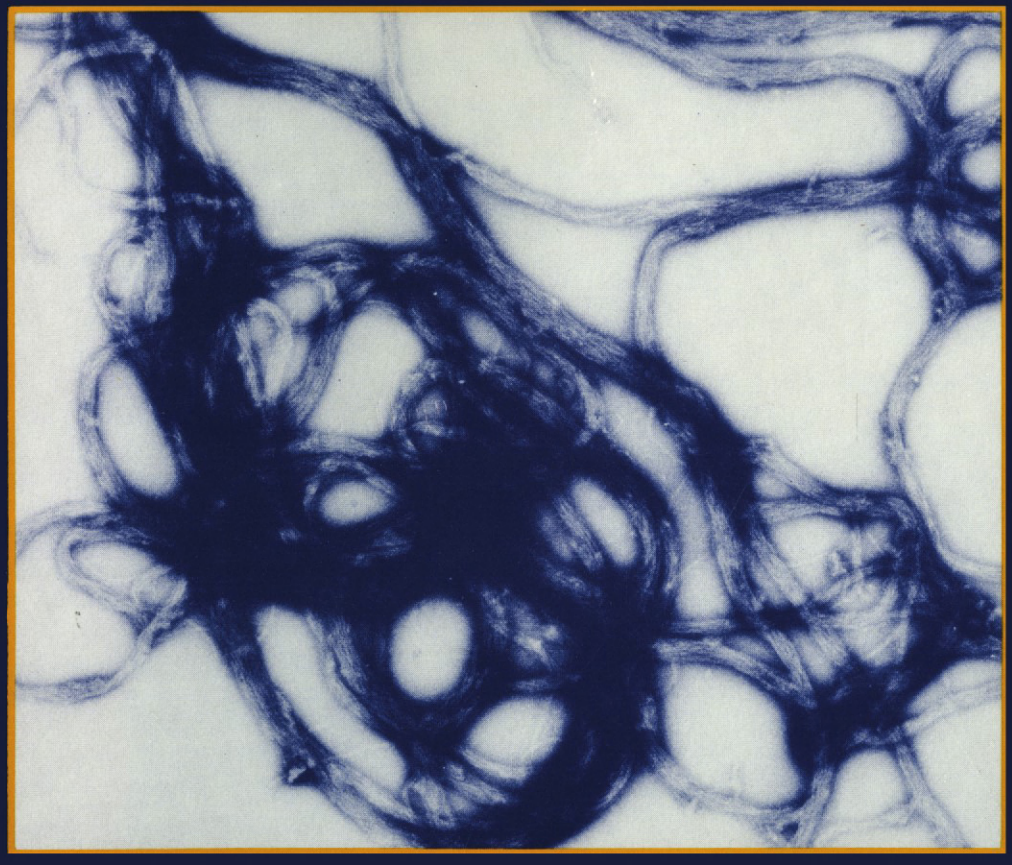
CELL and MOLECULAR BIOLOGY of the CYTOSKELETON



Edited by
Jerry W. Shay

CELL and MOLECULAR BIOLOGY of the CYTOSKELETON

CELL and MOLECULAR BIOLOGY of the CYTOSKELETON

Edited by

Jerry W. Shay

University of Texas Health Science Center
Dallas, Texas

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Cell and molecular biology of the cytoskeleton.

Includes bibliographies and index.
1. Cytoskeleton. I. Shay, Jerry W. [DNLM: 1. Cytoskeleton. QH 603.C96 C393]
QH603.C96C45 1986 574.87'5 86-4912
ISBN-13: 978-1-4612-9269-2 e-ISBN-13: 978-1-4613-2151-4
DOI: 10.1007/978-1-4613-2151-4

© 1986 Plenum Press, New York
Softcover reprint of the hardcover 1st edition 1986
A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

All rights reserved

No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise, without written permission from the Publisher

Contributors

Hans Bloemendal, Department of Biochemistry, University of Nijmegen, 6525 EZ Nijmegen, The Netherlands

Don W. Cleveland, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Ursula Euteneuer, Department of Zoology, University of California, Berkeley, California 94720

Stephen R. Farmer, Department of Biochemistry, Boston University Medical School, Boston, Massachusetts 02118

Donald E. Fosket, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Elaine Fuchs, Departments of Molecular Genetics and Cell Biology, and Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Annette Füchtbauer, Developmental Biology Unit, University of Bielefeld, 48 Bielefeld, Federal Republic of Germany

Norbert Geisler, Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany

W. Sue T. Griffin, Department of Cell Biology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

David Hirsh, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

Bernd Höner, Developmental Biology Unit, University of Bielefeld, 48 Bielefeld, Federal Republic of Germany

Brigitte M. Jockusch, Developmental Biology Unit, University of Bielefeld, 48 Bielefeld, Federal Republic of Germany

Michael Krause, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

Douglas Marchuk, Departments of Molecular Genetics and Cell Biology, and Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Louis C. Morejohn, Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108

Marcelle R. Morrison, Department of Neurology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Wim Quax, Department of Biochemistry, University of Nijmegen, 6525 EZ Nijmegen, The Netherlands. *Present address:* Department of Microbiology, Gist-brocades, 2600MA Delft, The Netherlands

Dennis R. Roop, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Peter M. Steinert, Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Angela Tyner, Departments of Molecular Genetics and Cell Biology, and Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Klaus Weber, Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany

Christine Wiegand, Developmental Biology Unit, University of Bielefeld, 48 Bielefeld, Federal Republic of Germany

Preface

Cell and Molecular Biology of the Cytoskeleton focuses on the three major fibrous proteins in the cytoplasm that are collectively known as the cytoskeletal system. These polymorphic cytoskeletal proteins are microtubules (25-nm diameter), microfilaments (6-nm diameter), and intermediate filaments (10-nm diameter). Microtubules consist of tubulin and several well-characterized microtubule-associated proteins (MAPs) such as MAP1, MAP2, and tau. Microfilaments consist of actin and associate with actin-binding proteins, including alpha-actinin, filamin, myosin, tropomyosin, vinculin, and others. Intermediate filaments consist of at least five different tissue-specific classes, including desmin or skeletin (muscle), prekeratin (epithelial), vimentin (mesenchymal), neurofilament (nerve), and glial acidic fibrillary protein (astrocytes).

In this volume distinguished researchers in the field cover the interaction of these fibrous proteins, not only with each other and other cytoplasmic components, but also with such biological processes as cell shape changes, growth, motility, secretion, and division. These comprehensive reviews explore the cytoskeleton's molecular, biochemical, and structural properties with an emphasis on their manifestation in the living cell.

Jerry W. Shay

Dallas, Texas

Contents

Chapter 1
Probing the Cytoskeleton by Microinjection
Brigitte M. Jockusch, Annette Füchtbauer, Christiane Wiegand, and Bernd Höner

1. Introduction 1
2. The Microtubule System 2
   - 2.1. Analyzing the Equilibrium between Tubulin and Microtubules 2
   - 2.2. Studying Microtubular Dynamics 3
   - 2.3. Analyzing Microtubule-Bound Organelle Transport 5
   - 2.4. Introducing Antitubulins 6
3. The Microfilament System 9
   - 3.1. Studying Actin Dynamics 9
   - 3.2. Studying the Dynamics of Actin-Associated Proteins 13
   - 3.3. Reorganizing Microfilament Patterns by Introducing Structural Proteins 14
   - 3.4. Introducing Phalloidin 19
   - 3.5. Using Antibodies against Microfilament Proteins 20
4. The Intermediate Filament System 27
   - 4.1. Introducing Antibodies 27
   - 4.2. Introducing mRNA 29
5. The Coated Vesicle System 29
6. Conclusion 30

References 32

Chapter 2
Structural Aspects of Intermediate Filaments
Norbert Geisler and Klaus Weber

1. Introduction 41
2. Domain Organization of IF Proteins 42
3. Neurofilaments 46
4. Organization of the Sequences along the Rod Domain 48
5. Comparison of the Six Nonepithelial Proteins along the Rod Domain 49
6. Structural Characterization of the Rod 51
7. From the Rodlike Protofilament to the 10-nm Filament 53
8. Structure and Function of the Terminal Domains in IF Proteins . 58
   8.1. The Involvement of Headpieces in Filament Formation 58
   8.2. Structural Characterization of the Tailpieces 60
   8.3. Are the Carboxyterminal Tailpieces Appendages Communicating between Stiff Filaments and the Cytoplasm? 62
9. Addendum 64

References 64

Chapter 3
The Structure and Evolution of Intermediate Filament Genes
Dennis R. Roop and Peter M. Steinert

1. Introduction 69
   1.1. Correlation of Keratin Gene Expression with Differentiation State 70
   1.2. Correlation of Keratin Subunit Structure with Differential Expression 71
2. Structure of Intermediate Filament Genes 73
3. A Model for the Evolution of Intermediate Filament Genes 75
   3.1. Evolution of the Rod Domains of Intermediate Filament Genes 75
   3.2. Evolution of the End Domains 78
4. Conclusion 81

References 81

Chapter 4
Differential Expression of the Genes Encoding the Keratins of Cultured Human Epidermal Cells
Elaine Fuchs, Douglas Marchuk, and Angela Tyner

1. Introduction 85
2. The Structure of the 8-nm Keratin Filaments of Epidermis 86

3. The Genes Encoding the Human Epidermal Keratins 94
4. Structure of the Two Types of Keratin Genes and Their Relation to Other Intermediate-Filament Genes 97
5. Regulation of the Type I and Type II Keratin Genes 100
References 103

Chapter 5
Organization and Expression of the Vimentin and Desmin Genes
Wim Quax and Hans Bloemendal

