and pathways to the essential chemistry of life dictated by these different beginnings. Then we look at possible scenarios of chemical evolution that must have occurred before life itself. Finally, we consider how

primitive (read “simpler”) biochemistries could have evolved into the present-day metabolisms shared by all existing life forms…, including its shared genetic code!

347 What any Life Origins Scenario Must Explain

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain how organic molecules would capture chemical energy on a *prebiotic earth*.
2. list the essential chemistries required for life and why they might have been *selected* during chemical evolution.
3. discuss the different fates of prebiotically synthesized organic *monomers* and *polymers* and how these fates would influence the origins of the first cells on earth.
4. compare and contrast two scenarios for *extraterrestrial origins* of organic molecules.
5. summarize the arguments against Oparin’s *primordial soup* hypothesis.
6. summarize the evidence supporting origins of life in a *non-reducing* earth atmosphere.
7. compare the *progenote* and the *LUCA*.
8. discuss the evidence suggesting an origin of cellular life in the *late Hadean eon*.
9. describe how life might have begun in deep ocean vents – compare the possibilities of life beginning in *black smokers* vs. *white smokers*.
10. argue for and against an *autotroph-first* scenario for cellular origins.
11. explain why some investigators place significance on the early origins of free energy storage in *inorganic proton gradients*.
12. define *autocatalysis*, *co-catalysis* and *co-catalytic sets*; provide examples.
13. define *coevolution*.
14. describe the significance and necessity of *coevolution* before life. In what ways is coevolution a feature of living things? Explain.

20.2 Thinking about Life’s Origins: A Short Summary of a Long History

By all accounts, the earth must have been a very unpleasant place soon after its formation! For that reason, the period from 4.8 to 4.0 billion years ago is called the ***Hadean Eon***, after Hades, the hell of the ancient Greeks!

Until recently, geological, geochemical and fossil evidence suggested that life arose between 3.8 and 4.1 billion years ago. The 2017 discovery of evidence for life in 3.95 billion year-old sedimentary rocks in Labrador points to an even earlier origin of life, (see From Canada Comes the Oldest Evidence of Life on Earth). In fact, questions about life’s origins are probably “as old as the hills…” or at least as old as the ancient Greeks! We only have records of human notions of life’s origins dating from biblical accounts and, just a bit later, from Aristotle’s musings. While Aristotle did not suggest that life began in hell, he and other ancient Greeks did speculate about life’s origins by *spontaneous*

generation, in the sense of *abiogenesis* (*life* originating from *non-life*). He further speculated that the origins of life were gradual. Later, the dominant theological accounts of creation in Europe in the middle ages muted any notions of origins and evolution. While a few mediaeval voices ran counter to strict biblical readings of the creation stories, it was not until the Renaissance in the 14th-17th century that an appreciation of ancient Greek *humanism* was reawakened, and with it, scientific curiosity and the ability to engage in rational questioning and research.

You may recall that Louis Pasteur in the mid-19th century put to rest any lingering notions of life forming from dead (e.g., rotten, or fecal) matter. He showed that life would not form in sterilized nutrient solutions unless the broth was exposed to the air. Fewer know that much earlier, Anton Van Leeuwenhoek (the 17th century sheriff of Delft, amateur lens grinder and microscopist who first described pond water bacteria and protozoan animalcules) had already tested the notion of spontaneous generation. By observing open and sealed containers of meat over time, he became convinced that 'large' animals like fleas and frogs did not arise *de novo* from putrid meat or slime. He also declared that insects come from other insects, and not from the flowers that they visited.

No lesser a light than Charles Darwin suggested in 1859 that life might have begun in a "*warm little pond, with all sorts of ammonia and phosphoric salts, light, heat, electricity, &c., present, that a proteine compound was chemically formed ready to undergo still more complex changes*." He even realized that these chemical constituents would not have survived in the atmosphere and waters of his day, but must have done so in a prebiotic world. In *On the Origin of Species,* he referred to life having been 'created'. There, Darwin was not referring to a biblical basis of creation; he clearly meant that life originated "*by some wholly unknown process*" at a time before which there was no life. Finally, Pasteur's 1861 contribution was the irrefutable, definitive proof that 'invisible' *microbial life* likewise did not arise by spontaneous generation. Thus for creatures already on earth, they could only arise by ***biogenesis*** *(life-from-life)*, the opposite of *abiogenesis,* a term that now applies to only the first origins of life! Among Darwin's friends and contemporaries were Charles Lyell and Roderick Murchison, both geologists who understood much about the slow geological changes that shaped the earth. Darwin was therefore familiar with the concept of extended periods of geological time, amounts of time he believed was necessary for the natural selection of traits leading to species divergence.

Fast-forward to the 1920s when J.H.B.S. Haldane and A. Oparin offered an hypothesis about the life's origins based on notions of the chemistry and physical conditions they believed might have existed on a ***prebiotic earth***. Their proposal assumed that the earth's atmosphere was hot, hellish and reducing (i.e., filled with inorganic molecules able to give up electrons and hydrogens). There are more than a few hypotheses for which

chemicals were already present on earth, or that formed when the planet formed about 4.8 billion years ago. We'll start our exploration with Oparin and Haldane's *reducing atmosphere*. Then we look at possibility that life began under *non-reducing conditions*, with passing reference to a few other ideas!

348 Early Ideas to Explain the Origins of Life

20.3 Formation of Organic Molecules in an Earthly *Reducing Atmosphere*

A prerequisite to prebiotic chemical experimentation is a source of organic molecules. Just as life requires energy (to do anything and everything), converting inorganic molecules into organic molecules requires an input of *free energy*. As we have seen, most living things today get free energy by oxidizing nutrients or directly from the sun by photosynthesis. Recall that *all* the chemical energy sustaining life today ultimately comes from the sun. But before there were cells, how did organic molecules form from inorganic precursors? Oparin and Haldane hypothesized a reducing atmosphere on the prebiotic earth, rich in inorganic molecules with *reducing power* (like H_2, NH_3, CH_4, and H_2S) as well as CO_2 to serve as a carbon source. The predicted physical conditions on this prebiotic earth were:

- lots of water (oceans).
- hot (no free O_2).
- lots ionizing (e.g., X-, γ-) radiation from space, (no protective ozone layer).
- frequent ionizing (electrical) storms generated in an unstable atmosphere.
- volcanic and thermal vent activity.

20.3.1 Origins of Organic Molecules and a Primordial Soup

Oparin suggested that abundant sources of free energy fueled the reductive synthesis of the first organic molecules to create what he called a "primeval soup". No doubt, he called this primeval concoction a "soup" because it would have been rich in chemical (nutrient) free energy. Urey (who had won the 1934 Nobel Prize in Chemistry for discovering deuterium) and Miller tested the prediction that under Haldane and Oparin's prebiotic earth conditions, inorganic molecules could produce the organic molecules in what came known as the *primordial soup*. In their classic experiment a mix of inorganic molecules provided with an energy source were reduced to very familiar organic molecules, supporting the Oparin/Haldane proposal. Their experiment is illustrated below in Fig. 20.4.

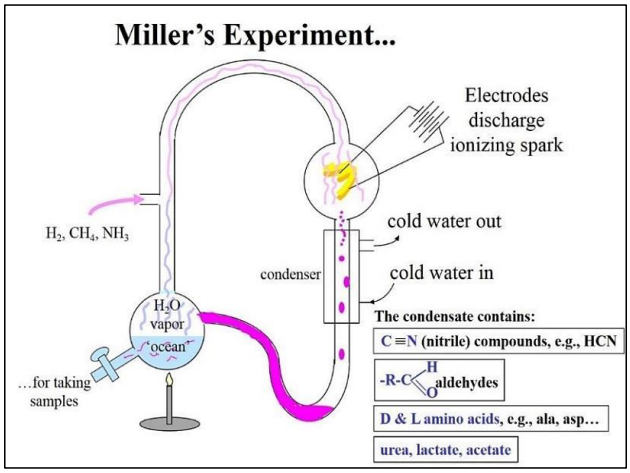


Fig. 20.4: The Miller & Urey experiment: organic molecules seen in living things today can be made in the lab under *reducing* conditions like those expected in a prebiotic earth atmosphere.

Miller's earliest published data indicated the presence of several organic molecules in their *ocean* flask, including a few familiar metabolic organic acids (lactate, acetate, several amino acids…) as well as several highly reactive *aldehydes* and *nitriles*. The latter can interact in spontaneous chemical reactions to form organic compounds. Later analyses further revealed purines, carbohydrates and fatty acids in the flask. Later still, 50 years after Miller's experiments (and a few years after his death), some un-analyzed sample collection tubes from those early experiments were discovered. When the contents of these tubes were analyzed with newer, more sensitive detection techniques, they were shown to contain additional organic molecules not originally reported, including 23 amino acids (to read more, click Surprise Goodies in the Soup!).

Clearly, the thermodynamic and chemical conditions proposed by Oparin and Haldane could support the *reductive synthesis* of organic molecules. At some point, Oparin and Haldane's evolving chemistries would have to have been internalized inside of semipermeable aggregates (or boundaries) destined to become cells. Examples of such structures are discussed below. A nutrient-rich primordial soup would likely have favored the genesis of *heterotrophic* cells that could use environmental nutrients for energy and growth, implying an early evolution of fermentative pathways similar to glycolysis. But, these first cells would quickly consume the nutrients in the soup, quickly ending the earth's new vitality!

To stop life from becoming a dead-end experiment, one must propose the early evolution of some populations of cells that could capture free energy from inorganic molecules (***chemoautotrophs***) or even sunlight (***photoautotrophs***). As energy-rich

organic nutrients in the 'soup' declined, ***autotrophs*** (e.g., photoautotrophs that could split water using solar energy) would be selected. Photoautotrophs would *fix* CO_2, reducing it with H^- ions from water. Photoautotrophy (***photosynthesis***) would thus replenish carbohydrates and other nutrients in the oceans and add O_2 to the atmosphere. At first, oxygen would have been toxic to most cells, but some of those cells already had the ability to survive oxygen. Presumably these spread, evolving into cells that could *respire*, i.e., use oxygen to *burn* environmental nutrients. Respiratory metabolism must have followed hard on the heels of the spread of photosynthesis. After photosynthesis emerged, sometime between 3.5 and 2.5 billion years ago (the Archaean Eon), photosynthetic and aerobic cells and organisms achieved a natural balance to become the dominant species in our oxygen-rich world.

20.3.2 The Tidal Pool Scenario for an Origin of Polymers and Replicating Chemistries

In this scenario, prebiotic organic monomers are concentrated in tidal pools in the heat of a primordial day, followed by dehydration synthesis and polymerization. The formation of polymer linkages is an 'uphill' reaction requiring free energy. Very high temperatures (the ***heat of baking***) can link monomers by dehydration synthesis in the laboratory and may have done so in tidal pool sediments to form random polymers. This tidal pool scenario, shown below in Fig. 20.5 further assumes the dispersal of these polymers from the tidal pools with the ebb and flow of high tides.

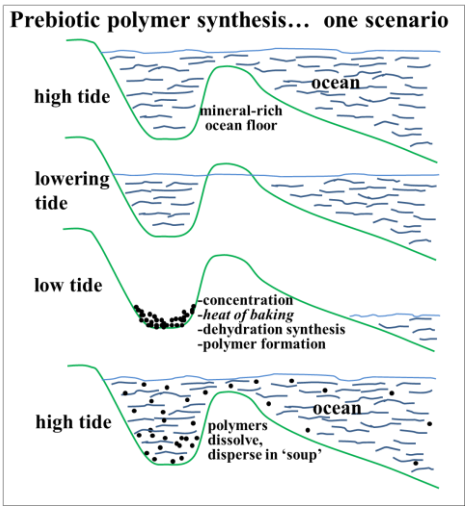


Fig. 20.5: Scenario for the synthesis of prebiotic polymers in tidal pools.

The concentration of putative organic monomers at the bottom of tidal pools may have offered opportunities to catalyze polymerization, even in the absence of very high heat. Many metals (nickel, platinum, silver, even hydrogen) are inorganic catalysts, able to speed up many chemical reactions. The heavier metals were likely to exist in the earth's crust as well as in the sediments of primordial oceans, just as they do today. Such mineral aggregates in soils and clays have been shown to possess catalytic properties. Furthermore, metals (e.g., magnesium, manganese…) are now an integral part of many enzymes, consistent with an origin of biological catalysts in simpler aggregated mineral catalysts in ocean sediments.

Before life, the micro-surfaces of mineral-enriched sediment, if undisturbed, might have catalyzed the same or at least similar reactions repeatedly, leading to related sets of polymers. Consider the possibilities for RNA monomers and polymers, based on the assumption that life began in an RNA world (Fig. 20.6).

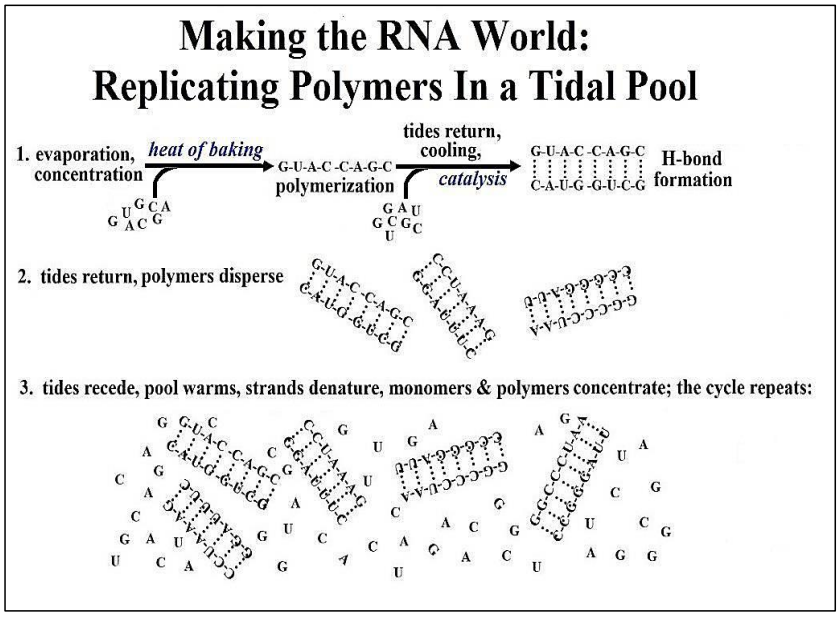


Fig. 20.6: Scenario for the synthesis of redundant prebiotic nucleic acid polymers in tidal pools.

The result predicted here is the formation not only of RNA polymers (perhaps only short ones at first), but of H-bonded double-stranded RNA molecules that might effectively replicate at each cycle of concentration, polymerization and dispersal.

Heat and the free energy released by these same reactions could have supported polymerization, while catalysis would have enhanced the fidelity of RNA replication.

Of course, in the tidal pool scenario, repeated high heat or other physical or chemical attack might also degrade newly formed polymers. But what if some RNA double strands were more resistant to destruction? Such early RNA duplexes would accumulate at the expense of the weaker, more susceptible ones. Only the *fittest* replicated molecules would be selected and persist in the environment. The environmental accumulation of structurally related, replicable and stable polymers reflects a prebiotic chemical ***homeostasis*** (one of those properties of life!).

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Overall, this scenario hangs together nicely, and has done for many decades. But there are now challenging questions about the premise of a prebiotic reducing environment. Newer evidence points to an earth atmosphere that was not at all reducing, casting doubt on the idea that heterotrophs were the first cells on the planet. Recent proposals posit alternative sources of prebiotic free energy and organic molecules that look quite different from those assumed by Oparin, Haldane, Urey and Miller.