1. The Discovery of Intermediate Filaments 109
2. The Diversity of IF Subunits 110
3. The Genes Encoding IF 114
4. Expression of the Cloned Desmin Gene 118
   4.1. In Lens Cells 118
   4.2. Expression of the Hamster Desmin Gene in Human Epithelial (HeLa) Cells 121
5. Chromosome Localization of the Human Vimentin and Desmin Gene 125
References 127

Chapter 6
Actin: A Regulator of Cell Growth and Differentiation
Stephen R. Farmer

1. Introduction 131
2. Cell Morphology and Growth and Differentiation 131
   2.1. Cell Configuration 131
   2.2. Cytoskeletal Organization 133
3. Regulation of Actin Gene Expression 135
4. Is Actin a Protooncogene? 141
5. Mechanisms by Which Actin May Regulate Cell Growth and Differentiation 143
References 146

Chapter 7
The Actin Genes in Caenorhabditis elegans
Michael Krause and David Hirsh

1. Introduction 151
2. Genomic Organization 152
3. Sequence Analysis 153
4. Transcription 158

5. Translational Products 163
6. Identification of Actin Mutants 165
7. Actin Gene Organization in Revertants of Actin Mutants 167
8. Transcription in Revertants 169
9. Future Prospects 175
References 176

Chapter 8
Implications of Microtubule Polarity for Microtubule Function
Ursula Euteneuer

1. Introduction 179
2. Background and Methods for Determining MT Polarity 179
3. MT Polarity and Spindle Function 187
4. MT Polarity and Intracellular Motility 193
References 196

Chapter 9
Molecular Mechanisms Controlling Tubulin Synthesis
Don W. Cleveland

1. Introduction 203
2. Apparent Autoregulatory Control of Tubulin Synthesis in Animal Cells 204
   2.1. Tubulin Synthesis in Animal Cells Is Sensitive to the Apparent Pool of Unpolymerized Subunits 204
   2.2. Possible Molecular Levels for Regulating Tubulin Synthetic Rates 207
   2.3. Transient Induction of Tubulin Synthesis by Interferon ... 213
3. Programmed Synthesis of Tubulin during Flagellar Growth in Unicellular Organisms 213
   3.1. Regulated Synthesis of Tubulin Achieved through a Combination of Transcriptional Control and mRNA Stability in *Chlamydomonas* 214
   3.2. Identification of Candidate DNA Sequences That Mediate Tubulin Regulation in *Chlamydomonas* 218
   3.3. The Paradigm of Tubulin Regulation in *Naegleria* 219
   3.4. Altered Tubulin Regulation in the Life Form Switch of the Parasitic Protozoa *Leishmania* 219
4. Programmed Synthesis of Tubulin during the Cell Cycle 220
   4.1. The Synchronous Cell Cycle of *Physarum* 220
   4.2. Cell Cycle Regulation in Other Eukaryotes 221
5. Summary 222
References 223

Chapter 10

Regulation of Tubulin Expression in Brain

Marcelle R. Morrison and W. Sue T. Griffin

1. Introduction 227
2. Tubulin Proteins 228
   2.1. Different Isoforms of α- and β-Tubulins 228
   2.2. Differential Localization of Brain α- and β-Tubulin Isoforms 229
   2.3. Evidence for Posttranslational Modification 231
   2.4. Developmental Regulation of Tubulin Isoforms 232
3. Tubulin Genes in Higher Eukaryotes 234
   3.1. α-Tubulin Genes 234
   3.2. β-Tubulin Genes 234
4. Expression of α-Tubulin mRNAs 235
   4.1. *In Vitro* Translation Products of α-Tubulin mRNAs 235
   4.2. Hybridization Analysis of Different α-Tubulin mRNAs 235
5. Expression of β-Tubulin mRNAs 237
   5.1. *In Vitro* Translation Products of β-Tubulin mRNAs 237
   5.2. Hybridization Analysis of β-Tubulin mRNAs 240
   5.3. *In Situ* Hybridization of Chick β2-Tubulin cDNA in Rat Cerebellum 244
6. Conclusions 245
References 251

Chapter 11

Tubulins from Plants, Fungi, and Protists: A Review

Louis C. Morejohn and Donald E. Fosket

1. Introduction: Scope and Objectives 257
2. Isolation and Identification of Tubulin 258
   2.1. Assays for Tubulin 259
   2.2. Ion-Exchange Chromatography 260
   2.3. Drug Affinity Methods 262
   2.4. Microtubule Polymerization and Depolymerization 263
   2.5. Copolymerization with Heterologous Tubulins 265
   2.6. Tubulin-Rich Organelles 265
   2.7. Technical Problems 268
3. Tubulin Dimer Structure 271
   3.1. Amino Acid Sequences 271
   3.2. Electrophoretic Resolution of α and β Subunits 275
   3.3. Peptide Mapping Patterns 279
   3.4. Immunology 282
   3.5. Subunit Microheterogeneity 284
   3.6. Posttranslational Modifications 287

3.7. Multiple Tubulin Genes 290
3.8. Membrane-Associated Tubulin 292
4. Antimicrotubule Drugs 293
4.1. Colchicine Effects *in Vivo* 294
4.2. Colchicine Effects *in Vitro* 296
4.3. Antimicrotubule Herbicides 300
4.4. Benzimidazole Derivatives 304
4.5. Taxol 306
4.6. Other Agents 309
5. Summary and Future Directions 309
References 311

Index 331

CELL and MOLECULAR BIOLOGY of the CYTOSKELETON

1

Probing the Cytoskeleton by Microinjection

Brigitte M. Jockusch, Annette Füchtbauer, Christiane Wiegand, and Bernd Höner

Dedicated to Professor Georg Melchers, on the occasion of his 80th birthday

1. Introduction

Animal cells employ about 20–35% of their total protein synthesis to construct the cytoskeleton—an intracellular cage of complex structures involved in cellular motility, like intracellular transport, exo- and endocytosis, protoplasmic streaming, locomotion, cellular polarity, anchorage, and cell division. Commonly, the entity of the cytoskeleton is divided into three fibrillar systems: microtubules, microfilaments, and intermediate (10-nm) filaments. In this chapter, we include the coated vesicle system, since it fulfills the criteria most commonly used to define cytoskeletal elements: it is composed of structural proteins that form supramolecular structures via self-assembly, it is involved in transport phenomena, and it associates with cellular membranes as well as with other cytoskeletal systems.

Whereas the cytoskeleton is intimately involved in cellular dynamics, the cytoskeletal elements are dynamic structures themselves. Their organization as well as their functions are, for example, subject to changes within the cell cycle, the developmental stage, and the cellular environment. The mechanism directing these changes probably involves protein:protein interactions with weak affinities guided by subtle changes in the ionic milieu and posttransla-

Brigitte M. Jockusch, Annette Füchtbauer, Christiane Wiegand, and Bernd Höner • Developmental Biology Unit, University of Bielefeld, 48 Bielefeld, Federal Republic of Germany.

tional protein modification. To study the molecular basis of these processes, one has to analyze synchronized cell populations or single cells.

Microinjection of specific drugs, structural proteins, or specific antibodies into single tissue culture cells has proven a valuable approach to this problem. Many cells have been shown to tolerate this experimental procedure quite well. The development of microscale methods (e.g., minigels and fast protein liquid chromatography) permits the biochemical analysis of approximately 100 cells, a number that can easily be microinjected within 5 min. Within the past few years, this technique has therefore become increasingly popular and has already answered many questions about the intracellular organization, the reversible assembly of cytoskeletal structures, and the functional requirement of individual components in specific motile processes. In this chapter, we shall discuss the most important results pertaining to the organization and function of the cytoskeleton obtained from microinjection with glass capillaries. We will concentrate on tissue culture cells but will compare data obtained with these cells with similar studies on lower eukaryotic cells.

2. *The Microtubule System*

2.1. *Analyzing the Equilibrium between Tubulin and Microtubules*

The dynamic character of intracellular microtubules is well known and is the basis of multiple but very specific functions in determining cell shape, polarity, and direction of migration of whole cells as well as of intracellular particles (for review, see Dustin, 1985). Since Weisenberg's first experiments with polymerization of microtubules from subunits (Weisenberg, 1972), much has been learned about the conditions for and the control of microtubule polymerization *in vitro* (see, for example, Kirschner, 1978; Timasheff and Grisham, 1980; Hill and Kirschner, 1982). Although there is no reason to believe that the principles of microtubule assembly are in any way different *in vivo* as compared to the *in vitro* situation, there must be many factors controlling microtubule-associated motility processes in the living cell, involving particles like microtubule organizing centers (MTOCs) and kinetochores, proteins like microtubule-associated proteins (MAPs), GTP, or ions (for review, see De Brabander *et al.*, 1985a).

A coarse regulation of microtubule dynamics may be achieved by controlling the amount of building blocks: From experiments with microtubule inhibitors, it was evident that the depolymerization of microtubules is inversely correlated with tubulin synthesis (cf. Cleveland and Kirschner, 1982). Microinjection of tubulin subunits has strongly supported the interpretation of the experiments cited: it is the pool size of free tubulin subunits which is monitored by the cell and is used to regulate tubulin synthesis, and probably assembly. CHO cells injected with homologous or heterologous tubulin in quantities to raise the presumed intracellular tubulin pool (2 mg/ml) by 100%

reduce their tubulin synthesis to approximately 10% of the control level (Cleveland *et al.*, 1983). The role of free calcium ions (Ca^{2+}) as a potential control factor for the *in vitro* polymerization of microtubules was discovered 13 years ago (Weisenberg, 1972) and is well accepted today. Microinjection of Ca^{2+} into mitotic echinoderm eggs has supplied good evidence that Ca^{2+} may indeed play such a role in the dynamics of cellular microtubules: at an intracellular concentration of 3–7 μM, mitotic spindle fibers were rapidly depolymerized. The velocity of this process (depolymerization of a half-spindle in less than 1 sec postinjection, p.i.) indicates that the injected Ca^{2+} may cause fracture of microtubules rather than loss of subunits from one end. The effect is specific for CA^{2+} and does not spread over the entire egg but seems locally restricted. This finding suggests rapid sequestering of free Ca^{2+} from the cytoplasm into a vesicular system, functionally reminiscent of the sarcoplasmic reticulum in striated muscle (Kiehart, 1981).

2.2. *Studying Microtubular Dynamics*

Incorporation of building blocks into microtubules and microtubular growth could be directly visualized by injecting fluorochome-tagged tubulin or MAPs into tissue culture cells (Keith *et al.*, 1981; Scherson *et al.;* 1984). In this type of experiment, it is extremely important to ascertain that the labeled probe is not damaged in its affinity for the assembly site and is still fully and reversibly polymerization competent. Fluorochromes that have been used successfully to label microtubule or microfilament proteins include NH-reactive (e.g., fluorescein isothiocyanate, FITC, lissamin-rhodamine B sulfhydrylchloride, RB 200 SC, dichlorotriazinyl amino fluorescein, DTAF) and SH-reactive (e.g., iodoacetamide derivatives of tetramethylrhodamine or fluorescein molecules) (cf. Taylor and Wang, 1980; Kreis and Birchmeier, 1982; Glacy, 1983a,b). The results obtained with different probes will be discussed in detail in the relevant section. Both the fluorochrome and the mode of labeling the protein must be carefully chosen, depending on the protein used. For example, tubulin labeled with DTAF was readily incorporated into cellular microtubules, but DTAF-labeled brain MAPs were not (Keith *et al.*, 1981; Scherson *et al.*, 1984). However, iodoacetamido fluorescein (IAF)-coupled MAPs associated effectively with microtubules (Scherson *et al.*, 1984). In this case, attacking the sulfhydryl groups of the MAPs with IAF apparently was tolerable, but modifying the amino agroups with DTAF probably interfered with affinity of the MAPs for tubulin. Moreover, labeling of MAPs with IAF within the assembled microtubule resulted in MAPs with better assembly competence than labeling them in the disassembled state, probably because the tubulin binding sites of MAPs are protected in the assembled microtubule (Scherson *et al.*, 1984).

For observation of cytoskeletal dynamics with fluorochromed building blocks, it is furthermore important to minimize damage to cellular metabolism by using weak illumination with suitable intensifying systems and a temperature-controlled environment. However, even with all the precautions

mentioned, it cannot be excluded that fluorochromed subunits behave differently from nonderivatized molecules. For example, such modified molecules seem to have a faster turnover than the endogenous counterparts (Zavortink *et al.*, 1979).

DTAF-labeled tubulin, injected into mouse fibroblasts and gerbil fibroma cells at the same concentration that drastically reduces tubulin synthesis (see Cleveland *et al.*, 1983), was incorporated into cellular microtubules within 15 min p.i. This system was used to study elongation of microtubules into cellular extensions. Monitoring the growth of labeled microtubules with video intensifying equipment revealed a growth rate of 1 μm/min at 37°C (Keith *et al.*, 1981).

DTAF- and FITC-labeled brain tubulins have been injected into sea urchin eggs (Salmon *et al.*, 1983; Wadsworth and Sloboda, 1983; Hamaguchi *et al.*, 1985). The fluorescent probes, introduced into fertilized eggs, were rapidly incorporated into the growing spindle. When cells were injected in metaphase or early anaphase, the label appeared within 20–30 sec in the spindle fibers, indicating a very rapid exchange between microtubules and subunit pools in this system (Hamaguchi *et al.*, 1985). Similar conclusions can be drawn from photobleaching experiments: after bleaching of fluorescent spindle areas in metaphase with an argon laser beam, full fluorescence recovery was measured within 1 min. This process is considerably slower than the values obtained for free diffusion of labeled tubulin in the cytoplasmic matrix (7 μm/sec). However, it is still much faster than can be explained by exclusive addition of labeled subunits at the ends of the 5- to 7-μm-long spindle microtubules (Salmon *et al.*, 1983). Thus, these data point to the rather unorthodox possibility that subunits may be incorporated all along the length of spindle microtubules (Salmon *et al.*, 1983; Hamaguchi *et al.*, 1985). In another study, DTAF-labeled tubulin appeared rapidly in spindle asters, but failed to outline the spindle proper (Wadsworth and Sloboda, 1983). The discrepancy of these results must await further studies for explanation.

Injection studies with DTAF-labeled tubulin into mitotic PtK cells show that the results obtained on the dynamic behavior of tubulin subunits in dividing sea urchin eggs are of general relevance, at least for animal cells: the probe was incorporated within seconds into metaphase microtubules. Photobleaching recovery showed that mobility within spindle fibers is also very high, while incoporation of the labeled probe as well as recovery of fluorescence after photobleaching in interphase microtubuli is much slower (in the order of several minutes, Saxton *et al.*, 1983).

IAF-labeled MAPs were introduced into PtK_2 and chicken gizzard cells, and into a mouse x rat neuronal hybrid cell line (NBr10-A). The protein preparation was enriched for MAP2, a polypeptide that is probably specific for neuronal cells (cf. Littauer and Ginzburg, 1984), but was found to readily associate with cytoplasmic microtubules in the nonneuronal cells cited previously (Scherson *et al.*, 1984). Another study describes that IAF-labeled MAPs associate also rapidly with spindle fibers in mitotic PtK_2 cells, without interfering with mitosis (Vandenbunder and Borisy, 1983). In conjunction

with fluorescence photobleaching recovery measurements, the microinjected fluorochromed MAPs were also used to determine treadmilling velocities along individual microtubules: in analogy to the findings with fluorochromed tubulin, it was found that there is slow fluorescence recovery (half-time approximately 5 min) of the bleached spot (diameter approximately 0.5 μm), but no or a very slow (less than 4 μm/hr) migration of the fluorochrome-tagged molecules along microtubules (Scherson *et al.*, 1984). These results allow several interpretations: either treadmilling is not an important feature of microtubules in the living cell, or fluorochromed MAPs arrest treadmilling, or photobleaching induces microtubular breaks that are healed by reannealing fractured microtubules with tubulin subunits and MAPs from cytoplasmic pools.

A caveat for the interpretation of photobleaching experiments may be deducted from another study with DTAF-labeled MAPs: when this probe was bleached after incorporation into microtubules, gel analysis demonstrated that the bleached DTAF can cross-link neighboring protein molecules. DTAF-labeled tubulin, when bleached, lost its capacity for reversible assembly (Leslie *et al.*, 1983).

2.3. Analyzing Microtubule-Bound Organelle Transport

Some of the rules governing intracellular transport of organelles along axoplasmic or cytoplasmic microtubules have recently been elucidated by microinjecting exogenous particles into various cell types. Polystyrene beads of 370- to 500-nm diameter, when injected through glass capillaries into giant axons of a crab, moved rapidly in a saltatory fashion in the antegrade direction. Velocity and mode of transport were indistinguishable from those of antegradely transported endogenous organelles (Adams and Bray, 1983). Injection of somewhat smaller (260-nm), fluorescein coupled carboxylated polystyrene beads into tissue culture cells (BS-C-I and PtK_2 cells) in combination with video intensification optics gave essentially the same results (Beckerle, 1984). Such experiments indicate that, at least in these systems, exogenous particles, like endogenous ones, associate with a microtubule-bound transport mechanism. Transportation is dependent on ATP as well as on intact microtubules and requires a negative surface charge of this particle. Not surprisingly, the particles must not exceed an upper size limit (800 nm).

Microinjected gold particles (polyethylene glycol stabilized) were also found to be transported along microtubules in PtK_2 cells. With a video intensifier camera and electronic background subtraction, reflections of polarized light can be recorded even from particles much smaller (20–40 nm) than the resolution limit of the light microscope. Again, in all parameters tested (frequency of saltations, directionality and velocity of transport, travel distances) these tiny particles introduced into the cell behave like endogenous organelles (De Brabander *et al.*, 1985a,b).

The question as to whether the force generator in these transports may be dyneinlike is still under debate: there are conflicting reports on the effect

of vanadate, a presumedly specific inhibitor of dynein ATPase (cf. Beckerle and Porter, 1982; Forman, 1982; Buckley and Stewart, 1983).

2.4. Introducing Antitubulins

Microinjection of antibodies against tubulin into tissue culture cells has led to interesting observations on structural stability, topography of subunits, and cellular functions of microtubules. The results obtained naturally differ with the antibody employed. A rat monoclonal antibody raised against yeast tubulin (YL 1/2, Kilmartin *et al.*, 1982) was found to react with the tyrosylated carboxy-terminus of α-tubulin (Wehland *et al.*, 1983). Blocking this epitope *in vitro* by antibody binding does not interfere with microtubule polymerization. Microtubules in the living cell were decorated by the antibody in interphase and mitotic cells. Thus, the tyrosylated carboxy-terminus of α subunits must be exposed at the microtubule surface in cytoplasmic and spindle microtubules (Wehland *et al.*, 1983). An inhibitory effect of this antibody on endogenous organelle and particle transport was seen at rather high antibody-tubulin ratios. On the assumption of a tubulin concentration of approximately 2 mg/ml (cf. Hiller and Weber, 1978), an injected volume of approximately 5–10%, and an antibody concentration of 20 mg/ml in the injection solution, an intracellular antibody:tubulin ratio of approximately 1 should be obtained. Since only about 15% of total α-tubulin is tyrosylated (Thompson, 1982) and probably only 50% of it is microtubule bound, much less of this particular antibody should be required to saturate the relevant epitopes along the microtubule. Concentrations of 6 mg/ml and above were found to inhibit saltatory movement of endogenous organelles, to induce microtubular bundles, and to interfere with mitosis (Wehland and Willingham, 1983). Injected at very high concentrations (20 mg/ml), the antibody not only caused the microtubule system to collapse but also dispersed the Golgi stacks into small vesicles. The same antibody, when adsorbed to colloidal gold particles and injected into PtK_2 cells, prevented saltatory movement of these particles, even when only a few particles were injected (approximately 30 per cell, De Brabander *et al.*, 1985b). These results suggest that blocking the tyrosylated carboxy-termini of α-tubulin subunits within the microtubule is incompatible with organelle or particle transport.