20.4 Origins of Organic Molecules in a *NON-Reducing Atmosphere*

A prebiotic non-reducing atmosphere is based on several assumptions: (1) The early earth would have had insufficient gravity to hold H_2 and other light gasses; thus "outgassing" would have resulted in a loss of H_2 and other reducing agents from the atmosphere. (2) Geological evidence suggests that the earth's oceans and crust formed early in the Hadean Eon, just a few hundred million years after formation of the planet. (3) Studies of 4.4-billion-year-old (early Hadean Eon) Australian *zircon* crystals suggest that their oxidation state is the same as modern day rocks, meaning that the early Hadean atmosphere was largely N_2 and CO_2, a distinctly *non-reducing* one! Solid geological evidence of cellular life dates to 3.5-3.95 billion years ago (i.e., the *Archaean Eon*). Softer evidence of microbial life exists in the form of graphite and other 'possible' remains as old as 4.1 billion years ago, near the end of the Hadean Eon. If this is true, the discovery of an oxidizing Hadean atmosphere at least 3 billion years earlier (4.4 billion years ago) argues that life began in a non-reducing atmosphere. Therefore, regardless of whether life began 3.5 or even 4.1 billion years ago, the evidence suggests that life's beginnings had to contend with a non-reducing environment. A colorized image of this Australian zircon is shown in Fig. 20.7 (below)

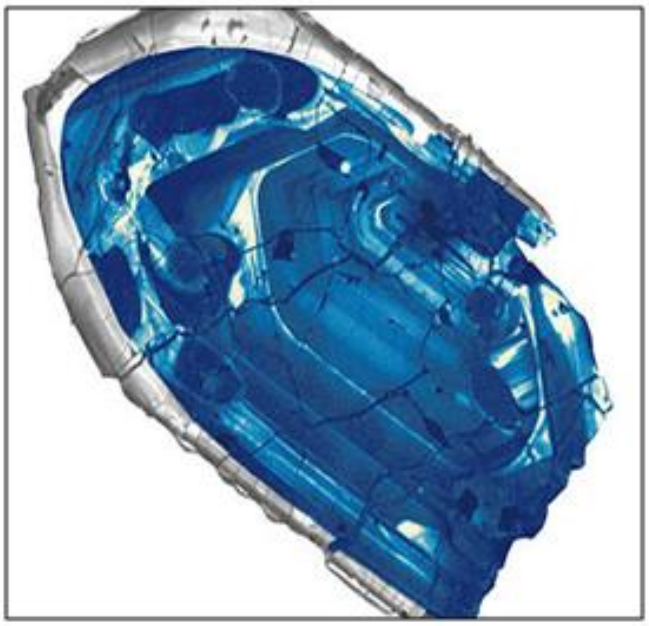

Fig. 20.7: Analysis of this Australian *zircon* supports the presence of an oxidizing atmosphere on a prebiotic earth. Photo Courtesy of J.W.Valley et al.

Before we look more closely at other evidence of life origins under non-reducing conditions, let's consider the ***Panspermia***, the possibility that life came to earth from extraterrestrial sources and a related hypothesis that prebiotic organic molecules came from ***extraterrestrial*** sources. Then we'll return to the question of how cells might have formed in localized, favorable ***terrestrial*** environments.

20.4.1 Panspermia – an Extraterrestrial Origin of Earthly Life

Panspermia posits that life itself arrived on our planet by hitchhiking on comets or meteorites. Since these are unlikely to have sustained life in space, they must have been a kind of interstellar 'mailbox' into which dormant life forms were deposited. The cells in the mailboxes must have been *cryptobiotic*. Examples of cryptobiosis exist today (e.g., bacterial spores, brine shrimp!). Once delivered to earth's life-friendly environment, such organisms would emerge from dormancy, eventually populating the planet. There is however, no evidence of dormant or cryptobiotic life on comets or meteorites, and no hard evidence to support *Panspermia*. On the other hand, there is evidence at least consistent with an extraterrestrial source of organic molecules, and

plenty to support more terrestrial origins of life. In any case, notions of *Panspermia* (and even extraterrestrial sources of organic molecules) just beg the question of the conditions that would have led to the origins of life elsewhere!

Not a favored scenario, panspermia is still intriguing in the sense that it is in line with a likelihood that organic molecules formed soon after the *Big Bang.* Moreover, if ready-made organic molecules and water were available, we can expect (and many do!) that there is life on other planets. This possibility has stimulated discussion and serious funding of programs looking for signs of extraterrestrial life. It supported the earlier *Search for Extraterrestrial Intelligence* (SETI) program based on the assumption that life not only exists elsewhere, but that it evolved high level communication skills (and why not?)! More recently NASA funded *Rover*'s search for signs (and discovery) of signs of water on Mars. For a fascinating story about meteorites from Mars that contain water and that are worth more than gold, click Martian Obsession.

And then there is this! *Maybe, just maybe* we have ***exported*** earthly life to comets when they bumped into us and skittered away instead of crashing and burning (evidence suggests this is so). And *maybe, just maybe* they scraped up a few upper atmospheric microbes along their way to other outer-space encounters (colonies of such microbes exist). And *maybe, just maybe* such encounters could have led to exoplanetary life. Read about the possibilities and realities at Maybe_just_maybe. Finally *maybe, just maybe*, our attempt to share the gift of life can explain organic molecules on some space objects that come near or actually visit us from time to time!

20.4.2 Extraterrestrial Origins of Organic molecules

Even if life did not come to us ready-made, could organic molecules have arrived on earth from outer space? They are abundant, for example in *interstellar clouds*, and could have become part of the earth as the planet formed around 4.8 billion years ago. This suggests that there was no need to create them *de novo*. Meteorites, comets and asteroids are known to contain organic molecules, and could have brought them here during fiery impacts on our planet. Comet and meteorite bombardments would have been common 3.8 or more billion years ago. In this scenario the question of how (*not on earth!*) free energy and inorganic molecular precursors reacted to form organic molecules…, is moot!

A related hypothesis suggests that those fiery hits themselves provided the free energy necessary to synthesize organic molecules from inorganic ones… a *synthesis-on-arrival* scenario. With this hypothesis on the one hand, we are back to an organic oceanic primordial soup. On the other, some have suggested that organic molecules produced in this way (not to mention any primordial life forms) would likely have been destroyed by the same ongoing impacts by extraterrestrial bodies; witness the

relatively recent dinosaur extinction by an asteroid impact off the coast of Mexico some 65.5 million years ago.

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20.5 Organic Molecular Origins Closer to Home

Deep in the oceans, far from the meteoric bombardments and the rampant free energy of an oxygen-free and ozone-less sky, deep-sea hydrothermal vents would have been spewing reducing molecules (e.g., H_2S, H_2, NH_4, CH_4), much as they do today. Some vents are also high in metals such as lead, iron, nickel, zinc copper, etc. When combined with their clay or crustal substrata, some of these minerals could have provided catalytic surfaces to enhance organic molecule synthesis. Could such localized conditions have been the focus of prebiotic chemical experimentation leading to the origins of life? Let's look at two kinds of deep-sea hydrothermal vents: ***volcanic*** and ***alkaline***.

20.5.1 Origins in a High-Heat Hydrothermal Vent (*Black Smoker*)

The free energy available from a volcanic hydrothermal vent would come from the high heat (temperatures ranging to 350ºC) and the minerals and chemicals expelled from the earth's mantle. Fig. 20.8 (below) shows a volcanic hydrothermal vent.

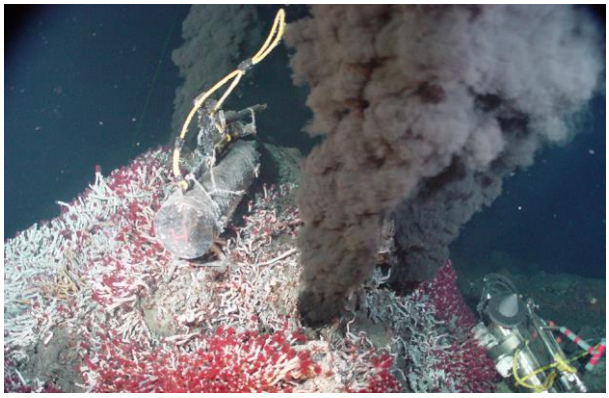


Fig. 20.8: An oceanic volcanic hydrothermal vent or *black smoker*.

Conditions assumed for prebiotic volcanic hydrothermal vents could have supported the catalytic synthesis of organic molecules from inorganic precursors (see Volcanic Vents and organic molecule formation). Mineral catalysts would have been inorganic,

i.e., nickel, iron, etc. Chemical reactions tested include some that are reminiscent of biochemical reactions in chemoautotrophic cells alive today. Günter Wächtershäuser proposed the Iron-sulfur world theory of life's origins in these vents, also called "black smokers". These vents now spew large amounts of CH_4 and NH_4 and experiments favor the idea that iron-sulfur aggregates in and around *black smokers* could provide catalytic surfaces for the prebiotic formation of organic molecules like methanol and formic acid from dissolved CO_2 and the CH_4 and NH_4 coming from the vents.

Wächtershäuser also realized that prebiotic selection acted not so much on isolated chemical reactions, but on aggregates of *metabolic reactions*. We might think of such metabolic *aggregates* as biochemical pathways or multiple integrated pathways. Wächtershäuser proposed the selection of cyclic chemical reactions that released free energy that could be used by other reactions. This prebiotic ***metabolic evolution*** of reaction chemistries (rather than a simpler chemical evolution) would have been essential to the origins of life. A variety of extremophiles (e.g., thermophilic archaea) now living in and around *black smokers* seems to be testimony to black smoker origins of life.

While the idea of selecting metabolic pathways has great merit, there are problems with a life-origins scenario in volcanic hydrothermal vents. For one thing, their high temperatures would have destroyed as many organic molecules as were created. Also, the extremophilic archaea now found around these volcanic vents cannot be the direct descendants of any cells that might have originated there. Woese's phylogeny clearly shows that archaea share a lineage with eukaryotes (not eubacteria). Thus, extremophilic cellular life originating in the vents must have given rise to the more temperate/moderate LUCA first…before then dying off ! In this case, extremophiles alive today would have had to evolve independently from already diversified organisms living in cooler, calmer waters, eventually re-colonizing the thermal vents!

Such twists and turns over time militate against an extremophiles-first origins scenario. Given these concerns, recent proposals focus on life origins in less extreme ***alkaline hydrothermal vents***.

20.5.2 Origins in an Alkaline Deep-Sea Vent (*White Smoker*)

Of the several scenarios discussed here, an origin of autotrophic life in *alkaline vents* is one of the more satisfying alternatives to a soupy origin of heterotrophic cells. For starters, at temperatures closer to 100ºC -150ºC, alkaline vents (***white smokers***) are not nearly as hot as are black smokers. An *alkaline vent* is shown in Fig. 20.9.

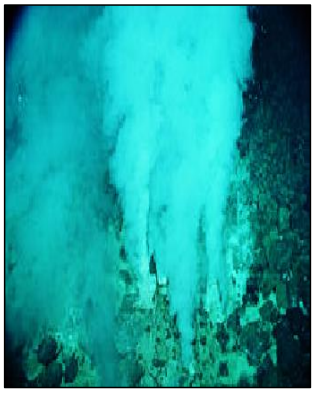


Fig. 20.9: An oceanic *volcanic hydrothermal* vent, or *White smoker*.

Other chemical and physical conditions of alkaline vents are also consistent with an origins-of-life scenario dependent on *metabolic evolution*. For one thing, the interface of alkaline vents with acidic ocean waters has the theoretic potential to generate many different organic molecules [Shock E, Canovas P. (2010) *The potential for abiotic organic synthesis and biosynthesis at seafloor hydrothermal systems*. Geofluids 10 (1-2):161-92)].

In laboratory simulations of alkaline vent conditions, the presence of dissolved CO_2 favors ***serpentinization***, a reaction of water and heat with *serpentinite*, an iron-containing mineral found on land and in the oceanic crust. Fig. 20.10 is a sample of serpentinite.

Serpentinite

Fig. 20.10: S*erpentinite* from Deer Lake in upper Michigan. It is a mineral also found in the oceanic crust that under conditions found in an alkaline vent, can form methane from CO_2 .

Experimental serpentinization produces hydrocarbons and a warm aqueous oxidation of iron produces H_2 that could account for abundant H_2 in today's *white smoker* emissions. Also, during serpentinization, a mineral called *olivine* [$(Mg^{+2}, Fe^{+2})_2SiO_4$] reacts with dissolved CO_2 to form methane (CH_4). So, the first precondition of life's earthly origins, the energetically favorable creation of organic molecules, is possible in alkaline vents.

Proponents of cellular origins in a late-Hadean non-reducing ocean also realized that organic molecules formed in an alkaline (or *any*) vent would disperse and be rapidly neutralized in the wider acidic ocean waters. Somehow, origins on a non-reducing planet had to include some way to contain newly formed organic molecules from the start, and a way to power further biochemical evolution. What then, were the conditions in an alkaline vent that could have contained organic molecules and led to metabolic evolution and ultimately, life? Let's consider an intriguing proposal that gets at an answer!

The porous rock structure of today's alkaline vents provides micro-spaces or micro-compartments that might have captured alkaline liquids emitted by *white smokers*. It turns out that conditions in today's alkaline vents also support the formation of hydrocarbon ***biofilms***. Micro-compartments lined with such biofilms could have formed a primitive prebiotic membrane against a rocky "cell wall", within which alkaline waters would be trapped. The result would be a natural *proton gradient* between the *alkaline* solutions of organic molecules trapped in the micro-compartments and the surrounding *acidic* ocean waters. Did all this happen?

Perhaps! Without a nutrient-rich environment, ***heterotrophs-first*** is not an option. That leaves only the alternate option: an ***autotrophs-first*** scenario for the origins of life. Nick Lane and his coworkers proposed that proton gradients were the selective force behind the evolution of early metabolic chemistries in the alkaline vent scenario (Prebiotic Proton Gradient Energy Fuels Origins of Life). Organized around biofilm compartments, prebiotic structures and chemistries would have harnessed the free energy of the *natural proton gradients*. In other words, the first protocells, and then cells, may have been ***chemoautotrophs***.

Last but not least, how might chemoautotrophic chemistries on a non-reducing planet have supported polymer formation, as well as polymer replication? Today we see storage and replication of information in nucleic acids as separate from enzymatic catalysis of biochemical reactions. But are they all that *separate*? If replication is the faithful reproduction of the information needed for a cell, then enzymatic catalysis ensures the redundant production of all molecules essential to make the cell! Put another way, if catalyzed *polymer* synthesis is the replication of the workhorse

molecules that accomplish cellular tasks, then what we call 'replication' is nothing more than the replication of nucleic acid *information* needed to faithfully reproduce these workhorse molecules. Was there an early coordinated, concurrent selection of mechanisms for the catalyzed metabolism as well as catalyzed polymer synthesis and replication? We'll return to these questions shortly, when we consider the origins of life in an RNA world.

Life-origins in a non-reducing (and oxygen-free) atmosphere raise additional questions. Would proton gradients provide enough free energy to fuel and organize life's origins? If so, how did cells arising from prebiotic chemiosmotic metabolism actually harness the energy of a proton gradient? Before life, were protocells already able to transduce gradient free energy into chemical free energy? When was ATP selected to hold chemical free energy from the start? Or was it selected from the start of metabolism? Alternatively, was the relief of the gradient coupled at first to the synthesis of other high-energy intermediate compounds with e.g., thioester linkages? Later on, how did cells formed in alkaline vents escape the vents to colonize the rest of the planet?

However proton gradient free energy was initially captured, the ***chemoautotrophic LUCA*** must already have been using membrane-bound proton pumps and an ATPase to harness gradient free energy to make ATP, since *all* of its descendants do so. Finally, when did photoautotrophy (specifically ***oxygenic photoautotrophy***) evolve? Was it a late evolutionary event? Is it possible that photosynthetic cells evolved quite early among some of the chemoautotrophic denizens of the white smokers, biding their time before exploding on the scene to create our oxygenic environment?

351 Life Origins in a Thermal Vent

20.6 Heterotrophs-First vs. Autotrophs-First: Some Evolutionary Considerations

In the alkaline vent scenario, chemiosmotic metabolism predated life. Therefore, the first chemoautotrophic cells did not need the fermentative reactions required by cells in a heterotrophs-first origin scenario. Even though all cells alive today incorporate a form of glycolytic metabolism, *glycolysis may in fact, not be the oldest known biochemical pathway*, as we have thought for so long.