The effect of introducing specific monoclonal mouse antibodies directed against either α- or β-tubulin, as well as injecting a polyclonal guinea pig antibody, on integrity and dynamics of the cytoskeleton has been described in another study (Blose *et al.*, 1984). These antibodies, injected in amounts to yield a 1:1 ratio of tubulin:antibody, had little effect on mitosis as well as on the distribution and integrity of intracellular microtubules, as seen in immunofluorescence and in electron microscopy. However, they caused a severe distortion of the intermediate filament system within the injected cells. The vimentin system expressed in the cell line used (gerbil fibroma) collapsed into either lateral aggregates or perinuclear caps within 1.5 hr p.i. Thus, it seems that those antibodies, by coating the microtubules, disrupt microtubule–inter-

mediate filament interactions, which have been postulated in many publications (cf. Geiger and Singer, 1980; Schliwa and Van Blerkom, 1981; Hirokawa, 1982). In this work, it is not excluded that the antibody injection may have led to a rapidly reversed (within 30 min p.i.) disruption of microtubules which, for technical reasons, would have escaped recording. This possibility is mentioned by the authors themselves (Blose *et al.*, 1984).

Polyclonal antibodies with high affinities for α- or β-tubulin have been raised by immunizing rabbits with hemocyanin (KLH)-coupled, SDS-denatured α- and β-tubulin, and affinity purification by cross-absortption. With these methods, antibodies with an affinity of approximately 10^{-8} M for half-saturation with α- or β-tubulin have been obtained (Mandelkow *et al.*, 1985a). In immunoblots with chymotryptic and tryptic tubulin digests, the α-specific antibody was found to react with the amino-terminus of the α polypeptide, the β-specific antibody bound to the carboxy-terminus of the β-tubulin. Thus, these antibodies can be used to identify the location of structural and functional domains of tubulin within the microtubule (Mandelkow *et al.*, 1983, 1985a,b). The effect of such antibodies (raised against β-tubulin, one β-specific moiety, another batch cross-reacting also with α-tubulin) was studied on microtubules polymerized *in vivo*, on microtubules in detergent-extracted cell models, and on microtubules in living cells. In all three systems, the antibodies disrupted microtubules very effectively. Microtubules, polymerized *in vitro* and stabilized by glycerol, were disrupted within 10 min at relatively high antibody:tubulin ratios (1:2). Cell models, incubated with antibody or living cells, injected with antibody, showed a rapid disintegration of microtubules (as monitored by immunofluorescence and electron microscopy) even with low antibody concentrations (varying between 1:10^3 and 1:10). In the living cell, this effect was concentration-dependent and fully reversible (Mandelkow *et al.*, 1983; Füchtbauer *et al.*, 1985). To monitor the rapid and reversible disintegration of microtubules on a background of breakdown products, the injected antibody had to be either quenched with unlabeled second antibody prior to immunofluorescence, or staining had to be performed with an unrelated antitubulin (Fig. 1). This was especially important for low antibody: tubulin ratios, where the transient effect was overcome by the cell within 1 hr after injection (Füchtbauer *et al.*, 1985). The effect of such antibody molecules on microtubules is probably comparable to the effect of actin-severing proteins on actin filaments: the cytoskeletal structure is disrupted by probes that have a higher affinity for the relevant building block than the building blocks have for each other. In the case of the antitubulins described here, their affinity for tubulin is by two orders of magnitude higher than the affinity of tubulin for tubulin (Gaskin *et al.*, 1974).

The rapid restoration of the microtubular pattern (within 1–4 hr p.i., depending on the amount of antibody injected) is probably due to reannealing of microtubular pieces, as well as to outgrowth of new microtubules from the MTOC in the perinuclear area. Tubulin:antitubulin complexes are probably cleared eventually from the cell. Since degradation of endogenous as well as of microinjected proteins varies considerably, depending on molecular

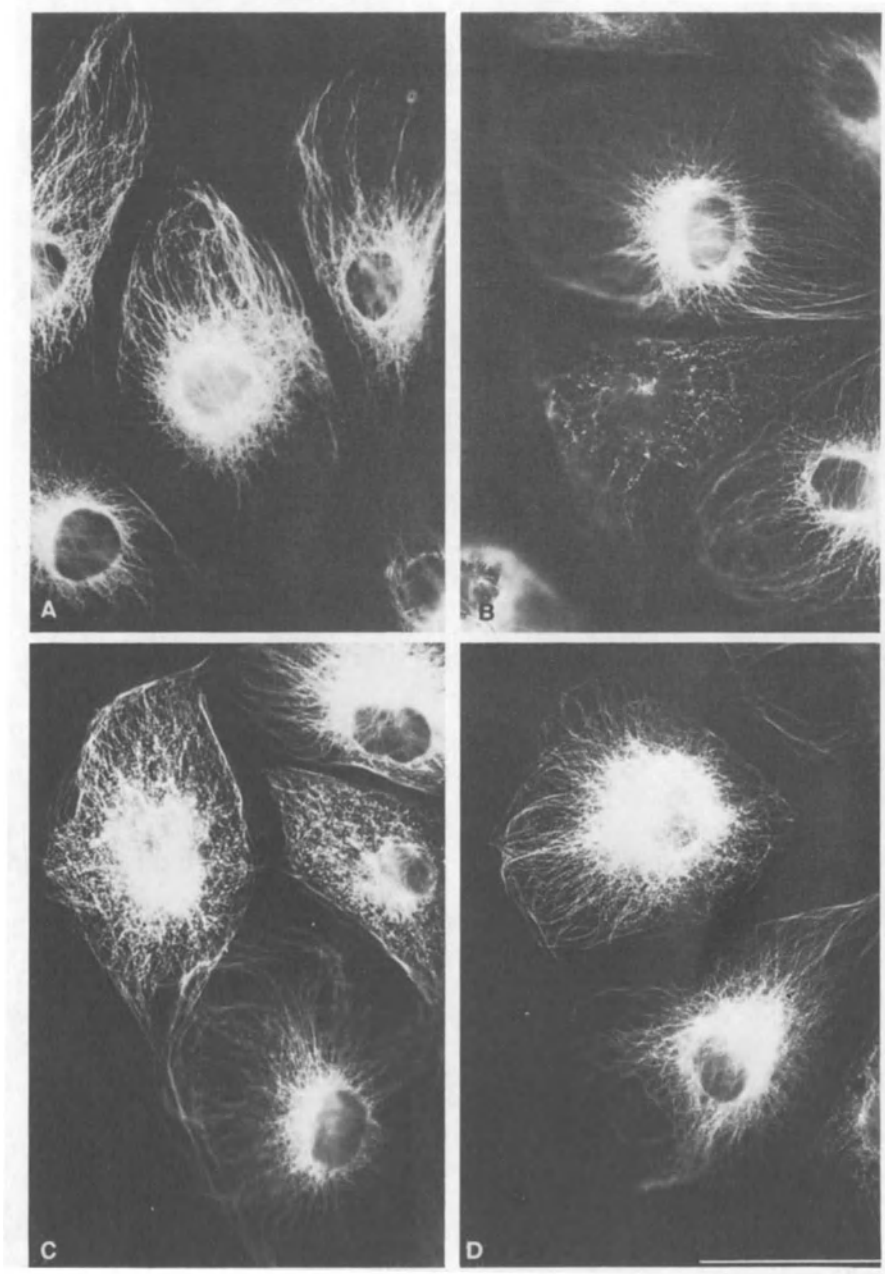


Figure 1. Effect of a polyclonal antibody specific for β-tubulin on cytoplasmic microtubules in PtK_2 cells. The antibody was raised in rabbits against SDS-denatured, hemocyanin-coupled β-tubulin from bovine brain. β-Tubulin-specific IgG was purified by affinity chromatography on β-tubulin sepharose (Mandelkow *et al.*, 1985b). PtK_2 cells were injected with either phosphate-buffered saline (PBS) (A) or 1 mg/ml anti-β-tubulin in PBS (B–D). Cells were fixed with formaldehyde and stained with a rat monoclonal antibody against α-tubulin (YL 1/2, see Wehland *et al.*, 1983) and affinity-purified rabbit-anti-rat IgG coupled to lissamine–rhodamine. Cells were processed this way at 0.5 hr (A, B), 2 hr (C), and 5 hr (D) after injection. Note the complete breakdown of microtubules into short pieces in B, and their recovery in C and D. Bar: 50 μm.

weight, isoelectric point, and susceptibility to proteases (cf. Goldberg and Dice, 1974; Goldberg and St. John, 1976; Stacey and Allfrey, 1977; Neff *et al.*, 1981), the degradative rate of the complexed tubulin is unknown. However, it seems unlikely that the injected cell can get rid of such aggregates within a period of 4 hr. Thus, removal of tubulin:antibody does not seem to be a necessary prerequisite for restoration of microtubules.

3. The Microfilament System

3.1. Studying Actin Dynamics

Like the microtubule system, the microfilament system in nonmuscle cells is characterized by highly dynamic behavior. This implies that building blocks must be readily exchanged between filamentous structures and subunit pools. Microinjection of fluorochrome-labeled subunits, analogous to a pulse label, is therefore also a suitable procedure to study microfilament dynamics.

This assumption was justified by the first experiments using IAF-labeled actin. Taylor and Wang carefully studied polymerizability, incorporability into actomyosin gels, and extent of myosin ATPase activity of the labeled actin and thus ascertained the validity of this probe (Taylor and Wang, 1978, 1980; Wang and Taylor, 1980).

Several studies used it to study actin dynamics in large single cells. Upon injection into the giant ameba *Chaos carolinensis,* the labeled actin was found distributed into both the exo- and endoplasm, while microplasmodia of *Physarum polycephalum* retained the injected probe in the ectoplasm (Taylor and Wang, 1978). In the early period of such microinjection experiments, kinetics of actin dynamics could only be followed indirectly, by examining individual cells for each time point. This may have been the reason for some unexpected results. For example, no increase in fluorescence was detected in the cleavage furrow of dividing sea urchin eggs labeled with IAF-actin. Since there is little doubt that contracting filament bundles are responsible for cytokinesis in all animal cells (cf. Schroeder, 1975), this result may be explained by the assumption that rapid changes in fluorescence may have escaped the recording of separate cells (Wang and Taylor, 1979).

Video intensifying systems greatly improved the fine analysis of actin dynamics in objects with an unfavorable geometry, such as amebae and eggs: fluorescent microfibrils were detected in the advancing pseudopods of IAF-labeled *Chaos carolinensis* (Taylor and Wang, 1980; Taylor *et al.*, 1980a), in the cortical layer and the uropod region of locomoting *Chaos* and *Amoeba proteus* cells (Taylor *et al.*, 1980a; Gawlitta *et al.*, 1980b), and in the cleavage furrow of dividing *A. proteus* (Gawlitta *et al.*, 1981b). In *A. proteus,* intense fluorescence was found underneath the cell membrane and surrounding the pulsating vacuole and phagocytotic vacuoles (Stockem and Gruber, 1983; Hoffmann *et al.*, 1984). The fluorescent structures (probably corresponding to actin filament aggregates) were found to fluctuate in intensity and change their positions as a consequence either of changes in natural cellular activities (Stockem

and Gruber, 1983) or of the application of drugs (procaine, dinitrophenol, puromycin, ouabain, and *N*-ethyl maleimide) (Hoffmann *et al.*, 1984).

The distribution of free Ca^{2+} in locomoting *Chaos carolinensis*, as measured by luminiscence of microinjected aequorin, suggested a Ca^{2+}-mediated constant actomyosin contraction at the uropod and rapid contraction pulses in advancing pseudopods (Taylor *et al.*, 1980b). In *A. proteus*, induced pinocytosis (which probably requires actomyosin contraction) was found correlated with free intracellular Ca^{2+} close to the plasma membrane, as measured with microinjected chlorotetracycline (CTC) and by monitoring the Ca^{2+}-CTC-fluorescence (Gawlitta *et al.*, 1980a).

The injection of fluorochrome-labeled skeletal muscle actin into flat tissue culture cells of avian and mammalian origin combined with video intensifying microscopy and, more recently, with fluorescence photobleaching recovery yielded a wealth of interesting data on the spatial and temporal distribution of actin in nonmuscle cells. Actin was usually labeled at the cystein residue 373 (Wang and Taylor, 1980) with IAF or with rhodamine derivatives (for example, iodoacetamido tetramethyl rhodamine, IATMR, or at reactive amino groups with lissamine rhodamine sulfonyl chloride, RB 200 SC). When these probes were injected into fibroblasts or epithelial cells, they were readily and rapidly (within 5–30 min) incorporated into all the various microfilament-rich structures previously identified in tissue culture cells: stress fibers (microfilament bundles), ruffles, microspikes, and the marginal region of the leading lamella (leading edge) of locomotive cells (Kreis *et al.*, 1979, 1982; Wehland and Weber, 1980; Wehland *et al.*, 1980b; Glacy, 1983b; Geiger *et al.*, 1984b; Wang, 1984; Sanger *et al.*, 1980a; Wang *et al.* 1985). Within 20–30 min. p.i., the label appeared homogeneously (in rare cases, periodically; cf. Sanger *et al.*, 1983a, 1984a) distributed over the entire length of stress fibers, including their terminal ends (focal contact regions).

Neither muscle (cardiac myocytes) nor nonmuscle (fibroblasts) cells could discriminate between different isoforms of actin: FITC-labeled muscle actin and RB 200 SC-labeled brain actin were equally well accepted by both cell types and were incorporated into all types of microfilament structures (cortical filaments of the leading edge, stress fibers, myofibrils; McKenna *et al.*, 1985).

The maximum intensity of label in the leading edge appeared around 5 min p.i. (Glacy, 1983b). The distribution and the local density of fluorescent actin in chicken gizzard cells was monitored with the optical detection system of a laser beam photobleaching apparatus. It was found that the leading edge and the stress fibers exhibited a 2–3.5 times higher degree of fluorescence than the interfibrillary cytoplasm (Kreis *et al.*, 1982). With this setup, RB 200 SC-actin could be monitored as an integral part of stress fibers up to 48 hr p.i. (Kreis *et al.*, 1982).

By bleaching of small areas (of 1.5-μm radius) with an argon laser beam in fluorescent regions of RB 200 SC-actin-injected cells and monitoring of the recovery, several conclusions on the dynamic behavior of actin in nonmuscle cells could be drawn (Kreis *et al.*, 1982; Geiger *et al.*, 1984b).

1. The translational diffusion coefficients of actin in different cellular

domains (interfibrillar region, stress fibers, focal contact areas, leading edge) were remarkedly uniform. The values obtained (2–3 cm/sec) were only half the size measured for fluorescent control proteins, such as BSA and immunoglobulins (Kreis *et al.*, 1982; Geiger *et al.*, 1984b). This indicates a reduced diffusion capability of the injected actin, caused either by polymerization into larger units or by association with preexisting structures.

2. The highest degree of recovery (fractional mobility) of bleached actin was found in the area of the leading edge (more than 70%; Kreis *et al.*, 1982). This, together with the rapid appearance of a high concentration of label within the leading edge (Glacy, 1983b), indicates that actin in this cellular domain is more dynamic than in the stress fiber or is more easily accessible to additional actin from a subunit pool.

3. The low percentage of recovery (less than 20%) and the relatively long recovery period (apparent half-time several minutes) measured in bleached spots within stress fibers and focal contact areas indicated that actin within these structures is relatively immobile, but exchange of subunits does occur to a certain degree with the surrounding cytoplasm.

This result is also supported by the finding that fluorochromed actin associates more slowly with stress fibers (within 30 min p.i.) than with filaments in the leading edge (cf. Glacy, 1983b; McKenna *et al.*, 1985).

4. The size of the bleached area along a given stress fiber had no influence either on amount or on velocity of recovery. Thus, neither rearrangement of actin filaments within the parallel bundle of the stress fiber nor treadmilling along them seem very important factors in recovery (Kreis *et al.*, 1982). The exact mode of incorporation of the labeled subunits remains unknown at present. Although stress fibers are dynamic structures that can rapidly and reversibly assemble during the cell cycle (cf. Sanger, 1975) or during spreading and locomotion (cf. Wang, 1984), it is surprising to find all of them incorporating the added exogenous actin within such short periods, since individual stress fibers, especially in stationary epithelial cells, can persist for many hours, even days. Even more surprising, perhaps, was the finding that myofibrils of cardiac cells incorporated IATMR-actin also within 5–20 min (Glacy, 1983a). The half-life of actin in nonmuscle cells is approximately 2.5 days; for sarcomeric actin in striated muscle it is 12–19 days (Rubenstein *et al.*, 1976). This excludes the possibility that the observed labeling of stress fibers and myofibrils is due to metabolic turnover.

Interestingly, two different labeling patterns of myofibrils with different probes were obtained: IATMR-actin labeled the entire I band, but not the Z disk, and in addition, associated with the M band (Glacy, 1983a), RB 200 SC- or FITC-labeled actins were found exclusively in the I-Z-I-region, indistinguishable from phalloidin staining (McKenna *et al.*, 1985).

In contrast, isolated myofibrils bound RB 200 SC-actin exclusively in the regions adjacent to the M bands, i.e. in positions were free myosin heads can be expected (Sanger *et al.*, 1984b). Thus, it seems that the pattern of incorporation of exogeneous actin into myofibrils in living cells depends on the fluorochrome used and differs from that of isolated myofibrils. This is in contrast to the findings with stress fibers, where addition of labeled actin to glyceri-

nated cell models resulted in the same patterns as were obtained with living cells (Sanger *et al.*, 1984a).

The reversible assembly and reorganization of stress fibers in motile embryonic chicken heart fibroblasts have been extensively studied using microinjected IATMR-actin (Glacy, 1983b; Wang, 1984; Wang *et al.* 1985). It was found that stress fibers generally started to assemble from the periphery of the cell (at the leading edge as well as at the trailing end of kite-shaped cells) and proceeded to grow unidirectionally. Nascent stress fibers were able to change orientation, to fragment, or to merge with each other. Studies with RB 200 SC-actin and chicken gizzard cells supported these conclusions and showed in addition that nascent focal contacts can also change their orientation, with respect to the long axis of the cell (Kreis *et al.*, 1984). Disassembly was also unidirectional, starting from either the proximal or the distal end (Wang 1984; Wang *et al.*, 1985). The birth of a stress fiber within the leading edge started with an intensely fluorescent spot which coincided with a nascent focal contact (Glacy 1983b; Wang, 1984). Even in rapidly advancing cells, the position of these attachment points between the cell and the substratum remained constant, while the leading edge protruded forward (Wang, 1984).