In support of a later evolution of glycolytic enzymes, those of the archaea show little structural resemblance to those of bacteria. If fermentative heterotrophy was a late evolutionary development, then LUCA and its early descendants would lack a well-

developed glycolytic pathway. Instead, the LUCA must have been one of many 'experimental' autotrophic cells, most likely a chemoautotroph deriving free energy from inorganic chemicals in the environment. To account for heterotrophy in the three domains of life, it must have evolved separately in the two antecedent branches descending from the last universal common ancestor of bacterial, archaeal and eukaryotic organisms. The evolution of similar traits (fermentative biochemical pathways in this case) in unrelated organisms is called ***convergent evolution***.

Fig. 20.11 below superimposes an *autotrophs-first* scenario and separate evolution of heterotrophy by the two branches of an autotrophic LUCA on a familiar phylogeny, tracing the spread of fermentative pathways in all living things.

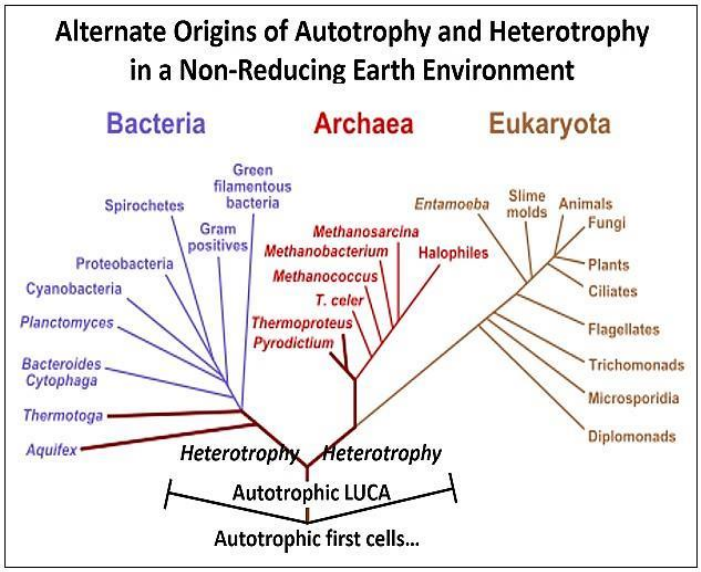


Fig. 20.11: Evolutionary (i.e., phylogenetic) tree of all living organisms reveals 3 domains of life: Bacteria (Prokarya), Archaebacteria (Archea) and Eukaryotes (Eukaryota).

20.7 A Summing Up

Speculation about life's origins begins by trying to identify a source of free energy with which to make organic molecules. The first cells might have been heterotrophs formed in a reducing earth environment, from which autotrophs later evolved. On the other hand, the earliest cells may have been autotrophs formed under non-reducing conditions in the

absence of a primordial soup. Then, only after these autotrophs had produced enough nutrient free energy to sustain themselves, did heterotrophs belatedly emerge. Evidence suggesting that the earth's atmosphere was a non-reducing one more than 4 billion years ago (soon after the formation of the planet), and suggesting life on earth 3.95 billion years ago favor metabolic origins of autotrophic life in a thermal vent, likely an alkaline one. Questions nevertheless remain about life-origins under non-reducing conditions. Even the composition of the prebiotic atmosphere is still in contention (see Non-Reducing earth-Not so fast!).

For now, let us put these concerns aside and turn to events that get us from the LUCA and its early descendants to the still more elaborated chemistries common to all cells today. The descriptions that follow are educated guesses about early pathways on the road to the familiar cellularity now on earth. They mainly speculate on the selection of catalytic mechanisms, replicative metabolism, the web of intersecting biochemical pathways, and the even more intricate chemical communications that organize cell function and complexity.

352 Phylogenetic Support for Autotrophs-First Origins of Life

20.8 Origins of Life Chemistries in an RNA World

Following the origins of organic monomers in a reducing environment in the tidal pool scenario, the energy for polymer formation came from cycling temperatures on an overheated earth. In that scenario, we considered how chains of nucleotides might have been synthesized and even replicated to form populations of nucleic acids with similar sequences. But if the prebiotic environment was non-reducing, where would the energy have come from to make any polymers, let alone ones that could replicate themselves? If you guessed that the energy was provided by a proton gradient between biofilm-enclosed acidic proto-cells and an alkaline ocean…, you would have been right! In this case, polymers would have been synthesized in enclosed spaces, and not in tidal pools only to be dispersed and diluted in the wider oceans. But then, how would replicative, informational and catalytic chemistries have arisen from these organic monomers and polymers? Polypeptides would have formed, but they have no inherent chemical or structural basis for self-replication. Unlike polypeptides, we saw in describing the tidal pool scenario that polynucleotides (nucleic acids) do! In fact, evidence is accumulating to support the hypothesis that life originated in a ***RNA world***:

- Today's RNAs include ***ribozymes*** that catalyze their own replication (e.g., self-splicing introns).
- Some RNAs are part of ***ribonucleoproteins*** with at least ***co-catalytic activity*** (recall ribosomes, spliceosomes and the secretory signal recognition particle).

- ***Retroviruses*** (e.g., HIV) store their genetic information in *RNA genomes* that may have been integral to the emergence of cellular life.

Ribozymes, ribonucleoprotein structures and retroviruses may be legacies of a prebiotic RNA world. In fact, in an '*in vitro* evolution study', self-replicating ribozyme polymerases in a test tube become more efficient at replicating a variety of increasingly longer and more complex RNAs over time. For more about these autocatalysts, click Artificial Ribozyme Evolution Supports Early RNA World.

There are hypothetical *RNA world scenarios* for the origins of replicating, catalytic polymers, and even a real organic chemical ***autocatalyst*** that can catalyze its own synthesis. So, which may have come first? A self-replicating RNA or some other self-replicating molecule, even a self-replicating organic molecule? Arguably, chemical evolution of an autocatalytic RNA is a stretch, but at least one organic molecule, **A**mino-**A**denosine **T**riacid-**E**ster (AATE), is a present-day self-replicating *autocatalyst*. Could an organic molecule like AATE have been a prebiotic prelude to the RNA world? Fig. 20.12 shows the structure and replication of AATE.

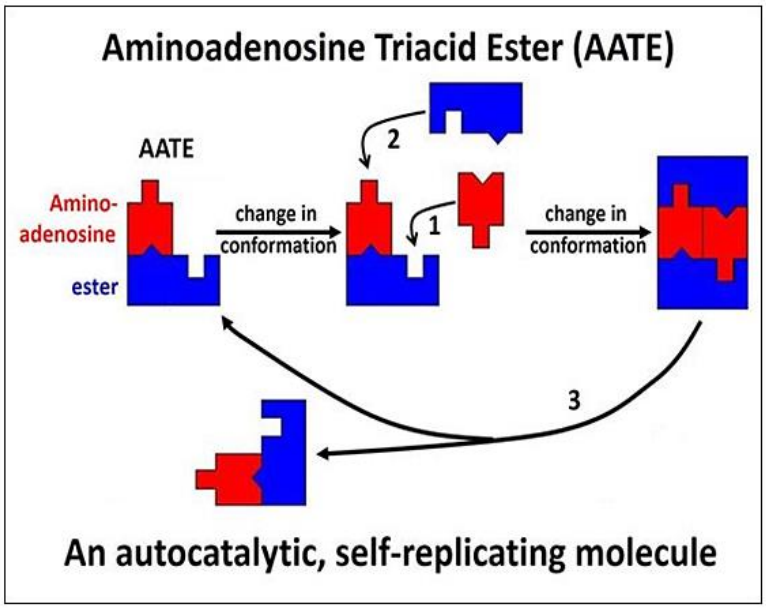


Fig. 20.12: *Aminoadenosine triacid ester* (AATE) catalyzes its own replication by the mechanism suggested here.

The replicative reaction proceeds in the following steps:

- The aminoadenosine triacid ester binds another molecule of aminoadenosine.
- The two aminoadenosines, now in opposite orientations, can attract and bind a second ester.
- After bond-rearrangements, the molecule separates into two molecules of AATE.

This reaction is catalytic because the stereochemistry of the reacting molecules creates an affinity of the aminoadenosine ester molecule first for another free aminoadenosine molecule, and then for a second free ester. The structure formed allows (i.e., catalyzes) linkage of the second aminoadenosine and ester followed by the separation of both AATE molecules. Subtle, sequential changes in the molecular conformation of the molecules result in the changes in affinities of the molecules for each other. In the replicative reaction, the AATE, free ester and free aminoadenosine concentrations would drive the reaction. Could AATE-like molecules have been progenitors of autocatalyzed polymer replication? *Could replication of a prebiotic AATE-like molecule have led to an RNA world?* Could primitive RNAs have been stabilized by binding to short prebiotic peptides, becoming forerunners of ribozymes? The possibility of a prebiotic AATE-like molecule is intriguing because the 'triacid' includes a nucleotide base, the purine adenosine! On the other hand, the possibility of prebiotic replicating RNA-peptide complexes implies the origins of life in an ***RNA-Protein world*** (rather than exclusively RNA-world)! Whether life began in an RNA world or an RNA-protein world, catalyzed replication is of course another property of life.

353 AATE: An Autocatalytic, Self-Replicating Organic Molecule

20.9 Experimental Evidence for an RNA World

So far, we have discussed circumstantial evidence and at least one candidate for a precursor to an RNA world. But could the chemistries necessary to link RNA bases into ribonucleic acids bases have existed under conditions assumed to exist on earth at the time of life's origins? Consider the following study…

Investigators probing an *RNA world* hypothesis for the origins of life reported the requirements for RNA replication in a test tube. Adding salt, magnesium ions, a bit of RNA primer and A, U, G and C (precursor bases to RNA) to a buffered basic solution… resulted in RNA synthesis! The replication reaction was slow and prone to error. Nevertheless, RNA *was* synthesized against the primer under these conditions which mimic aquatic candidates for life's beginnings, supporting an RNA world scenario. The study further found that adding the base inosine to the reactions increased the rate as well as the accuracy of RNA replication under what were otherwise the same conditions.

Of course, this is not what happens today! First of all, the bases are part of nucleosides and nucleotides that are the actual precursors of replication. Also, while inosine is in fact found in some tRNAs, it does not result from the use of inosine nucleotide precursors, but from the chemical modification (deamination) of adenine bases already in the transcript as seen in Fig. 20.13 (below).

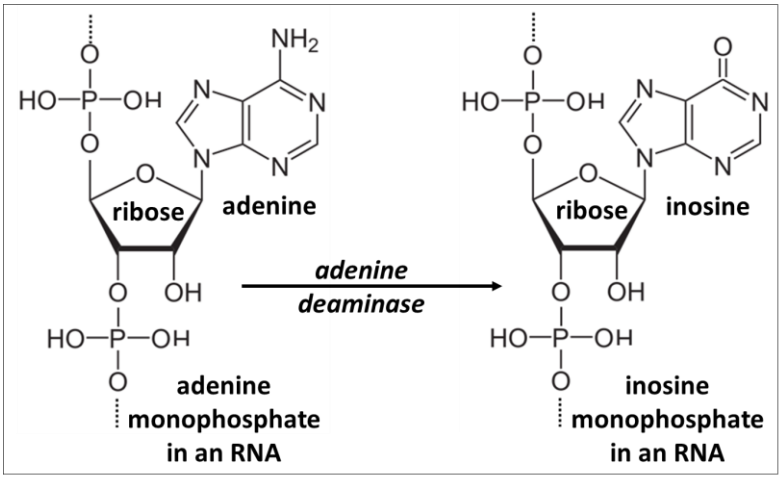


Fig. 20.13: The deamination of adenine in a ribonucleotide chain to inosine in a tRNA by the ***adenine deaminase*** enzyme.

OK, calling inosine the "missing ingredient" in life's origins might be hyperbole. Still, if cell-free RNA synthesis using nucleobases is more efficient with inosine than without it, could inosine have participated in an early RNA world at life's origins, to be later replaced by our DNA world? Read more possible roles for inosine or other unusual bases in an prebiotic RNA world at https://www.livescience.com/64355-missing-ingredient-in-origin-of-life.html?utm_source=notification.

20.10 Molecules Talk: Selecting Molecular Communication and Complexity

In our complex human society, we define ***communication*** by its ***specificity***. Without a careful choice of words, our speech would at best, be a source of magnificent misunderstanding…, or just plain babel! What does this mean for prebiotic chemistries? In terms of prebiotic chemical evolution, selection by definition would have favored the protective accumulation of longer-lived molecular aggregates. Over time, the same

selective imperatives would create webs of such aggregates, increasing the *range and specificity* of molecular interactions in a challenging environment. If this were to have occurred in an enclosed proto-cellular space, it would have resulted in a primitive *molecular communication* and the potential for a growing ***complexity*** (another property of life!). In fact, all of the properties of life must have accompanied the achievement of more and more complex intermolecular communication. Simply put, a prebiotic (or for that matter a cellular) genetic change that alters the rate of one catalytic reaction (if not destructive) will drive the selection of changes in components of other, interconnected metabolic chemistries. If *molecular communication* required the evolution of catalytic specificity, then the final elaboration of complexity and order as a property of life further requires the selection of mechanisms of ***regulation*** and ***coordination***.

20.10.1 Intermolecular Communication: Establishment of Essential Interconnected Chemistries

Earlier, we suggested that inorganic catalyst precursors to biological enzymes were probably minerals embedded in clay or other substrata, providing surfaces that would naturally aggregate organic molecules and catalyze repetitive reactions. Either the initial objects of prebiotic selection included stable monomers and polymers outside or as seems more likely, inside proto-cells. Later, chemical selection would have favored polymers that enhanced growth and reproduction of successful aggregates. These polymers were likely those that catalyzed their own synthesis, perhaps collaborating with inorganic catalytic minerals. The result would be the elaboration of a web of *interconnected chemical reactions* between molecules with high affinity for each other, thereby increasing the specificity of those reactions. In the context of life origins and evolution, ***co-catalysis*** describes the activities of these interconnected metabolic reactions.

As noted, high-affinity interactions are inherently ***protective***. During prebiotic chemical and/or metabolic evolution, protected stable ***molecular assemblies*** would be targets of selection. Continuing co-evolution of catalysts, substrates and co-catalytic reaction sets would lead to more and more sophisticated *molecular communication*. Once established, efficient biochemical reaction sets would be constrained against significant evolutionary change. Any change (mutation) that threatened this efficiency would mean the end of a prebiotic chemical (or for that matter, cell) lineage! This explains why we find common pathways for energy-generation (e.g., autotrophic and fermentative), reproduction (replication), and information storage and retrieval (DNA, RNA, protein synthesis) in all of LUCA's descendants. In other words, an organizing principle must have been selected to enable communication between molecules and their reactions. Such complex and effective communication requires ***coordination***.

In fact, effective communication is *defined* by coordination, the capacity to make *chemical decisions*. Selection of molecular aggregates that sequestered metabolic reactions behind a semipermeable membrane ensures that only certain molecules communicate with each other. This sequestration is likely to have occurred repeatedly during chemical evolution, beginning with the synthesis of larger, polymeric molecules and possibly, an aggregation of primitive lipoidal molecules. We can think of increasingly effective catalysis in an enclosed environment as ***a conversation mediated by good speakers***! Thus, *coordination* is a property that started with prebiotic chemistry and likely co-evolved with life itself.

20.10.2 Origins of Coordination

Let's look some possible structures churning around in the prebiotic chemistry set that might have self-assembled and sequestered compatible chemistries of life. Along with the alkaline vent *biofilm compartment,* ***coacervates***, ***proteinoid microspheres*** and ***liposomes*** have been considered as possible progenitors of biological membranes. Each can be made in the laboratory. They are demonstrably semipermeable, and in some cases can even replicate! The production of coacervates, proteinoid microspheres and liposomes is summarized in Fig. 20.14.

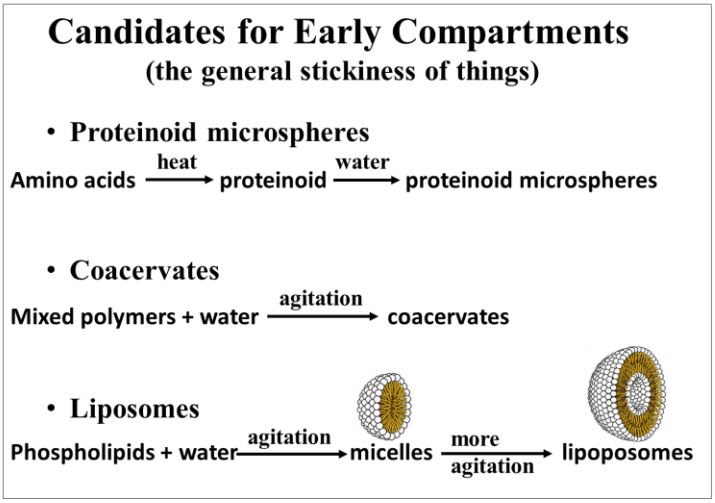


Fig. 20.14*: Proteinoid microspheres*, *coacervates* and *liposomes* can all be made in a laboratory and are candidates for boundary structures that could have protected early prebiotic organic molecules and reactions. Click the link at https://www.flickr.com/Candidates for Early Compartments to see micrographs of these synthetic compartments.

Oparin had proposed that the action of sunlight in the absence of oxygen could cause ionized, oppositely charged organic molecules (e,g, amino acids, carbohydrates, etc.) to form droplets from organic molecules in his primordial soup. These ***coacervates*** were actually produced in 1932, visualized by microscopy and demonstarted to be a semi-permeable compartment. They even behaved as if they were able to grow and reproduce (also as Oparin originally suggested they might).

In the 1950s, Sidney Fox produced ***proteinoid microspheres*** from short peptides that formed spontaneously from aqueous amino acid solutions heated to dryness (not unlike what happens in the tidal pool scenario of polymer formation from organic monomers). These can be seen by light and electron microscopy.

While ***liposomes*** are easily made in a laboratory, it isn't clear that they existed on a pre-biotic earth. Nevertheless, cell membranes must have had acquired their phospholipid bilayer structure by the time of LUCA since we all have them! Prior to LUCA (perhaps in or soon after formation of *our* progenote), chemical rearrangements must have occurred to enable incorporation of a phospholipid bilayer into whatever starting boundary life started with.

We have already considered the ***biofilm*** proposed for cellular origins in an alkaline vent. The formation of such biofilms would have separated acidic ocean protons from the interior of such protocells, creating a proton gradient. Such a gradient could have driven the early evolution of chemiososis as a means of capturing chemical energy, complete with the eventual selection of ATP synthases and the enzymes of proton transport, again because all cells descendent from LUCA posess these biochemistries.

Of course, proteinoid microspheres, coacervates, biofilm-based 'membranes and liposomes are not alive, and are therefore not cells. But one or another of them must have been where the enhanced ***coordination of molecular communication*** required for life began.


354-2 Protected Molecular Communication-Semipermeable Membranes


An important ***take-home message*** here is that whatever the original structure of the first cells, they arose soon after the organic chemical prerequisites of life began to acquire familiar metabolic functions. We need to see chemical and structural progress to cellularity as concurrent metabolic evolutionary events. At some point, selection of sequestered biochemistries led to ***protocells***, then to the first cell or cells, each with all of the properties of life.

Finally, selection of highly specific communication between cellular molecules allowed cells themselves to talk to one another, engage in group activities, and eventually join together to form multicellular organisms. Multicellularity is of course a characteristic of many if not most eukaryotes. But watch a great TED Talk on bacterial intercellular communication by Dr. Bonnie Bassler at Intercellular Communication in Bacteria.

20.10.3 An RNA World: Origins of Information Storage and Retrieval

Let us accept for now that molecular communication began concurrently with the packaging of interconnected co-catalytic sets into semipermeable structures. Then the most 'fit' of these structures were selected for efficient coordination of meaningful, timely chemical messages. Ultimately, coordination requires ***information processing*, *storage* and *retrieval***, something we recognize in Francis Crick's *Central Dogma* of information flow from DNA to RNA to protein. Cells and organisms do coordination quite well, but what do its beginnings look like? The answer may lie in the pre-biotic RNA world we discussed earlier. Fig. 20.15 below is a statement of *The Central Dogma*, modified to account for reverse transcription and the behavior of retroviruses.

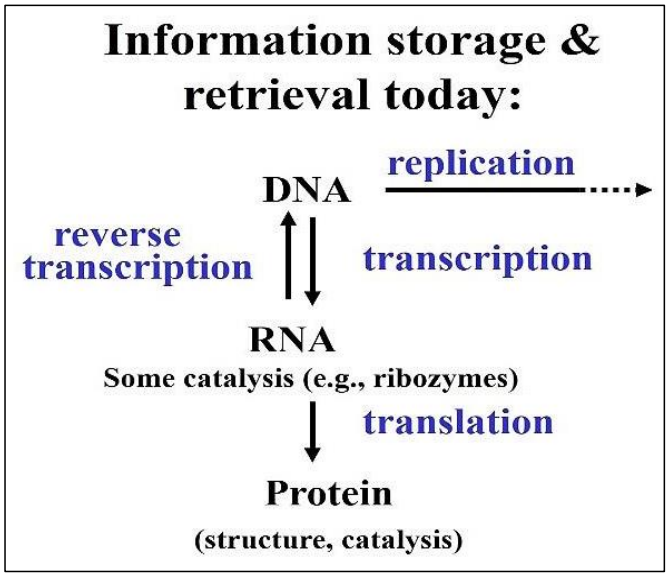


Fig. 20.15: Genetic information flows from DNA to RNA to protein (the *Central Dogma*) but can also flow from RNA to DNA by reverse transcription.

We do not really know how cells came to rely on DNA to store, pass on and mobilize genetic information, but we have presented reasons to believe that the first replicating nucleic acid was RNA, creating an *RNA world*. Here is the evidence that leads us to this conclusion.

- Based on the stem-and-loop and other structures that form when RNA molecules undergo internal H-bonding, we know that RNAs can take on varied and intricate shapes.
- Diverse conformations are consistent with the evolution of specificity in the interaction of RNAs with themselves and/or with other molecules in the prebiotic environment.
- RNAs, either alone as *autocatalysts* (for example, self-splicing mRNAs) or in catalytic ribonucleoprotein complexes (e.g., ribosomes) exist in cells today.
- Some of these RNAs (specifically rRNAs), have a long phylogenetic heritage, shared by cells in all three domains of life.

The propensity of single stranded RNA molecules to fold based on internal H-bonding can lead to those diverse three-dimensional shapes (tertiary structure). These structures could have interacted with other molecules in a prebiotic environment. Because they could be replicated according to different prebiotic scenarios, the same RNAs could also pass on simple genetic information contained in their base sequences. The combination of informational and catalytic properties in a single molecule is illustrated in Fig. 20.16 below.

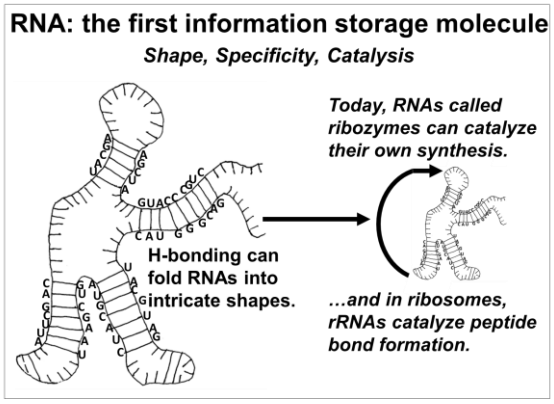


Fig. 20.16: RNA Sequences contain genetic information, and in retroviruses, they are molecules of inheritance. As single stranded molecules, RNAs can fold into 3-dimensional shapes, creating specific shapes that can act as catalysts (e.g., ribozymes). Thus RNAs can combine information storage with catalytic activities.

The capacity of RNAs to be catalysts and warehouses of genetic information at the same time speaks to an efficient candidate for the first *dual* or *multi-purpose* polymer, a property that is not known and cannot be demonstrated for DNA. Read more about the proposed 'RNA worlds' in which life may have begun in *Cech T.R. 2012* (*The RNA Worlds in Context; Cold Spring Harbor Perspectives in Biology; Cold Spring Harbor, NY: Cold Spring Harbor press; 4:a006742e*).

355 Self-Replication: Information, Communication & Coordination

20.10.4 From Self-Replicating RNAs to Ribozymes to Enzymes; From RNA to DNA

What might RNA catalysis beyond self-replication have looked like in simpler times? Consider the interaction between a two hypothetical RNAs and different hypothetical amino acids bound to each (Fig. 20.17 below).

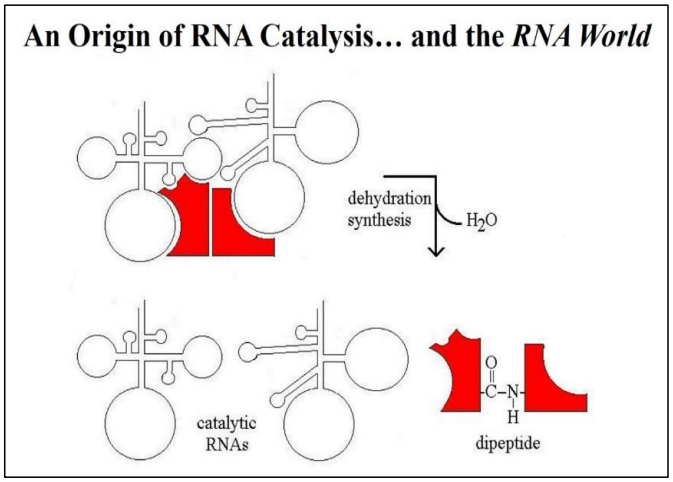


Fig. 20.17: Hypothetical origin of RNA catalysis, suggesting how some early (prebiotic) RNAs may have evolved to catalyze peptide bond formation between amino acids.

The binding of each RNA to its amino acid would be a high affinity, specific interaction based on charge and shape complementarity. Likewise, the two RNAs seen in the illustration must have a high affinity for each other, also based on chemical and physical complementarities. One can even envision some strong H-bonding between bases in the two RNAs that might displace intra-strand H-bonding (not shown in the illustration here).

The result is that the two amino acids are brought together in a way that catalyzes peptide bond formation. This will require an input of free energy (recall that peptide bond is one of the most energy intensive reaction in cells). For now, assume a chemical energy source and let us focus on the specificities required for RNA catalytic activity.

We know now that tRNAs are the intermediaries between nucleic acids and polypeptide synthesis. So it's fair to ask how the kind of activity illustrated above could have led to the tRNA-amino acid interactions we see today. There is no obvious binding chemistry between today's amino acids and RNAs, but there may be a less obvious legacy of the proposed bindings. This has to do with the fact that the genetic code is universal, which means that any structural relationship between RNA and amino acids must have been selected early (at the start!) of cellular life on earth. Here is the argument.

1. The code is indeed universal (or nearly so)
2. There is a correlation between the chemical properties of amino acids and their codons, for example:
    - Charged (polar) amino acids are encoded by triplet codons with more G (guanine) bases.
    - Codons for uncharged amino acids more often contain a middle U (uracil) than any other base.

These correlations would mean that an early binding of amino acids to specifically folded RNAs was replaced in evolution by enzyme-catalyzed covalent attachment of an amino acid to a 'correct' tRNA, such as we see today.

What forces might have selected separation of the combined template/informational functions from most of the catalytic activities of RNAs? Perhaps it was the selection of the greater diversity of structure (i.e., shape) that folded polypeptides can achieve, compared to folded RNAs. After all, polypeptides are strings of 20 different amino acids compared to the four bases that make up nucleic acids. This potential for molecular diversity would in turn accelerate the pace of chemical (and ultimately cellular) evolution. A scenario for the transition from earlier self-replicating RNA events to the translation of proteins from mRNAs is suggested in Fig. 20.18 (below).

Adaptor RNAs in the illustration will become tRNAs. The novel, relatively unfolded RNA depicts a presumptive mRNA. Thus, even before the entry of DNA into our RNA world, it is possible to imagine the selection of the defining features of the genetic code and mechanism of translation (protein synthesis) that characterizes all life on the planet. Next, we consider "best-speculations" of how RNA-based information storage and catalytic chemistries might have made the evolutionary transition to DNA-based information storage and predominantly protein-based enzyme catalysis.

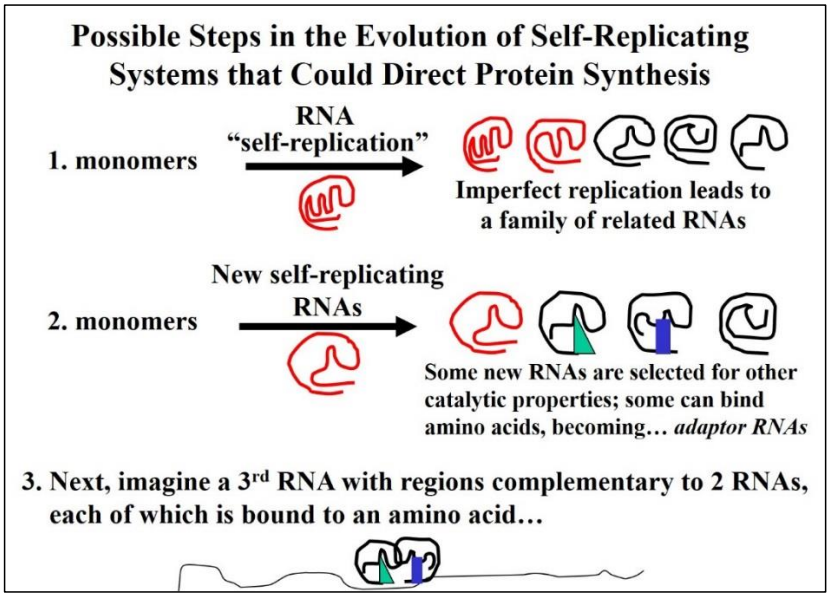


Fig. 20.18: Suggested steps in evolution from an RNA world to our DNA world. The first of these steps would be to divorce the self-replication activity of RNAs from their information storage function, as illustrated here (see text for details).

20.10.4.1 Ribozymes Branch Out: Replication, Transcription and Translation

The term *co-catalysis* could very well describe biochemical reactions in which a catalyst accelerates a chemical reaction whose product feeds back in some way on its own synthesis. We saw this in action when we discussed allosteric enzyme regulation and the control of biochemical pathways. Catalytic feedback loops must have been significant events in the evolution of the *intermolecular communication* and *metabolic coordination* required for life. Here we consider some scenarios for the transition from an RNA world to something more recognizable as today's nucleic acid information storage and protein-based catalytic metabolism.

If early RNAs catalyzed their own replication, they were functioning as primitive ribozymes. If some of these ribozymes bound and polymerized amino acids, they may have catalyzed the synthesis of short peptides. What if some of the polypeptides occasionally bound to their shapely catalytic RNAs and enhanced the catalytic properties of the aggregate? What if one of these enhanced the rate of synthesis of its own RNA, or evolved to catalyze other reactions useful to life?

Such a structure might presage the ribosome, which is a ribonucleoprotein with catalytic properties. If later in evolution, a peptide changed just enough to accomplish the catalysis on its own, it might dissociate from its RNA, flying solo as a protein enzyme catalyst. This idea is illustrated below in Fig. 20.19.