Although the mode of incorporation of fluorochromed subunits into stress fibers and myofibrils is still not clear, the same techniques (injection of labeled actin, photobleaching, recovery measurements with an intensifying and image processing system) allowed precise investigation of the growth of microspikes, which are highly dynamic structures within the leading edge, containing a core of actin filaments. Following fluorescence recovery after photobleaching of an IATMR-actin labeled microspike, it was found that incorporation of new fluorescent subunits was a polar process: it started near the edge of the cell and subsequently moved from the distal to the proximal region, at a constant rate of 0.8 μm/min (Wang, 1985). These results are consistent with other data demonstrating that actin filaments face the plasma membrane with their positive ends, and that new subunits are preferentially inserted at that site (Tilney *et al.*, 1981). Thus, in contrast to the results obtained with stress fibers, here the concept of treadmilling (cf. Wegner, 1976) can explain both growth of actin filaments and protrusion of the plasma membrane at the leading edge.

The dynamics of the actin traffic between the cytoplasm and the nucleus have also been investigated with microinjected fluorochrome-labeled probes. While the question of endogenous nuclear actin and its possible functions has raised controversial discussions in the past decade (cf. Jockusch *et al.*, 1974; Comings and Harris, 1976; Goldstein *et al.*, 1977; LeStourgeon, 1978; Clark and Rosenbaum, 1979; Scheer *et al.*, 1984), several studies documented that addition of dimethylsulfoxide (DMSO) to the growth medium led to the appearance of actin filament bundles in the nucleus of amebae, slime molds, and mammalian cells (Fukui, 1978; Fukui and Katsumaru, 1979, 1980; Sanger *et al.*, 1980b, 1983b). When epithelial cells were microinjected with fluorescent actin and allowed to incorporate this probe into their stress fibers, subsequent addition of 10% DMSO to the growth medium caused the disruption of the

fluorescent stress fibers. The liberated actin molecules were used to build up massive actin filament bundles inside the nucleus, while other components of the stress fibers such as alpha-actinin, myosin, and tropomyosin remained in the cytoplasm (Sanger *et al.*, 1980a; Wehland *et al.*, 1980b). The significance of this readily reversible traffic between two cellular compartments for unperturbed cells remains unclear. However, this result emphasizes that stress fibers are indeed highly dynamic and that a massive, but selective, transport of cytoskeletal proteins within the cell can occur within several minutes.

3.2. Studying the Dynamics of Actin-Associated Proteins

Microinjection of tissue culture cells with fluorochromed actin-binding proteins has been carried out so far with chicken gizzard alpha-actinin (labeled with tetramethylrhodamineisothiocyanate, TMRITC; iodoacetamidotetramethylrhodamine, IATMR; or lissamine rhodamine, RB 200 SC), with IAF-labeled smooth muscle (gizzard) tropomyosin, and with FIIC- and RB 200 SC-labeled gizzard vinculin (Feramisco, 1979; Feramisco and Blose, 1980; McKenna *et al.*, 1984; Wang *et al.*, 1985; Wehland and Weber, 1980; Burridge and Feramisco, 1980; Geiger *et al.*, 1984b; Kreis *et al.*, 1984). Smooth muscle TMRITC-alpha-actinin was found incorporated into stress fibers of injected fibroblasts in the characteristic periodic fashion, into ruffling membranes and foci of polygonal microfilament networks. Thus, the injected protein, like injected actin, was recruited into all cellular areas, where enrichment of the endogenous protein had been previously recorded by immunofluorescence (cf. Lazarides and Burridge, 1975). However, in addition, an intense labeling of nuclei was found, which suggests that the TMRITC-labeled probe was overconjugated and bound unspecifically to nuclear structures. RB 200 SC-alpha-actinins were used to study the dynamics of alpha-actinin in stress fibers, focal contacts, and myofibrils (Geiger *et al.*, 1984b; Kreis *et al.*, 1984; McKenna *et al.*, 1984; Wang *et al.*, 1985). The recovery of fluorescence after photobleaching small spots within stress fibers showed, in analogy to actin, a reduced mobility of stress fiber-bound alpha-actinin as compared with other cellular domains. Recovery of the fluorescence in Z disk of myofibrils in injected muscle cells takes much longer (several hours) than in stress fibers (approximately 10 min). This result probably reflects the difference in stability between muscle Z disks and Z-like dense bodies in stress fibers of nonmuscle cells.

Fluorochromed tropomyosin, as could be expected from immunofluorescence studies (Lazarides, 1976a,b), was incorporated into stress fibers and excluded from the leading edge (Wehland and Weber, 1980), but even image intensification failed to show the fine punctate pattern with cell-type specific periodicities revealed by antibody staining (cf. Lazarides, 1976a,b; Sanger *et al.*, 1983a).

Fluorochromed vinculin concentrated predominantly in contact areas (Burridge and Feramisco, 1980; Geiger *et al.*, 1984b; Kreis *et al.*, 1984). Again, this result supports the general conclusion that fluorochrome-labeled sub-

units of the different microfilament structures are readily accepted by the living cell and faithfully used at their proper destination: by immunofluorescence, vinculin had been originally discovered as a major constituent of the cytoplasmic face of focal contacts (Geiger, 1979).

Studies involving microinjection of fluorochromed derivatives and quantitation of fluorescence intensities of all three major components of this region revealed data on their ratios and local distribution: actin, the most abundant constituent, was found by a factor of 100 more concentrated in focal contact areas than vinculin and alpha actinin (Kreis *et al.*, 1984). The fractional recovery after fluorescence photobleaching (indicating protein mobility) was around 40% for vinculin and alpha-actinin in focal contact regions, that is, about twice the value found for actin (see above, Geiger *et al.*, 1984b).

3.3. Reorganizing Microfilament Patterns by Introducing Structural Proteins

Microinjection of actin-associated proteins has also been used to experimentally alter the endogenous pattern of microfilament organization in tissue culture cells. This is especially effective in cases where the injected protein is known to act in substoichiometric amounts on actin filaments, as in the case of actin-capping and actin-severing proteins (for reviews on different classes of actin-binding proteins, see Korn, 1982; Weeds, 1982; Craig and Pollard, 1982; Jockusch, 1983). Two different capping proteins isolated from bovine brain (Kilimann and Isenberg, 1982; Wanger and Wegner, 1984) and from *Physarum polycephalum* (Maruta *et al.*, 1983) were injected into epithelial cells and fibroblasts. In fibroblasts, a rapid disruption of stress fibers, concomitant with disappearance of focal contacts (as observed by reflection contrast microscopy) and a contraction of the injected cells, was observed (Füchtbauer *et al.*, 1983; Jockusch and Füchtbauer, 1985). In epithelial cells, the marginal microfilament bundle proved more resistant to the action of the capping proteins, but radial stress fibers disappeared as in fibroblasts. As in fibroblasts, contraction was seen in single epithelial cells. However, if grown in a sheet, epithelial cells did not change their gross morphology, probably because they were held in position by junctional complexes formed with their neighbors (Füchtbauer *et al.*, 1983).

These phenomena were readily reversible, the times required for complete restoration of cell shape, stress fibers, and focal contacts being dependent on the amount of the injected capping proteins (Table 1). The time course of stress fiber disruption was consistent with the current hypothesis that the positive end of actin filaments is close to the plasma membrane and accessible to addition of new protein molecules: stress fibers started to disintegrate from their distal ends (Fig. 2), and focal contacts disappeared concomitantly (Jockusch and Füchtbauer, 1983).

Microinjection of an F-actin severing protein (pig or human plasma ADF; Harris *et al.*, 1980; Harris and Gooch, 1981; Chaponnier *et al.*, 1985) into fibroblasts and epithelial cells also led to a rapid and reversible disruption of stress fibers (Fig. 3). However, while the kinetics of these events were quite

Table 1. Correlation between the Intracellular Concentration of Injected Actin-Binding Proteins and the Recovery of Stress Fibers

Protein	Intracellular concentration (nM)[a]	Half-time of recovery ($t_{1/2}$, hr)[b]
Brain capping protein[c]	160	3.25
Brain capping protein[c]	80	3.0
Brain capping protein[c]	16	1.75
Actin depolymerizing factor (porcine serum)[d]	125	3.5
Actin depolymerizing factor (porcine serum)[d]	1.25	1.5
Actin depolymerizing factor (human serum)[e]	50	2.0
Actin depolymerizing factor (human serum)[e]	10	1.5

[a]These values were calculated from the following data: the molecular weight of the injected proteins (63,000 for brain capping protein, 91,000 for human and porcine actin depolymerizing factor), the protein concentration of the injection solution, and an injection volume corresponding roughly to 10% of the cellular volume.
[b]These figures were obtained from examining stress fibers by immunofluorescence at various time points after injection, counting the number of cells showing an intact stress fiber pattern. At least 100 cells were injected for each value.
[c]Kilimann and Isenberg, 1982.
[d]Harris *et al.*, 1980.
[e]Chaponnier *et al.*, 1985.