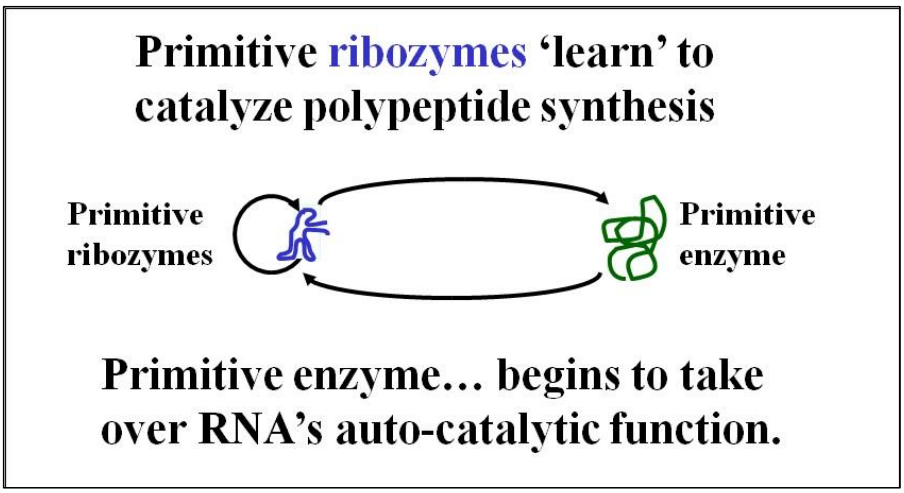


Fig. 20.19: If RNAs could bind peptides whose synthesis they catalyze, evolution could have selected ribozymes from some RNA-peptide complexes that did not separate. Later, the peptides themselves could have evolved to take over catalytic functions from ribozymes.

Selection favoring the synthesis of short oligopeptides and polypeptides is consistent with a catalytic diversification that led to the dominance of protein catalysts, i.e., enzymes. The primitive enzyme shown here must have been selected because at first, it assisted the autocatalytic replication of the RNA itself!

Over time, the enzyme would evolve along with the RNA. This co-evolution then eventually replaced autocatalytic RNA replication with the enzyme-catalyzed RNA synthesis we recognize as transcription today. In this scenario, self-splicing pre-mRNAs and ribozymes are surviving remnants of an RNA world!

356 Information Storage and Retrieval in an RNA World

Let's turn now to some ideas about *how* an RNA world could make the transition to the DNA-RNA-protein world we have today.

20.10.4.2 Transfer of Information Storage from RNA to DNA

The transfer of function from RNA to DNA is by no means a settled issue among students of life origins and early evolution. A best guess is that the elaboration of protein enzymes begun in the RNA world would lead to reverse transcriptase-like enzymes that copied RNA information into DNA molecules. The basic transfer scenario is illustrated below in Fig 20.20.

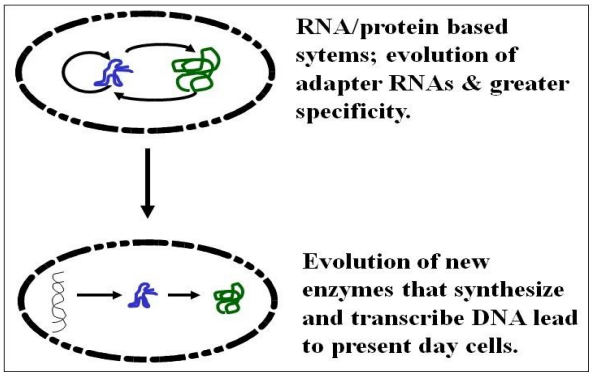


Fig. 20.20: A final evolutionary scenario gets us from the RNA world to a DNA world that governs most life on earth today.

DNA information may have been selected because DNA is chemically more stable than RNA. All cells alive today store information in DNA (only some viruses have an RNA genome). Therefore, transition to the use of DNA as an information molecule must have preceded the origin of life. At least, it must have occurred in the cells from which the LUCA arose. Details of this key change involve evolutionary steps yet to be worked out to everyone's satisfaction!

357 The Transition from an RNA World to a DNA World

20.11 The Evolution of Biochemical Pathways

The tale of the evolution of enzymes from ribozymes and of informational DNA from RNA, and the other metabolic chemistries behind prebiotic semipermeable boundaries is ongoing in cells today. Undoubtedly, early cellular metabolism involved only reactions crucial to life…, catalyzed by a limited number of enzymes. But, if evolution inexorably trends towards greater complexity of molecular communication and coordination…, in other words, towards increasingly refined regulation of metabolism,

how did the repertoire of enzymes get larger, and how did biochemical pathways become more elaborate? We answered the first question elsewhere, when we discussed gene duplication (e.g., by unequal crossing over). The duplicate genes encoding the same enzyme provided the raw material for new enzymes and new enzymatic functions.

Whether in cells or in prebiotic structures, we can hypothesize how a new chemical reaction could evolve. For example, assume that a cell acquires molecule **D** required for an essential function, from an external, environmental source. What happens if levels of **D** in the environment become *limiting*? Clearly, cells would die without enough **D**. That is, unless some cells in the population already have a duplicated, redundant gene that has mutated and now encodes an enzyme with the ability to make **D** in the cell. Such a cell might have existed with other cells without the mutation, but a **D**-limited environment would select the mutant cell for survival and reproduction. Imagine the scenario illustrated in Fig 20.21.

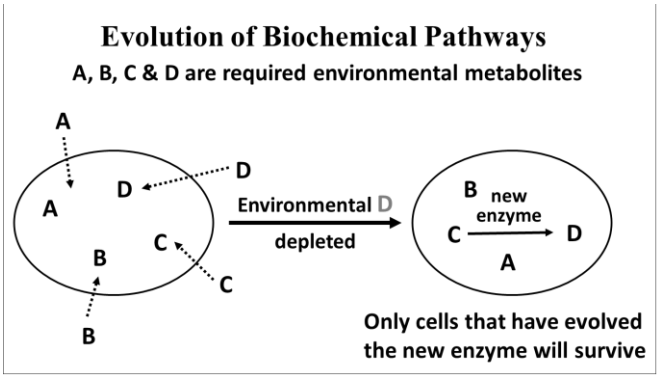


Fig. 20.21: One selective pressure that contributes to the complexity of biochemical pathways would be the depletion of a particular chemical resource, say molecule D, in the environment). The few cells in the population that happen to have an enzyme that can convert C to D will survive and proliferate.

358 Origins and Evolution of Biochemical Pathways

In a similar scenario, a mutation in a duplicated gene could result in a novel enzyme activity that can convert some molecule (e.g., C or D) in the cell into a new molecular product. If the new enzyme and molecular product do not kill or debilitate the cell, the cell might survive to be selected by some future exigency.

20.12 A Grand Summary and Some Conclusions

Our consideration of how life began on earth was intentionally placed at the end of this textbook, after we tried to get a handle on how cells work. Clearly any understanding of life origins scenarios is very much a matter of informed, if divergent speculations. Alternative notions for the origins of life entertained here all address events that presaged life under 'best-guess' hypothetical conditions. After trying to get a grip on prebiotic events, we asked how we got from what could have happened under a given set of prebiotic conditions to the cellular life we recognize today.

All proposals recognize that the first cells had all of the properties of life (including evolution itself). Starting with that common understanding, all arguable scenarios try to navigate pathways from primitive, less controlled chemistries to more regulated and coordinated metabolisms, in other words from chemical simplicity to biochemical complexity. The chemical and metabolic evolution that began before life may have overlapped in time with cellular evolution, at least until the LUCA. While chemical evolution was mainly a series of selections by the physicality of a prebiotic world, the evolution of life contends with both that physical world, and with life itself. LUCA, the universal common ancestor, had already escaped the RNA world, replicating DNA, transcribing RNA and translating mRNAs into polypeptides, all behind a semipermeable phospholipid bilayer. Whether a heterotroph or (increasingly more likely) an autotroph, LUCA used the energy of ATP to power all of its cellular work, as do its descendants. Thus, cellular evolution, in fact all life after the LUCA, is focused on continued selection of the complexities of metabolism that enables the spread and diversification of life from wherever it started.

The selection of chemistries and traits encoded by already existing, accumulated random neutral genetic changes continues to this day to increase the diversity of species and their spread to virtually every conceivable ecological niche on the planet. The overall take-home message of this chapter should be an understanding of how the molecular basis of evolution can help us understand how life may have begun and been sustained on earth (or anywhere for that matter). In turn, speculation about life's origins informs us about how the properties of life were selected under a set of reasonably likely prebiotic physical and chemical conditions.

Some iText & VOP Key Words and Terms

AATE	**deep sea hydrothermal vent**	**progenote**
abiogenesis	**Hadean eon**	**proteinoid microsphere**
adaptor RNA	**heat of baking**	**protocell**

alkaline hydrothermal vent	heterotrophs-first	reducing atmosphere
aminoadenosine triacid ester	ionizing radiation	retroviruses
Archean eon	Last universal common ancestor	ribonucleoproteins
autocatalysis	liposome	ribozymes
autotrophs-first	LUCA	RNA world
biofilm	metabolic evolution	Serpentinite
biogenesis	molecular communication	serpentinite
black smoker	non-reducing atmosphere	serpentinization
chemoautotrophs	ozone layer	spontaneous generation
chemoautotrophs	Panspermia	tidal pool scenario
coacervate	photoautotrophs	white smoker
co-catalysis	primordial soup	zircon

Epilogue

We began this textbook with a testimony to the marvels of what science can accomplish. In Chapter 1 we defined the scientific enterprise as both intuitive and disciplined and learned that the goal of learning science and its methods is to prepare us to ask creative questions and to answer them. In Chapter 1 we learned that prebiotic chemical evolution set the stage for life's origins and that evolution is a key property of life itself, leading inexorably to the incredible diversity of life on which Darwin predicated his theory. In Chapter 1 we noted the natural role of extinction in favoring diversity… and finally, how recent human activity accelerated the near extinction of the American bison and the final extinction of the passenger pigeon. The role of evolution in creating diversity is emphasized throughout the book. This is a moment to consider the threat of human activity to life on earth that has only increased since the last known passenger pigeon died in 1914. Here are some more recent threats.

- Big cities (notably Los Angeles) had a smog problem dating back to the 1940's. After much resistance to evidence that auto emissions were the cause, public acceptance came in the 1950s. Congress responded in 1963 by passing the first Clean Air Act and set some emission standards 2 years later. In 1975 the U.S. mandated catalytic converters on all new cars. California still has some of the worst air in the country. But "worst" isn't as bad as it used to be. Ozone levels in Los Angeles are just 40 percent of what they were in the mid-1970s, and that's with more than twice the number of cars." (From S. Gardener (1914) https://www.marketplace.org/battle-against-air-pollution/).

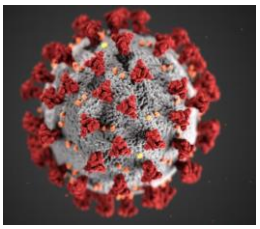
  Nevertheless, Los Angeles can still show us smog, as in the photo below:

By Diliff-Own work; CC-BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=464376

- Plagues, epidemics and pandemics have been with us since ancient times. More recent ones include a flu pandemic that may have started in Russia in 1889 that killed an estimated million people worldwide by 1890. WW I began 24 years later, ending in 1918 with 20 million (mostly men) dead. Then the influenza pandemic of

1918 wasted no time killing an estimated 50,000,000+ men and women worldwide, proving once again, that nature always gets the upper hand. The 2009 swine flu (*H1N1pdm09*) pandemic killed 100,000-500,000+ people worldwide. There were other recent gentle reminders that nature can still plague us (e.g., SARS in 2002-03, the 'bird flu in 2003-07). As if to drive home this message, in 2020 we have *COVID-19*, a respiratory disease caused by *SARS-CoV-2*, a novel corona retrovirus.

COVID-19 surfaced in China in late 2019, spreading rapidly. By March 11, 2020 with >250,000 infections world-wide and >10,000 dead, the World Health Organization (WHO) called *COVID-19* a pandemic. As of this writing (April, 2020), China and South Korea seem to have controlled the spread of *COVID-19*. But cases still double every few days in some countries (Italy, Iran, Spain, France…) and in U.S. states like Washington, New York and Louisiana, with predictions of worse to come. This is in spite of the sequencing of the *SARS-CoV-2* genome rapid development of PCR-based and serological tests for the disease. The latter are based on antibody-detection of viral antigens (for some details, see https://en.wikipedia.org/wiki/COVID-19_testing).



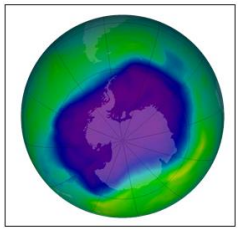
COVID-19 Viral Particle: Public Domain;
https://phil.cdc.gov/Details.aspx?pid=23313
(CDC/Alissa Eckert, MS; Dan Higgins, MAMS)

Our individual response is often to rally around our relatives, neighbors and even strangers to help them through tough times. We bring them food and medical supplies and keep up their spirits if they are in quarantine or physically isolated by their illness. Unfortunately as a group or polity, we are curiously remiss. For example, as *COVID-19* spread in China, a sometimes secretive government slowed recognition of a looming pandemic. While China took actions that seem to have contained *COVID-19*, the response beyond China's borders has been variable and reluctant. Timid governmental reactions have led to shortages of medical equipment and personnel, an economic crash, looming unemployment, public uncertainty, and ill-advised behavior (e.g., large gatherings that ignore *social distancing* prescriptions).

The good news is that corona viruses, including SARS-CoV-2 are more stable that other viruses such as HIV, which is known for mutating rapidly and evading the immune system. After many years of study, there are treatments for HIV, but still no vaccine. Unlike HIV, corona viruses a proofreading and correct replication errors, and we may soon have vaccines.

So, the *COVID19* pandemic will pass, but only after the damage already done by collaboration between the virus and us. How soon we emerge from this pandemic? How well will we cope with the next one? That will depend on how well we use available science, as well as how our leaders respond and how we all behave!

- Some of you may recall the polar ozone holes. It was discovered over the Antarctic in 1984 and a smaller one hovers over the Arctic.



The largest Ozone hole over the Antarctic lasted from 2000 to 2006 until it slowly began to close after CFCs were banned starting in 1987. The blue and purple colors define this 10.6 million square mile zone of ozone depletion. Public Domain; http://www.nasa.gov/vision/earth/lookingatearth/ozone_record.html

We watched the Antarctic ozone hole grow. Then we discovered that CFCs (chloro-fluorocarbons) were the cause. CFCs are a propellant that used to be in spray cans filled with things like hair spray and paint. They are also a refrigerant that was part of every household refrigerator. Since the international 1987 Montreal Protocol banned CFCs, more and more countries agreed to the ban. While the expanse of the Antarctic ozone hole has *slowly* receded, it still reopens every year. The Arctic ozone hole opens periodically and varies in size, the one that opened in the winter of 2019-2020, at three times the size of Greenland, was one of the largest (see the report at https://www.livescience.com/largest-ozone-hole-arctic). Concern over upper atmospheric ozone depletion continues, and now, with new information linking it to climate change (for more information, click Ozone Holes and Climate Change).

- Plastics are produced from petrochemicals (i.e., oil), often by oil companies themselves. Most everyday disposable plastics are *not* biodegradable.

Public Domain: From https://ocean_service_noaa.gov_facts/microplastics.html

Water bottles and milk jugs are typically recyclable. However, most other consumable plastics (straws, cups, plates, packing materials, particles in cosmetics and tooth-paste, grocery bags, clam-shells, tubs and other food wrappings…) are not, even when marked as recyclable with the 'chasing arrows'.

Public Domain, From Wikipedia

They just cost too much to sort, clean up and recycle. Some plastic waste ends up in landfills. Much was being shipped to the far east third world countries (e.g., Indonesia), intended for recycling. But it's too expensive to recycle even there. So some is incinerated and much ends up dumped into the Pacific Ocean.

This plastic debris now floats in *The Great Pacific Garbage Patch* (actually two patches), circulated by vortex current that distributes the plastic trash from the United States to Japan. Either as whole pieces or as degraded microplastics, the trash accumulates in the bodies of marine animals feeding around the patches. Toxic polychlorinated biphenyls (PCBs) that leach from plastics made before 2001 (when PCBs were effectively banned worldwide) also spread through the marine food chain. The floating patches can also block light from penetrating the ocean water, killing off photoautotrophic marine phytoplankton and algae, the photoautotrophic primary producers reduce atmospheric CO_2 and feed marine life.

Plastic debris has even settled more than 6000 feet to the bottom of the Pacific Ocean Marianna Trench. There, a new crustacean species was discovered in 2020, already having ingested a polyethylene-derived bit of plastic garbage. The new species was named *Eurythenes plasticus*!

CC-BY-SA; By Johanna Weston, Alan Jameson - Newcastle University, WWF Germany, Attribution, https://commons.wikimedia.org/w/index.php?curid=87860438. *For more, Read the article at* https://www.biotaxa.org/Zootaxa/article/view/zootaxa.4748.1.9 and listen to https://www.youtube.com/watch?v=QBqQi6xLdww.

- Fast forward to our last chapter where we revisited in detail physical and chemical conditions that could support life chemistries and the origins of life itself, noting that evolution began the pathway to the generation of new species and the spectacular diversity on display to everyone in magazines like National Geographic, Smithsonian and the like . By the 1960s, a few scientists saw a role of human activity in global warming. Now, 97% of scientists form the consensus that we caused a dramatic acceleration in climate change, raising global temperatures by 1^0 C. We are seeing stronger storms, more severe coastal flooding and inland droughts that most scientists view as the result.

  And, despite agreement on the human impact on climate change, international agreements have done little to slow increases in the greenhouse gas (90% CO_2 and CH_4) emissions that aggravate global warming. Tropical deforestation and the consumption of vehicle, residential and industrial fossil fuels continue with little restraint (check out https://www.epa.gov/ghgemissions/overview-greenhouse-gases for some more details.

  So, have we reached a temperature "tipping point" past which climate change is irreversible, ecological losses will drive extinctions and dramatically change the course of evolution, and above which, faith in solutions from science becomes a rush to cope? Is the recent spate of forest fires in California caused by climate change? Does an estimate of loss of a billion animals (not counting small critters like insects and worms…) in the southern Australia bush fires mean that we have reached or even surpassed that tipping point?

Left: Firefighters move in to protect properties from an out of control bushfire in SW Sydney, Australia. CC-BY-SA; From Helitak430, Dec. 2019, https://commons.wikimedia.org/Bushfire.jpg
Right: A large, out of control bushfire approaches the township of Yanderra. CC-BY-SA: From Helitak430 (Dec. 2019); https://commons.wikimedia.org/wiki/File:Yanderra_Bushfire.jpg#filelinks

With more or less pessimism, young people are asking these questions and making demands of their elders. Click Speaking truth at the UN to see Greta Thunberg's address to the United Nations 2019 Climate Action Summit. With more or less optimism we can hope that the children will find the answers.

Appendix I: List of Figures & Sources

Chapter 1

*Chapter Top-Art: Public Domain: l*eft, *R. Hooke, (1865) Micrographia';* middle, 19th century travelogue; right, Debbie Stinson photograph from https://notesfromstillsong.blogspot.com/2012/02/eremitical-life.html ; and https://www.pinterest.com/pin/138485757266449322/

Fig. 1.1: Evolution of three domains showing a closer relationship between archaebacteria and Eukaryotes. Illustration by G. Bergtrom

Fig. 1.2: Tobacco mosaic virus symptoms on a tobacco leaf.
USDA Forest Service, http://www.forestryimages.org/browse/detail.cfm?imgnum=1402027 and https://en.wikipedia.org/wiki/Tobacco_mosaic_virus#/media/File:Tobacco_mosaic_virus_symptoms_tobacco.jpg

Fig. 1.3: Transmission electron micrographs of giant viruses, the AIDS (HIV) virus and the bacterium *E. coli*. *K. casanovai* is at least the same size and *M. horridgei* is twice the size of the bacterium. All the giant viruses, even the mimivirus, dwarf HIV, a typical eukaryotic virus. Illustration by G. Bergtrom

Fig. 1.4: Transmission and scanning electron micrographs of the gram-negative *E. coli* bacterium (left and middle), with its basic structure illustrated at the right.

- CC0, From Peter Highton estate, Un. of Edinburgh:; https://commons.Wikimedia.org/w/index.php?curid=50418351
- CC-0,From photo by Eric Erbe, digital colorization by Christopher Pooley at USDA, EMU, ARS (ARS materials are in the public domain https://commons.wikimedia.org/w/index.php?curid=958857
- Illustration by G. Bergtrom

Fig. 1.5: Illustrated cross section of a dividing bacterium showing location of MreB cortical ring protein (purple). Public domain; Adapted From: https://en.m.wikipedia.org/wiki/File:FtsZ_Filaments.svg

Fig. 1.6: Carboxysomes in a cyanobacterium, as seen by transmission electron microscopy. CC-BY; From: http://en.wikipedia.org/wiki/File:Carboxysomes_EM.jpg

Fig. 1.7: Scanning electron micrograph of Halobacterium salinarium, a salt-loving bacterium. CC-BY; Adapted from: https://openi.nlm.nih.gov/imgs/512/185/3495301/PMC3495301_gbi0010-0424-f2.png

Fig. 1.8: Scanning electron micrograph of 'heat-loving' *Thermus aquaticus* bacteria. CC-0 (public domain) Adapted From: https://upload.wikimedia.org/wikipedia/commons/4/48/Thermus_aquaticus.JPG

Fig. 1.9: Illustration of the structural components of a typical animal cell. Source: Public Domain; From Mariana Ruiz, Image: Animal cell structure.svg https://commons.wikimedia.org/w/index.php?curid=4266142

Fig. 1.10: Illustration of the structural components of a typical plant cell. Source: Public Domain; From Mariana Ruiz, Image: Animal cell structure.svg, https://commons.wikimedia.org/w/index.php?curid=4266142

Fig. 1.11: LEFT - Transmission electron micrograph of an insect cell nucleus indicates the nucleolus(**n**)); RIGHT – Illustration of a nucleus showing chromatin (purple) and pores in the nuclear envelope (orange).

- Left ; From Bergtrom et al., (1977) J. Ultrastr. Res. 60:395-406: Research by G. Bergtrom
- Right; CC BY 3.0; Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". *WikiJ. Medicine 1(12).* DOI:10.15347/wjm/2014.010. Own work-https://commons.wikimedia.org/w/index.php?curid=28223971

Fig. 1.12: Simulated fluorescence micrograph showing the immunolocalization of antibodies against fibrillarin, coilin and ASF/SF2 protein to *nuclear bodies* (nucleolus, *Cajal Bodies* and nuclear speckles, respectively). Illustration by G. Bergtrom, adapted from a fluorescence micrograph in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC138913/12

Fig. 1.13: Fluorescence micrograph of a mitotic spindle treated with antibodies to chromosomal proteins (blue) and spindle fiber proteins (green). Public Domain; From: Afunguy-Transferred to Commons by Lije also using CommonsHelper; https://commons.wikimedia.org/w/index.php?curid=5148470

Fig. 1.14: To form a polysome, ribosomes (blue) assemble at the left on an mRNA molecule. As they move along the mRNA from left to right, they translate the message into a polypeptide (green), shown growing and emerging from ribosomes in the polysome. Illustration by G. Bergtrom.

Fig. 1.15: Transmission electron micrographs of cells from desiccated and fully hydrated *Selaginella lepidophylla* plants. Free ribosomes or ribosomal subunits in the desiccated cells (LEFT) appear to have organized to form polysomes in the hydrated plant cells (RIGHT). From Bergtrom et al. (1982) J. Ultrastr. Res 78:269-282. Research by G. Bergtrom

Fig. 1.16: An illustration of the differences between prokaryotic and eukaryotic ribosomes; Eukaryotic ribosomes and their subunits are larger and contain more proteins and larger ribosomal RNAs (rRNAs) than those of e.g., bacteria. The components were separated by sucrose density gradient centrifugation in which particles and macromolecules (like RNA) move through a sugar gradient at rates dependent on their mass (in effect, their size). Illustration by G. Bergtrom

Fig. 1.17: Transmission electron micrograph of an insect cell showing Golgi bodies (G). Adapted from Bergtrom and Robinson (1977) J. Ultrastr. Res. 60:395-405. Research by G. Bergtrom

Fig. 1.18: Illustration of 'packaged' protein traffic through a cell, from the RER to organelles (e.g., lysosomes) or to the plasma membrane for exocytosis (i.e., secretion). RER and Golgi vesicles are major sites for the modification (i.e., maturation) of packaged proteins. Illustration by G. Bergtrom

Fig. 1.19: Transmission electron micrograph of mitochondria (LEFT) and drawing of a mitochondrion (RIGHT).

- Electron micrograph from: Bergtrom et al. (1977) J. Ultrastr. Res. 60:395-405. Research by G. Bergtrom
- Public Domain; By Mariana Ruiz Villarreal LadyofHats, https://commons.wikimedia.org/w/index.php?curid=6195050

Fig. 1.20: Transmission electron micrograph of chloroplast that could have begun photosynthesizing (LEFT), and one that has photosynthesized long enough to accumulated starch granules (RIGHT). S, starch granule; T, thylakoids.

- Left: GNU Free Documentation License**;** By and3k & caper437-Own work by uploaders, https://commons.wikimedia.org/w/index.php?curid=7153916
- Right: CC-BY; Transmission electron micrograph of chloroplast [T=thylakoids, S = starch] ; Adapted from PLOS one: http://redoxbiologycenter.unl.edu/ee00b8d7-d7fc-43f8-bab9-5570b2dbd731.pdf

Fig. 1.21: Transmission electron micrograph of a leucoplast, a chloroplast that has become filled with starch granules (S). From Bergtrom et al. J. Ultrastr. Res. 78:269-282. Research by G. Bergtrom

Fig. 1.22: Illustration of assembly, structure and polarity of microtubules, microfilaments and intermediate filaments alongside fluorescence micrographs made using fluorescent antibodies against isolated microtubule, microfilament and intermediate filament proteins (TOP, MIDDLE and BOTTOM, respectively).

- CC-BY 2.0; Adapted from: http://bmcbiochem.biomedcentral.com/articles/10.1186/1471-2091-9-23 Open Access
- CC-BY-SA-2.5 and GNU.org; Adapted from https://en.wikipedia.org/wiki/media/MEF_microfilaments.jpg, By Y Tambe (Y tambe's file) via Wikimedia Commons
- CC-BY; Adapted from: www.plosone.org/article/info:doi/10.1371/journal.pone.0012115
- Illustrations by G. Bergtrom

Fig. 1.23: Transmission electron micrograph showing a flagellum (#1) emerging from a basal body (2). Number 3 is another basal body, this time in cross section. Public Domain, By Dartmouth Electron Microscope Facility, Dartmouth College – http://remf.dartmouth.edu/imagesindex.html , https://commons.wikimedia.org/w/index.php?curid=2787228

Fig. 1.24: The characteristic "9+2" arrangement of microtubules seen in cross-sections of eukaryotic cilia and flagella is maintained the *axoneme*, a structure remaining after removing the plasma membrane from isolated cilia or flagella. Illustration by G. Bergtrom

Fig. 1.25: A cell fractionation involving disruption of cells and the isolation of sub-cellular components, including organelles, ribosomes and a soluble fraction, the cytosol. Separation is achieved by successive centrifugations at different speeds (centrifugal forces) that sediment subcellular structures on the basis of mass; the lowest mass structures require the highest G-forces (fastest spin), requiring an ultracentrifuge. Illustration by G. Bergtrom

Fig. 1.26: Organelles isolated by cell fractionation from eukaryotic cells.

1. CC-BY-NC-ND-SA; Adapted from Hancock, 2009: https://openi.nlm.nih.gov/detailedresult.php?img=PMC2762040_pone.0007560.g001&query=isolated+nuclei&it=xg&req=4&npos=23
2. CC-BY-NC-ND-SA Adapted from Siekevitz and Palade , 1956: https://openi.nlm.nih.gov/detailedresult.php?img=PMC2854378_JCB_201002147R_GS_Fig1&query=isolated+rough+endoplasmic+reticulum&it=xg&req=4&npos=5
3. CC-BY-NC-ND-SA Adapted from Beznoussenko et al., 2015; https://openi.nlm.nih.gov/detailedresult.php?img=PMC4394477_ijms-16-05299-g005b&query=isolated+golgi+vesicles&it=xg&req=4&npos=1
4. CC-BY-NC-ND-SA; Adapted from Soubannier et al., 2012 https://openi.nlm.nih.gov/imgs/512/66/3530470/PMC3530470_pone.0052830.g001.png?keywords=absences
5. CC-BY-SA: Adapted from S. Saleh, https://commons.wikimedia.org/wiki/File:Chloroplast.jpeg
6. CC-BY-SA 4.0; Adapted from Boone et al. *Isolation of Plasma Membrane from HeLa Cell,* https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2107758/
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Chapter 2

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Fig. 2.1: The shell (Bohr) and orbital models of atoms respectively emphasize the energy of electrons and space occupied by the electrons moving around the atomic nucleus. Illustration by G. Bergtrom

Fig. 2.2: The *Periodic Table of the Elements*, emphasizing those found in living things (blue, purple, green or brown). Illustration by G. Bergtrom

Fig. 2.3: *Excited* electrons gain their energy by absorbing light. Absorbance of one *photon* causes an electron to 'jump' to the next shell further from the atomic nucleus. Excited electrons have *potential energy* that is released as the electron returns to *ground state*, releasing heat or light (*fluorescence*). Illustration by G. Bergtrom

Fig. 2.4: Non-polar covalent bonds vs. polar covalent bonds. Illustration by G. Bergtrom

Fig. 2.5: Electrons on the H atoms of water molecules are drawn close to the large, positively charged nucleus of the O atom. Thus, the H atoms 'lose' electrons and acquire a partial positive charge, while the oxygen atoms 'gain' those electrons and have a partial negative charge. The resulting polar covalent water molecules attract other water molecules. Illustration by G. Bergtrom

Fig. 2.6: Computer-generated space-filling (LEFT) and 'ribbon' models (RIGHT) of insulin structure. Source: CC-BY 2.5 http://commons.wikimedia.org/wiki/File:InsulinMonomer.jpg

Fig. 2.7: Ionic bonds in table salt (NaCl) crystals; Opposite charges on Na^+ and Cl^- hold the ions together in a regular crystalline array. Illustration by G. Bergtrom

Fig. 2.8: Table salt (NaCl) dissolving in water. Water's solvent properties result from its polar covalent structure, allowing interactions with Na^+ and Cl^- in the crystal. Illustration by G. Bergtrom

Fig. 2.9: Charged groups on the macromolecule (e.g., protein) attract the partial charges on water molecules, hydrating the molecule. Illustration by G. Bergtrom

Fig. 2.10: Structural and geometric *isomers* of hydrocarbons create molecules with the same chemical formula but different shapes. Illustration by G. Bergtrom

Fig. 2.11: The two different generic molecules (*enantiomers*) are mirror images of one another. They form from alternate arrangements of the same molecular groups around a *chiral* carbon. Illustration by G. Bergtrom

Fig. 2.12: Two straight chain forms of glucose (*d*-glucose and *l*-glucose) are enantiomers (optical isomers), differing in the arrangement of the H atom and the OH group around C5. Illustration by G. Bergtrom

Fig. 2.13: A generic monomer is linked to a growing polymer by water removal (*dehydration synthesis*); Water addition across the linkage between monomers (*hydrolysis*) breaks the polymer back down to monomers. Illustration by G. Bergtrom

Fig. 2.14: *Dehydration synthes*is forms peptide linkage (circled); *hydrolysis* is the reverse reaction. Illustration by G. Bergtrom

Fig. 2.15: When straight chain glucose forms a cyclic molecule in solution in water, C_1 becomes optically active (chiral), creating a racemic mixture of □□d)glucose and □(d)glucose enantiomers. Illustration by G. Bergtrom

Fig. 2.16: Polysaccharide sugar polymers form by dehydration synthesis: □□d)glucose monomers polymerize to form energy-storage molecules (e.g., starch, glycogen), while □-d-glucose monomers polymerize to form structural polysaccharides (starch, cellulose. Illustration by G. Bergtrom

Fig. 2.17: Two enantiomers (optical isomers) of Thalidomide (**R** and **S**) form in water. Public Domain; By Vaccinationist - (R)-(+)-Thalidomide & (S)-(−)-Thalidomide on PubChem, https://commons.wikimedia.org/w/index.php?curid=50750574