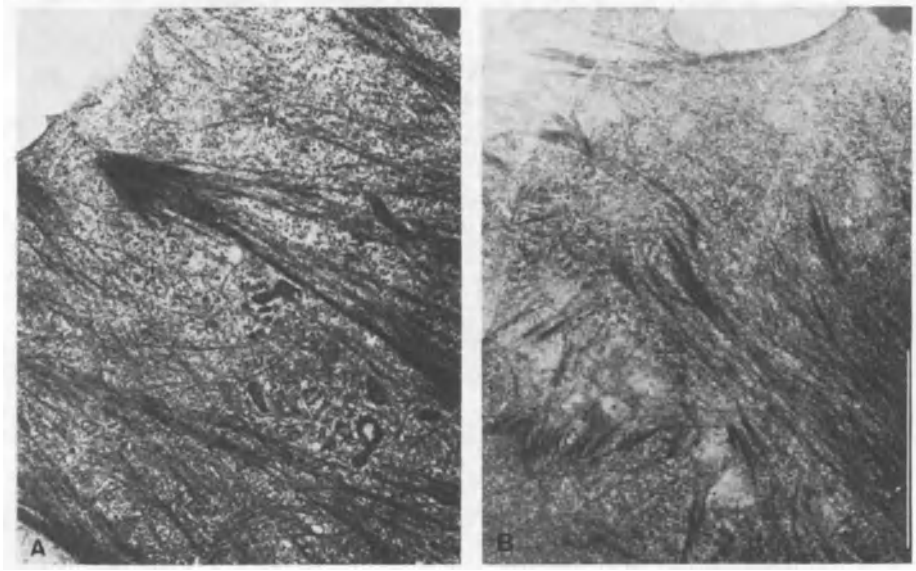


Figure 2. Effect of bovine brain capping protein (BCP) (Kilimann and Isenberg, 1982) on the terminal regions of stress fibers in PtK_2 cells. Cells were injected either with imidazole buffer (A) or with 0.02 mg/ml BCP in the same buffer (B), fixed, and processed for thin sectioning and electron microscopy 15 min after injection. The sections closest to the lower surface of the cells show a typical arrowhead-shaped terminal region of a stress fiber in (A), while in (B) this structure has completely disintegrated. Bar: 5 μm.

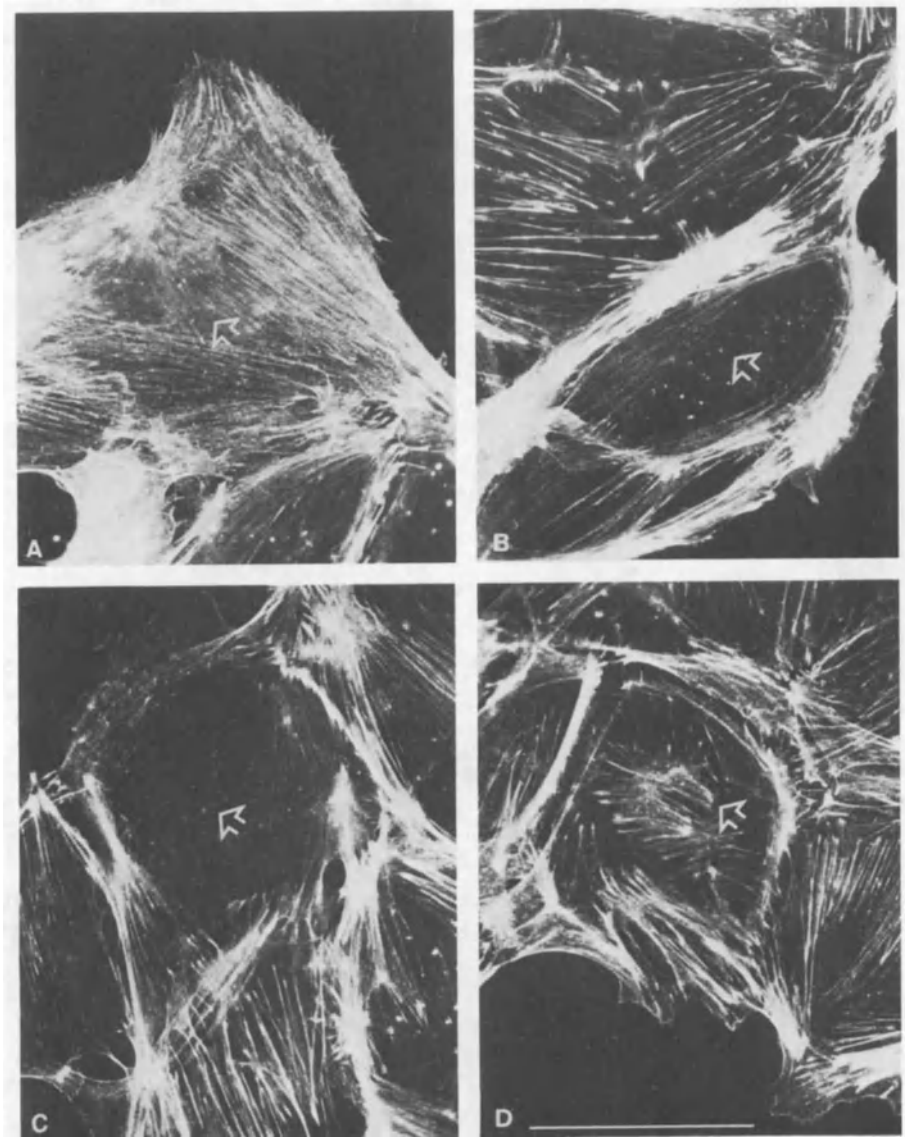


Figure 3. Effect of human actin depolymerizing factor (ADF) (Chaponnier *et al.*, 1985) on stress fibers in PtK_2 cells. Cells were injected with 0.02 mg/ml ADF in phosphate-buffered saline, fixed with formaldehyde at 40 min (A, B), 70 min (C), and 90 min (D) after injection and stained with rhodamine–phalloidin. Radial stress fibers of injected cells (arrow) have disappeared within the first hour after injection, but begin to reappear at approximately 90 min p.i. (Füchtbauer, Jockusch, Weeds, and Gabbiani, manuscript in preparation). Bar: 50 μm.

similar to the data obtained with capping proteins (see Table 1), there were also important differences:

1.The introduced ADF led to disruption of stress fibers starting at the site of injection, which was usually the perinuclear area. Disintegration then progressed from the proximal to the distal ends. Focal contacts were only moderately or not at all affected. Thus, even fibroblasts usually remained attached to the substratum and did not change their shape (Füchtbauer, 1984; Jockusch *et al.,* 1985b).

2. Whereas the capping proteins acted exclusively on stress fibers in the living cell, ADF also disrupted stress fibers in detergent extracted cell models (Füchtbauer *et al.,* 1983; Füchtbauer, 1984). These data suggest that actin severing proteins like ADF act on actin filaments via intrafilament insertion, probably because their affinity for actin subunits is higher than the affinities required for actin:actin interaction. On the other hand, the capping proteins employed, acting exclusively at the positive ends of actin filaments, may interfere with the dynamics of subunit exchange at that site. This process, naturally, takes place only in the living cell. Assuming that these interpretations are correct, one would predict that microinjection of a protein capping the negative ends of stress fiber filaments [like β-actinin (Maruyama and Sakai, 1981) or acumentin (Southwick and Hartwig, 1982)] should also act exclusively on living cells.

Microinjection in villin, an actin-binding protein found in microvilli of intestinal or renal epithelia, was carried out with nonpolarized epithelial tissue culture cells (PtK_2). Villin had been found to be Ca^{2+}-regulated in its interaction with actin; it contains several Ca^{2+}-binding sites (Hesterberg and Weber, 1983). At free Ca^{2+} concentrations above 10^{-6} M, villin *in vitro* acts like all other actin severing proteins (which generally seem to bind Ca^{2+}): it clips actin filaments into short pieces. At Ca^{2+} concentrations below micromolar, villin bundles actin filaments into tightly packed regular arrays (Bretscher and Weber, 1980; Howe *et al.,* 1980; Matsudaira and Burgess, 1982). When injected into PtK_2 cells, it was found to concentrate in the leading edge, where microfilament bundles occur in ruffles and microspikes (Bretscher *et al.,* 1981). No effect of this protein on stress fibers was seen. Since injection studies with two different ADF proteins have shown that binding sites for severing proteins are accessible within the stress fiber, nonaccessibility of binding sites for villin does not seem a plausible explanation for this result. A transient effect on stress fibers may have escaped recording, since cells were processed for immunofluorescence with antivillin and antiactin only 4 hr after injection. A more detailed study on the kinetics and the effect of villin association with actin might answer the question whether villin can transiently sever stress fiber filaments while leaving filaments in ruffling areas intact, which would require local differences in Ca^{2+} concentration.

The disruption of stress fibers by proteins capping the positive ends of actin filaments implies a functional importance of the focal contact areas and the precise organization of actin filaments within these areas for stress fiber

expression and cellular adherence (cf. Geiger *et al.*, 1984b). However, it is also possible that stress fiber expression requires actin filaments that are at least temporarily exempt from rapid polymerization cycles or highly dynamic rearrangements, processes that have been suggested for the leading lamella of locomoting tissue culture cells or migrating protozoans. One factor that might infer greater stability on actin filaments could be tropomyosin. The reasons for such an assumption are the following: (1) Tropomyosin has been found to stabilize actin filaments *in vitro* against spontaneous fragmentation (Wegner, 1982). (2) Transformed cells, which show predominantly a diffuse actin network and no or only a few stress fibers, express much less tropomyosin than their normal counterparts (Hendricks and Weintraub, 1981; Leonardi *et al.*, 1982). (3) While recent studies have revealed that several isotropomyosins already exist in normal nonmuscle cells, the pattern becomes even more complex in transformed cells, suggesting that transformation-induced changes in the actin organization are correlated with changes in tropomyosin gene expression (cf. Payne and Rudnick, 1984). Therefore, microinjection of tropomyosin into cells lacking stress fibers seemed an interesting approach to test the requirement of a "threshold" concentration of intracellular tropomyosin for the expression of stress fibers. Such experiments were carried out with transformed or tumor cells (Warren *et al.*, 1983; Jockusch *et al.*, 1985b). Smooth muscle (gizzard) tropomyosin, injected into transformed rat kidney cells, failed to induce stress fiber formation (Warren *et al.*, 1983). A more extensive study was carried out with an established line of rabbit tongue carcinoma cells (V2). These cells are characterized by constant locomotory activity. More than 90% are completely devoid of stress fibers and focal contacts. Actin and α-actinin were found predominantly concentrated within the leading lamella (Jockusch *et al.*, 1983). Chicken gizzard, rabbit skeletal muscle, and pig brain tropomyosins were injected into these cells at rather high concentrations: intracellular levels of tropomyosins in the order of 1 tropomyosin per 10 actin molecules were reached. Provided that V2 microfilaments can bind exogeneous tropomyosin at all, such concentrations should have been sufficient to induce changes in actin filament organization, cellular adherence, or motility, since each tropomyosin dimer should be able to cover six to seven actin subunits within the filament. No stress fiber formation was observed in the injected cells. In addition, their locomotory activity seemed unchanged (Jockusch *et al.*, 1985b). Thus, these results corroborate conclusions drawn from studies on the growth of nascent stress fibers with fluorochromed actin: they probably must start from their distal ends, within the area of focal contact. Stabilizing actin filaments may be a necessary factor for increasing the "life periods" of these structures, but is probably not sufficient to induce their expression.