Chapter 3

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Fig. 3.1: The four orders (levels) of protein structure. Primary, secondary and tertiary structures describe polypeptides

Fig. 3.2: Chemical characteristics of the 20 amino acids found in the proteins of cells. Illustrated by G. Bergtrom

Fig. 3.3: Partial polypeptide; the amino and carboxyl ends of a polypeptide define the polarity of the molecule, with positively charged amino and negatively charged carboxyl ends at physiological pH. Illustration by G. Bergtrom

Fig. 3.4: In the secondary structure of a polypeptide, more organized □-helical and □-pleated sheet structures are separated by less organized, random coil stretches of amino acids.
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Fig. 3.5: Tertiary structure is created by non-covalent *hydrophobic* amino acid interactions as well as *H-bonding* in the interior of a polypeptide, leaving charged (hydrophilic) amino acid side chains to interact with water on the exterior of a typical "globular" protein. Stable covalent disulfide bonds between cysteine amino acids help stabilize tertiary structures. Illustration by G. Bergtrom

Fig. 3.6: Disulfide bridges form in oxidation reactions between SH (sulfhydryl) groups on cysteine amino acids ('residues') in a polypeptide. Illustration by G. Bergtrom

Fig. 3.7: Light micrograph of a sickled erythrocyte (red blood cell). CC BY 3.0; By OpenStax College - Anatomy & Physiology, Connexions Web site; http://cnx.org/content/col11496/1.6/

Fig. 3.8: Mutations in a normal gene for a prion protein *(PrP^{c})* may produce some abnormally folded prion proteins (*PrP^{Sc}*). The misfolded *PrP^{Sc}* molecules interact with other (even normal) prions to mis-fold, precipitating *PrP^{Sc}* proteins into aggregated amyloid plaques. Adapted from: CC-BY-SA: M. R. Muntada (217) Prions: Special Proteins; https://allyouneedisbiology.wordpress.com/2017/11/12/prions-special-proteins/

Fig. 3.9: Steps in the formation of β-amyloid plaques. Amyloid precursor proteins (*APP*) are embedded in cell membranes (upper left). Enzymes digest APP, releasing β-amyloid protein fragments (middle panel). Unregulated accumulation of β-amyloid protein results in the formation of extracellular amyloid plaques.
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Fig. 3.10: Non-fluorescent immunostaining of *tau* proteins of an Alzheimer's brain reveals *tau tangles*. CC-BY-SA 3.0; From Wikimedia Commons User Patho; Neurofibrillary tangles in the Hippocampus of an old person with Alzheimer-related pathology, immunohistochemistry for tau protein.JPG

Fig. 3.11: The formation of tau tangles occurs in diseased neurons that cannot maintain normal microtubule structure; in the absence of stabilizing tau molecules microtubules degenerate and tau proteins clump. Public Domain; Adapted from: https://commons.wikimedia.org/wiki/File:TANGLES_HIGH.jpg

Fig. 3.12: The vertebrate hemoglobin molecule, consisting of 4 globin subunits (two □□and two □ polypeptides). Each globin is associated with a heme group bound to iron. GNU Free Documentation License; Adapted from: https://en.wikipedia.org/wiki/Hemoglobin

Fig. 3.13: The *Pleckstrin Homology* (PH) domains shown here are an example of common domains (maroon colored sequences) in two different proteins. Because they have a PH domain, both proteins can interact with cell signaling factors with roles in intercellular communication. CC BY-SA 3.0; By Fdardel - Own work, https://commons.wikimedia.org/w/index.php?curid=17659815

Fig. 3.14: Screen-shot of the NCBI protein structure database leading to 3D Macromolecular structures. http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html

Fig. 3.15: Screen-shot of NCBI protein structure database search leading to available macromolecular structures. http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html

Fig. 3.16: Screen-shot of NCBI protein structure database search results for human insulin. http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html

Chapter 4

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Fig. 4.1: The sum of all things contained in the universe (e.g., as mass and other, more familiar kinds of energy). In this simple "Venn" diagram, the universe is a closed system; nothing (mass, energy) can get into or exit the universe, consistent with the *First Law of Thermodynamics*. Illustration by G. Bergtrom

Fig. 4.2: Since even mass is a form of energy, the universe shown here consists of only two components, *entropy* and *free energy* (defined as available to do work), still consistent with the *First law of Thermodynamics*. Illustration by G. Bergtrom
Fig. 4.3: The reciprocal relationship between entropy and free energy graphed over time. If all activities in the universe proceed by increasing entropy, and if the universe consists *only* of free energy and entropy, then as entropy increases, free energy must be decreasing in the universe. Illustration by G. Bergtrom
Fig. 4.4: Basic design of a *bomb calorimeter*, with an inner chamber for conducting reactions, and an outer chamber containing water with a thermometer to measure temperature change (heat absorption or release, or □H) during the reaction. Illustration by G. Bergtrom

Chapter 5

Chapter Top-Art: Illustration by G, Bergtrom
Fig. 5.1: Illustration of the early *Lock-&-Key* mechanism for enzyme-substrate interaction, in which the substrate *key* fits an enzyme *lock*. Illustration by G. Bergtrom
Fig. 5.2: The *Induced Fit* mechanism of enzyme-substrate interaction. Initial interaction of substrate with enzyme based on mutual affinity causes an allosteric change in the enzyme. This results in the induced 'better' fit of substrate to enzyme, to be followed by catalysis. Illustration by G. Bergtrom
Fig. 5.3: Graph of change in free energy over time as chemicals A and B react. The high *Activation Energy* peak in the graph is the *free energy barrier* that A and B must overcome before much C and D can be made. This barrier is due mainly to thermal motion of the A and B molecules, with only rare encounters in relatively dilute solutions. Enzymes are even more efficient that inorganic catalysts in lowering the activation energy barrier to a reaction. Illustration by G. Bergtrom
Fig. 5.4: A generic biochemical pathway in which the final product of three enzymatic reactions accumulates. To prevent wasteful accumulation of the final product, the pathway is regulated, in this case, when some of the accumulating reaction product (E) binds to enzyme 1, blocking the reaction from A to form B. Illustration by G. Bergtrom
Fig 5.5: *Allosteric effectors* are small metabolites whose amounts in a cell reflect the cell's metabolic status. They bind to enzymes at a regulatory site, causing an *allosteric* (conformational) change in the enzyme that affects the shape of the *Active Site*, either inhibiting catalysis (as suggested here) or stimulating it. Illustration by G. Bergtrom
Fig. 5.6: *Saturation kinetics* of an enzyme-catalyzed reaction. At high substrate concentrations, all the active sites of all enzymes are occupied. Under these conditions, the reaction occurs at its fastest rate. Illustration by G. Bergtrom
Fig. 5.7: Experimental protocol for determining the kinetics of an enzyme-catalyzed reaction. Illustration by G. Bergtrom
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Fig. 5.9: This graph shows the slopes of the initial reaction rates (red) taken from the curves in the previous graph (Fig. 5.8). Illustration by G. Bergtrom
Fig. 5.10: This graph plots the initial reaction rates (slopes, or $\boldsymbol{v_0}$) for the reactions plotted in Fig. 5.9. The formula shown for this curve is that of a *rectangular hyperbola*. Illustration by G. Bergtrom

Chapter 6

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Fig. 6.1: The *flow of free energy* through life, from visible light to chemical energy (e.g., photosynthesis of glucose) to the high energy intermediates like ATP (fermentation or respiration) and finally to cellular work. Illustration by G. Bergtrom
Fig. 6.2: The energetics of ATP synthesis and hydrolysis. Illustration by G. Bergtrom
Fig. 6.3: Stoichiometry of glucose metabolism and ATP productions by glycolysis, the Krebs Cycle, electron transport and oxidative phosphorylation. Illustration by G. Bergtrom
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Fig. 6.5: Alternate Fates of Pyruvate: *Fermentation* (the anaerobic reduction to e.g., alcohol, lactate, etc.) or *Respiration* (aerobic oxidation of pyruvate using oxygen as a final electron acceptor, resulting in H_2O and CO_2 production). Illustration by G. Bergtrom

Fig. 6.6: In Reaction 1, phosphorylation of glucose to make glucose-6-P (G-6-P) consumes a molecule of ATP. If cellular energy needs are being met, G-6-Pwill be polymerized to make storage polysaccharides. The G-6-P can be retrieved by polysaccharide breakdown (hydrolysis).when the cells require nutrient energy; at that time, the G-6-P will resume glycolysis. Illustration by G. Bergtrom
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Fig. 6.11: Reactions 4 and 5 of glycolysis. In reaction 4, F1,6-di-P is split into dihydroxyacetone phosphate (DHAP) and glyceraldeghyde-3-P (G-3-P) by an aldolase enzyme. In reaction 5, DHAP is isomerized to G-3-P. Both reactions are endergonic, consuming free energy. These reactions mark the end of Stage 1 of glycolysis. Illustration by G. Bergtrom
Fig. 6.12: Reaction 6, a redox reaction catalyzed by G-3-P dehydrogenase, is freely reversible. G-3-P is oxidized to1,3 diphosphoglyceric acid (1,3 diPG), transferring electrons as a hydride (H^-) ion to NAD^+ to make NADH. Illustration by G. Bergtrom
Fig. 6.13: Reaction 7, a reversible, *exergonic*, *coupled* reaction generates ATP using free energy released by the hydrolysis of one of the phosphates from 1,3diPG. The mechanism of ATP synthesis here is called *substrate-level phosphorylation*. Illustration by G. Bergtrom
Fig. 6.14: Reaction 8 is a reversible, *endergonic* reaction catalyzed by a mutase. Mutases catalyze transfer of a chemical group from one part of a molecule to another; here, 3-PG is converted to 2PG. Illustration by G. Bergtrom
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Fig. 6.17: Free energy and ATP yields of complete glycolysis (a fermentation) and incomplete glycolysis (i.e., respiration). Percentages represent the (efficiency of ATP production. They are based on the ratios of the free energy captured as ATP to the free energy released by the different pathways for metabolizing glucose. Froom the data, incomplete glycolysis is a more efficient way to extract nutrient free energy. Illustration by G. Bergtrom
Fig. 6.18: Comparison of incomplete glycolysis to gluconeogenesis. The two pathways are essentially the reverse of one another, except for the *bypass enzymes* in gluconeogenesis (shown in green), required to get around biologically irreversible enzymes of glycolysis. Illustration by G. Bergtrom
Fig. 6.19: The *Cori cycle* reveals the relationship between glycolysis and gluconeogenesis. Lactic acid produced by complete glycolysis in active skeletal muscle goes to the liver where it could be converted to pyruvate, and then to glucose . CC-BY-SA 3.0; From https://commons.wikimedia.org/wiki/File:Cori_Cycle.SVG and GNU Free Documentation License User:Petaholmes, user:PDH, user:Eyal Bairey
Fig. 6.20: Control of gluconeogenesis by hormones of the *hypothalamic-pituitary axis*. A need for glucose for energy stimulates hypothalamic hormones that in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH then stimulates release of glucocorticoids rom the adrenal glands. Glucocorticoid hormones stimulate many tissues to control gluconeogenic glucose production as well as the use alternate nutrient fuels by many cells in the body. Illustration by G. Bergtrom
Fig. 6.21: Entry of pyruvate into mitochondrion, followed by its oxidation to acetyl-S-Coenzyme A (acetyl-S-CoA). Illustration by G. Bergtrom
Fig. 6.22: Pyruvate dehydrogenase catalyzes pyruvate oxidation to Ac-S-CoA, releasing a molecule of CO_2 and reducing NAD^+ to NADH. Illustration by G. Bergtrom
Fig. 6.23: Highlights of the Krebs Cycle. The first reaction is condensation of Acv-S-CoA and oxaloacetate (OAA) to form citric acid (citrate). Four of the reactions in the cycle are redox reactions that create reduced electron carriers (NADH, FADH2), while one reaction is coupled to GTP synthesis by substrate level phosphorylation. Illustration by G. Bergtrom

Chapter 7

Chapter Top-Art: Illustration by G, Bergtrom

Fig. 7.1: Overview of electron transport and oxidative phosphorylation. NADH and $FADH_2$ oxidation feed electrons into electron transport, releasing free energy that powers proton (H^+ ions) pumps to force H^+ ions out of the mitochondrion. The resulting H^+ gradient fuels ATP synthesis as the protons flow into the mitochondrial matrix through a regulated *ATP synthase* in the cristal membrane (at the right in the drawing) . Illustration by G. Bergtrom

Fig. 7.2: The flow of protons through the cristal membrane ATP synthase relieves the proton gradient, releasing free energy that fuels ATP synthesis in the mitochondrial matrix. Illustration by G. Bergtrom

Fig. 7.3: The plasma membrane ATPase of motile bacteria is a molecular motor that can e.g., spin a flagellum powered by protons flowing into the matrix to relieve a proton gradient. Public Domain; Adapted from LadyofHats - self-made. https://commons.wikimedia.org/w/index.php?curid=6219592

Fig. 7.4: Summary of carbon flow through life. Organic carbon is oxidized to release free energy and make ATP. Sunlight provides free energy for photosynthesis to turn inorganic carbon (CO_2) into organic carbon (e.g., glucose). Illustration by G. Bergtrom

Fig. 7.5: The *light dependent* and *light independent* ('dark') reactions of photosynthesis. The light-dependent reactions 'split' water, releasing oxygen and protons. The light-independent reaction 'fix' CO_2 in organic molecules. Illustration by G. Bergtrom

Fig. 7.6: Experimental design to test the hypothesis that photosynthesis is supported by absorption of light by chlorophyll pigments. Illustration by G. Bergtrom

Fig. 7.7: Graph showing the *action spectrum* of photosynthesis, showing that photosynthesis is supported across a wide range of visible light wavelengths. Illustration by G. Bergtrom

Fig. 7.8: Graph plotting the absorbance spectrum of purified chlorophylls, showing two main peaks of absorbance. Illustration by G. Bergtrom

Fig. 7.9: Low (LEFT) and high (RIGHT) power transmission micrographs of typical chloroplasts, thylakoid membranes and grana (thylakoid stacks). GNU Free Documentation License**;** By and3k & caper437-Own work by uploaders, https://commons.wikimedia.org/w/index.php?curid=7153916

Fig. 7.10: Graph superimposing the action spectrum of photosynthesis over the absorbance spectra of different plant pigments. The multiple absorbance peaks of different chloroplast pigments are consistent with the action spectrum. Illustration by G. Bergtrom

Fig. 7.11: The 'Z-scheme' of photosynthesis. Photosystems I and II (PSI, PSII) pigments absorb light energy that excites electrons captured by electron acceptors. Electrons excited from PSI reduce NADPH, the starting point of the light-independent reactions. Electrons excited out of PSII come from water ('splitting it to release oxygen). They will flow down a photosynthetic electron transport chain to replace electrons lost from PSI. Along the way, free energy from those excited electrons fuels ATP synthesis. Illustration by G. Bergtrom

Fig. 7.12: *Cyclic Photophosphorylation* cycles electrons excited from PSI along part of the photosynthetic electron transport chain, bypassing PSII and returning the electrons to PSI. ATP made during Cyclic photophosphorylation is used for cellular work when there is no need or capacity to make more sugar. Illustration by G. Bergtrom

Fig. 7.13: Overview of the *Calvin Cycle* for carbon fixation in *C3* (most) plants. Three RuBP molecules *fix* 3 CO_2 molecules, catalyzed by ribulose bisphosphate carboxylase –oxygenase (RUBISCO). The three 6C molecules produced split into six 3C carbohydrates, becoming six glyceraldehyde-3-P (GA3P in the illustration). Five of these go on to regenerate 3 RuBP molecules.; the 6^{th} GA3P waits for the Calvin Cycle to repeat, producing another GA3P. Two GA3Ps from 2 turns of the cycle are substrates for glucose synthesis. Illustration by G. Bergtrom

Fig. 7.14: *Calvin Cycle* Arithmetic: Making one glucose and regenerating 5 RubP molecules. Illustration by G. Bergtrom

Fig. 7.15: Photorespiration is a C3 plant strategy to survive a dry spell or a heat wave. Water-starved C3 plants resort to *fixing* oxygen using RuBP. This reaction is catalyzed by RUBISCO, the same enzyme that fixes CO_2 to RuBP, but using its oxygenase activity. Illustration by G. Bergtrom

Fig. 7.16: Crassulacean Acid Metabolism in CAM plants. To survive in arid climates and minimize water loss, CAM plants o[pen their stomata and fix CO_2 in oxaloacetate (OAA) that is converted to malic acid to be stored until daylight. Stomata close at daybreak to conserving water, while malic acid releases CO_2 to be *re-fixed* to make glucose. Illustration by G. Bergtrom

Fig. 7.17: *C4 Photosynthesis* pathway reactions in tropical plants. C4 plants separate their 'dark' reactions into 2 cell types. *Mesophyll* cells can keep stomata open part of the day, allowing CO_2 into the cells where it is fixed to PEP to make OAA and then malate. Unlike CAM plants, the malate is not stored, but transferred to *bundle sheath* cells where it can release CO_2 to be *re-fixed* by the Calvin Cycle. Illustration by G. Bergtrom

Chapter 8

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Fig. 8.1: The experiments of F. Griffith demonstrating the existence of a chemical that could transfer a genetic trait (i.e., virulence) between bacteria (see text for details). Illustration by G. Bergtrom

Fig. 8.2: The experiments of O. Avery et al. demonstrating for the first time that the chemical stuff of genes, of inheritance is DNA (see text for details). Illustration by G. Bergtrom

Fig. 8.3: Life cycle of a bacteriophage: The phage *coat* remains attached to a cell after infection, but the chemicals inside the phage enter the infected cell. Illustration by G. Bergtrom

Fig. 8.4: The experiments of Alfred Hershey and Martha Chase demonstrating that the chemical stuff of viral genes is DNA (see text for details). Illustration by G. Bergtrom

Fig. 8.5: *Photo 51*, the X-Ray crystallograph of DNA taken by Rosalind Franklin revealed 3 crucial molecular dimensions that ultimately led to the double helical DNA model. *Fair Use* (Ref. Wikipedia); By Source (WP:NFCC#4), https://en.wikipedia.org/w/index.php?curid=38068629; https://en.wikipedia.org/wiki/Photo_51

Fig. 8.6: The 3 molecular dimensions from Franklin's Photo 51 was best explained by proposing a DNA double helix (LEFT). Model building by Watson and Crick revealed the antiparallel structure of the double helix (RIGHT).

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Fig. 8.7: Three possible modes of DNA replication could be imagined. Illustration by G. Bergtrom

Fig. 8.8: Meselson and Stahl predict experimental outcomes based on 3 possible modes of DNA replication. Illustrated by G. Bergtrom

Fig. 8.9: Light micrograph of a human female chromosome stained for banding patters, showing the *centromere* constriction and defining *telomeres* (chromosome ends). Public Domain; Adapted from National Human Genome Research Institute; Cropped from Human male karyotype.jpg, https://commons.wikimedia.org/w/index.php?curid=40960106

Fig. 8.10: Spread of human mitotic cell chromosomes. CC BY-SA 3.0; By Steffen Dietzel - Own work, https://commons.wikimedia.org/w/index.php?curid=1369763

Fig. 8.11: Computer-colorized human *karyotype*. Public Domain; Adapted From https://commons.wikimedia.org/w/index.php?curid=7853183C Courtesy: National Human Genome Research Institute - Found on :National Human Genome Research (USA); copied from wikipedia:en.

Fig. 8.12: Transmission micrograph of *euchromatin* and *heterochromatin* in the nucleus. From Bergtrom et al., (1977) J. Ultrastr. Res. 60:395-406; Research by G. Bergtrom

Fig. 8.13: Low salt fractionation of interphase nuclei yields 10nm nucleosome *beads on a string*.

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Fig. 8.14: High salt chromatin extraction from nuclei, or high salt treatment of 10 nm filaments yields 30 nm *solenoid* structures, essentially coils of 10 nm filaments.

- Electron micrograph of nucleus, From Bergtrom et al., (1977) J. Ultrastr. Res. 60:395-406: Research by G. Bergtrom
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Fig. 8.15: Five different levels (orders) of chromatin structure (see text for deatiils). CC-BY-SA 3.0; Adapted From https://en.wikipedia.org/wiki/Chromatin**.**

Fig. 8.16: Short times of DNase I digestion of 10 nm filaments leaves behind shortened *beads on a string*. A long time of DNase I digestion releases single nucleosomes associated with some DNA. Illustration by G. Bergtrom

Fig. 8.17: Electrophoresis of nucleosomal proteins from digested 10 nm filaments reveals five different *histones* with the stoichiometry shown. Gel illustration by G. Bergtrom; nucleosome illustration adapted from CC-BY-SA drawing on https://commons.wikimedia.org/wiki/File:Chromatosom.png
Fig. 8.18: Acid extraction of chromatin to remove histones and leave more basic non-histone proteins behind. See original micrograph at https://www.flickr.com/photos/185778834@N06/49368658231/ Illustration by G. Bergtrom.
Fig. 8.19: Bacterial *conjugation* (sex in bacteria): The *F plasmid (*Fertility plasmid) can transfer bacterial chromosomal DNA from the F+ mating strain of *E. coli* to an opposite, F^- mating strain, leading to a measure of genetic diversity. Illustration by G. Bergtrom
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Chapter 9

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Chapter 12

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Chapter 15

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Fig. 15.18:. Automated DNA sequence chromatograph readout. CC BY 3.0; Adapted from Intechopen book (Book chapters are distributed under the Creative Commons Attribution 3.0) https://www.intechopen.com/books/nucleic-acids-from-basic-aspects-to-laboratory-tools/nucleic-acid-isolation-and-downstream-applications
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Fig. 15.29: Electrophoretic *DNA fingerprint*. Public domain; By James Tourtellotte, photo editor of CBP Today[1], http://www.cbp.gov/xp/cgov/newsroom/multimedia/photo_gallery/afc/laboratories/13_5flab_5fhiresa.xml (file Cbp13_5flab_5fhires.jpg, part of America's Frontline Photography / CBP Laboratories Photography), https://commons.wikimedia.org/w/index.php?curid=2875876
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Fig. 17.25: Glycocalyx protein interactions; roles in cell-cell recognition and attachment. Illustration by G. Bergtrom
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Fig. 17.27: *Migration* and *attachment* of cells during development requires the synthesis of membrane proteins that will recognize and bind proteins on the membranes and glycocalyx of other cells, forming cell junctions and enabling *tissue formation.* At the same time, the specificity of these membrane proteins prevents incorrect cell-cell associations. Integrin is an early cell membrane protein 'receptor' that interacts with many proteins to form a glycocalyx and then respond to cell-surface proteins of other cells. Illustration by G. Bergtrom

Fig. 17.28: Density-dependent inhibition (aka. Contact Inhibition) occurs when cells multiplying and spreading on a surface cease dividing when there is no more room on the surface. Cancer cells have lost the property of contact inhibition and keep growing over one another in layers. Loss of contact inhibition is correlated with an absence of gap junctions in cancer cells. Illustration by G. Bergtrom
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Chapter 18

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Fig. 18.1: Fluorescence micrograph of the mitotic spindle treated with antibodies to chromosomal proteins (blue) and spindle fiber proteins (green). Public Domain; From: Afunguy-Transferred to Commons by Lije also using CommonsHelper; https://commons.wikimedia.org/w/index.php?curid=5148470
Fig. 18.2: Illustration of assembly, structure and polarity of microtubules, microfilaments and intermediate filaments alongside fluorescence micrographs made using fluorescent antibodies against isolated microtubule, microfilament and intermediate filament proteins (TOP, MIDDLE and BOTTOM, respectively).
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Fig. 18.3: Illustration and immunofluorescence microscopic localization of microtubules, microfilaments and intermediate filaments in cells.
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Fig. 18.4. Transmission electron micrograph of the characteristic *9 triplet* microtubule array of *centrioles* and *basal bodies*. From: Chernov et el. (2008) BMC Biochemistry2008; 9:23: Public Domain; http://commons.wikimedia.org/wiki/File:Spindle_centriole;_embryonic_brain_mouse_-_TEM.jpg
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Fig. 18.8: Transmission electron micrographs of *axonemes* show that microtubules are made up of a ring of 13 tubulins (LEFT). Microtubules in cilia or flagella are arranged in typical 9+2 arrays (MIDDLE). The enlarged cross-section illustrates specific microtubule-associated proteins (*MAPs*) that maintain the 9+2 structure, including dynein motors that powers motility.

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- Illustration of axoneme components by G. Bergtrom

Fig. 18.9: Roles of *dynein* and *kinesin* in retrograde (backwards) and anterograde (forward) movement of cargo vesicles along microtubules (see text for details).
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Fig. 18.10: Removing the membrane from isolated cilia or flagella leaves behind the *axoneme*; when provided ATP, both the isolated structures and demembranated axoneme will beat as the ATP hydrolyzed. Illustration by G. Bergtrom

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Fig. 18.12: Comparison of ciliary beat with propeller like generation of flagellar wave motion (see text for details).
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Fig. 18.13: Light micrograph of skeletal muscle stained to showing characteristic striations. CC-BY: From OpenStax: Anatomy and Physiology; https://openstax.org/books/anatomy-and-physiology/pages/10-1-overview-of-muscle-tissues

Fig. 18.14: Skeletal muscle organization and the anatomy of a muscle cell *sarcomere* (see text for details).

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Fig. 18.15: Transmission **e**lectron micrograph and a corresponding illustration showing sarcomere shortening, consistent with the sliding of filaments during skeletal muscle contraction.

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Fig. 18.16: The skeletal muscle *Contraction Paradox*: Given ATP, *glycerinated* muscle fibers contract and pull a weight. When all the ATP is hydrolyzed the fiber can't stretch unless more ATP is added. Illustration by G. Bergtrom

Fig. 18.17: Overview of the isolation of actin thin filaments (still on Z-Lines) from myosin thick filaments. Illustration by G. Bergtrom

Fig. 18.18: Reconstitution of actin thin filaments (on Z-Lines) with myosin filaments. Illustration by G. Bergtrom

Fig. 18.19: Structure of a skeletal muscle myosin filament and the myosin monomer. Shown is ***myosin II***, the thick filament that spans both sides of the H zone in a sarcomere (upper). The head-&-tail structure of a myosin monomer is shown in the high magnification electron micrograph and is illustrated in the cartoon (lower). The myosin monomer is itself a polymer of four polypeptides.

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Fig. 18.20: Digestion of purified myosin monomers with enzymes that hydrolyze peptide bonds between specific amino acids produces an *S1 head* and a *tail* fraction with different properties.

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Fig. 18.21: Illustration of myosin decoration by myosin monomer S1 fragments, showing opposing polarity of actin filaments on opposite sides of the Z-line. Illustration by G. Bergtrom

Fig. 18.22: Steps in the *Micro-contraction Cycle* explain the muscle contraction paradox (see text for details). Illustration by G. Bergtrom
Fig. 18.23: The ability of flexible myosin heads to bend and change conformation is consistent with their proposed activity during the micro-contraction cycle. CC-BY 4.0; Adapted from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0093272 By H. Sugi et al. (2014) PLoS ONE 9(6): e93272; doi:10.1371/journal.pone.0093272
Fig. 18.24: Innervation leads to Ca++ release from *sarcoplasmic reticulum* to regulate contraction (see text for details).
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Fig. 18.25: Skeletal muscle thin filaments consist of actin associated with troponins and tropomyosin. These actin-associated proteins participate in the response to Ca++ ions to regulate interactions with myosin.
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Fig. 18.26: Ca++ ions initiate uncovering myosin binding sites on actin by binding to *troponin-C*, resulting in allosteric changes in the troponins and ultimately tropomyosin. Illustration by G. Bergtrom
Fig. 18.27: Structure of *titin* and its location in the sarcomere (see text for details)
- CC-BY 4.0: Colorized micrograph of titin adapted from Zsolt Mártonfalvi and Miklós Kellermayer (2014): http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0085847
- Illustration by G. Bergtrom

Fig. 18.28: Elasticity of skeletal muscle shown by the location of anti-N2A-linked and anti-120/122-linked nanogold particles in sarcomeres. Note the increased separation of the PEVK domains targeted by the antibodies as a muscle fiber is increasingly stretched. This titiin elasticity facilitates skeletal muscle relaxation. [Copyrighted free use; Adapted from https://commons.wikimedia.org/w/index.php?curid=764619, By User:Sameerb (en:WP; Author User:Sameerb in English WP), via Wikimedia Commons. Adaptations included adding simulated nanogold particles and elongating parts of the sarcomere to create an image replicating results of work from the laboratory of Wolfgang Linke.
Fig. 18.29: In the anti-actin immunofluorescence micrograph of fibroblasts, actin localizes with stress fibers which help maintain cell shape (LEFT). Actin also localizes in lamellipodia and retraction fibers in migrating fibroblasts (RIGHT), orienting in the direction of movement.
- Adapted from CC BY-SA 3.0 https://commons.wikimedia.org/w/index.php?curid=378394 By Y Tambe - Y Tambe's file
- Illustration by G. Bergtrom

Fig. 18.30: The molecular structure of stress fibers; myosin as well as other actin-binding proteins interact with actin in non-muscle cell motility. Illustration by G. Bergtrom
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Chapter Top-Art: Public Domain: https://pixabay.com/photos/microscope-sea-urchin-egg-splitting-1276131/
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- Electron micrographs, CC-BY; Adapted from: V. Wilhelmi et al. (2013) Zinc Oxide Nanoparticles Induce Necrosis and Apoptosis in Macrophages in a p47phox- and Nrf2-Independent Manner. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0065704
- Illustrations: Public Domain; File adapted from a German Wikipedia entry: https://commons.wikimedia.org/wiki/File:Apoptosis.png or http://de.wikipedia.org/wiki/Bild:Apoptose-german.png

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Chapter Top-Art: Public Domain photos; **https://pixabay.com/illustrations/sperium-cum-sperm-dark-winner-326157/** and **https://pixabay.com/photos/halloween-pumpkin-scary-giant-309296/**
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Valley, J. W. et al. (2014) Hadean age for a post-magma-ocean zircon confirmed by atom-probe tomography, Nature Geoscience vol. 7, p 219-223; http://dx.doi.org/10.1038/ngeo2075; Used by permission of JW Valley, University of Wisconsin - Madison; http://geoscience.wisc.edu/geoscience/people/faculty/ john-valley/john-valley-incle-on-zircons/

Fig. 20.8: An oceanic volcanic hydrothermal vent, or black smoker. Public domain: https://oceanservice.noaa.gov/facts/vents.html

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Fig. 20.10: S*erpentinite* from Deer Lake in upper Michigan. It is a mineral also found in the oceanic crust that under conditions found in an alkaline vent, can form methane from CO_2. Public Domain, USGS publication. From Bornhorst & Johnson (1993). Geology of volcanic rocks in the south half of the Ishpeming Greenstone Belt, Michigan. *USGS Bulletin* 1904-P. 13 pp.; http://www.jsjgeology.net/Deer-Lake-Peridotite.htm

Fig. 20.11: Evolutionary (i.e., phylogenetic) tree of all living organisms reveals 3 domains of life: Bacteria (Prokarya), Archaebacteria (archaea) and Eukaryotes (Eukaryota). Public Domain; Adapted from: https://en.wikipedia.org/wiki/Phylogenetic_tree

Fig. 20.12: *Aminoadenosine triacid ester* (AATE) catalyzes its own replication by the mechanism suggested here. Illustration by G. Bergtrom

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Illustration by G. Bergtrom

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Prebiotic Proton Gradient Energy Fuels Origins of Life
Non-reducing earth-Not so fast!
Artificial Ribozyme Evolution Supports Early RNA World
Intercellular Communication in Bacteria
Enzyme Evolution on a Cooling Planet
Stephen Hawking on the Evolution of Artificial Intelligence