Pancreatic DNAase I, a protein that rapidly forms complexes of high affinity with G actin and, much more slowly, depolymerizes F actin (cf. Lazarides and Lindberg, 1974; Hitchcock *et al.*, 1976), was microinjected into locomoting *A. proteus* cells. Within a few seconds p.i., the injected region appeared "relaxed," exhibiting a disorganization of protoplasmic streaming

within this area and invasion of streaming material from other regions of the cell. Thus, if DNAase I was injected into the contracting tail region, the uropod, a reversal of endoplasmic streaming was observed. At later times (10–15 min), in the entire cell, both streaming and locomotion stopped, and the cortical layer of microfilaments seemed drastically reduced (Wehland *et al.,* 1979). Thus, in these large cells, the injected protein acts in two phases: there is an immediate effect at the injection site, which is followed by much slower processes involving the whole cell.

Shifting the intracellular equilibrium between actin and myosin by injecting myosin is not feasible, since skeletal smooth and nonmuscle myosins would precipitate at the injection site, when confronted with the intracellular ionic milieu. However, the HMM fragment of skeletal muscle myosin, which is soluble at physiological salt concentration, has been injected into amphibian blastomeres (Meeusen *et al.,* 1980). No effect of this fragment was seen on cleavage. However, when the *N*-ethylmaleimide (NEM) derivate of HMM was injected, cytokinesis and wound contraction at the injection site were inhibited. NEM–HMM is a competitor for myosin in its binding to actin, but does not dissociate from actin in the presence of Mg–ATP. Injection of 1 μg NEM–HMM into one blastomere of the two-cell stage embryo, close to the expected second division, allowed the injected blastomere one more cleavage. However, the following cleavages were incomplete, since the cleavage furrow could not proceed through the injection site. These experiments, like microinjection of antimyosin (see Section 3.5), prove experimentally the involvement of actomyosin in cytokinesis, and also in other cortical contraction processes like wound closure.

3.4. Introducing Phalloidin

Phalloidin, one of the numerous poisons produced by the mushroom genus *Amanita,* reacts stoichiometrically and with high affinity with actin subunits in filaments, thus stabilizing the filaments and shifting the equilibrium in G/F actin solutions toward F actin (cf. Wieland, 1975). The drug, a cyclic bipeptide, does not penetrate the cell membrane, but seems to be taken up selectively by hepatocytes (Gabbiani *et al.,* 1975). When it was microinjected into fibroblasts or PtK_2 epithelial cells, a concentration-dependent reorganization of microfilaments was observed (Wehland *et al.,* 1977): injection solutions containing 0.2–1 mM phalloidin caused aggregation of actin filaments into needlelike, permanent structures (at 0.2 mM phalloidin), a slowdown (at 0.5 mM phalloidin), or a stop (at 1.0 mM phalloidin) of locomotory activity, and, at the highest concentration, dissolution of stress-fiber-bound actin, contraction, and growth inhibition of the injected cells. The needlelike aggregates, in addition to actin, contained myosin, filamin, alpha-actinin, and tropomyosin (Wehland *et al.,* 1980a). Thus, in this system, no local separation of these actin-binding proteins from actin took place, in contrast to the induction of nuclear actin bundles by DMSO (see Section 3.1). With a fluorescent derivate of phalloidin (FITC phalloidin), the binding of the drug could be directly

monitored in the living cell. When the probe was injected at concentrations too low to induce the needlelike structures, it was seen incorporated into stress fibers. Interestingly, actively ruffling lamellipodia were able to exclude the drug. Only cold-paralyzed cells (at 10°C) absorbed FITC phalloidin into the cortical microfilament webs of the leading edge (Wehland and Weber, 1981).

Since no such exclusion was found for injected actin or alpha-actinin (see Sections 3.1 and 3.2), this result appears specific for phalloidin. It seems noteworthy that staining fibroblasts with 7-nitrobenz-2-oxa-1,3-diazole (NDB)-coupled phallacidin, an acidic relative of phalloidin, gave somewhat different results; in this case, the drug was introduced into the cells by lysolecithin permeabilization, and NDB phallacidin labeled the cortical microfilaments in the leading edge (Barak *et al.*, 1980). However, it cannot be excluded that these cells were damaged by the lysolecithin treatment.

Phalloidin was also injected into invertebrate cells. In *A. proteus* and *Physarum polycephalum*, it inhibited protoplasmic streaming and induced the appearance of contracting filamentous structures (Götz von Olenhusen and Wohlfarth-Bottermann, 1979; Kukulies and Stockem, 1985). As in mammalian tissue culture cells, locomotion in the ameba stopped and the internal actin distribution was altered (Stockem *et al.*, 1978; Götz von Olenhusen and Wohlfarth-Bottermann, 1979; Hoffmann *et al.*, 1984).

NDB phallacidin, introduced into the alga *Chara australia* by lysolecithin permeabilization, did not interfere with cytoplasmic streaming. Such different results may be explained by differences in sensibility between species, in the intracellular concentrations of the drug, or in the affinities of the drugs (NDB phallacidin or phalloidin) for filamentous actin.

When introduced into sea urchin eggs prior to fertilization, phalloidin inhibited the movement of the egg nucleus toward the pronucleus. Thus, fusion of the nuclei could not take place, and development stopped. When phalloidin was injected after fertilization, cleavage was inhibited (at intracellular concentrations above 20 μM). Unexpectedly, nuclear division was also blocked (Hamaguchi and Mabuchi, 1982).

3.5. Using Antibodies against Microfilament Proteins

Antibodies against actin-associated proteins have also been used to study microfilament organization and dynamics. Affinity-purified antibodies have been injected in concentrations up to 20 mg/ml, which results in an intracellular concentration as high as 2 mg/ml. The concentrations required to see a cellular response, however, vary grossly with the antibody employed. This result probably reflects differences in the intracellular concentration and accessibility of the target protein, i.e. the antigen, as well as in the affinity and the epitope specifity of the antibody. In contrast to studies on the effect of antibodies on microtubuli (see Chapter 1) and intermediate filaments (see Chapter 3), until now microfilaments have usually been probed with polyclonal, affinity-purified antibodies, not with monoclonal antibodies.

Most antibodies against actin have not been able to disturb the cytoplas-

matic actin organization of injected cells, even when high concentrations were used (cf. Jockusch and Füchtbauer, 1985). This may be explained by the assumption that the cell contains much more actin than is needed for microfilament networks or bundles at any time during the cell cycle. Thus, a transient decrease in actin concentration caused by the formation of actin–antibody complexes may be tolerated. There is only one brief report on an actin antibody, which was microinjected with loaded erythrocyte ghosts into PtK_1 cells and was found to disorganize the cortical microfilament web (Daley and Eckart, 1980).

However, the situation is completely different with respect to nuclear actin. The presence of actin in the nucleus has already been discussed (see Section 3.1). In amphibian oocytes, an intranuclear concentration of 3–4 mg/ml has been determined (cf. Clark and Rosenbaum, 1979; Krohne and Franke, 1980; Gounon and Karsenti, 1981). At least some of this nuclear actin is associated with the loops of lampbrush chromosomes (Karsenti *et al.*, 1978). Intracellular injection of antiactin (8 ng/nucleus) into *Xenopus* oocytes effectively blocked chromosome condensation on meiotic divisions (Rungger *et al.*, 1979).

An unexpected result was obtained when antiactin was injected into nuclei of the amphibian *Pleurodeles waltlii:* the loops of lampbrush chromosomes, which are indicative of active transcription of protein genes, collapsed and were retracted. The effect was concentration- and time-dependent: with 2 ng of antibody corresponding to an approximate actin–antibody ratio of 1:400, a total retraction of loops was found 4–6 hr p.i. This effect was antibody-specific (antibodies against other cytoskeletal proteins had no effect) and specific for transcription activity of RNA polymerase II (nucleolar gene transcription by RNA polymerase I was not affected). Microinjection of actin severing proteins from *Physarum* and smooth muscle had the same effect as antiactin (Fig. 4; Scheer *et al.*, 1984). These experiments suggest a very specific role of actin in protein transcription, an assumption supported by previous findings that purified RNA polymerase II is tightly associated with actin (Smith *et al.*, 1979) and that faithful *in vitro* transcription by RNA polymerase II requires actin as a cofactor (Egly *et al.*, 1984). The precise function of actin in the transcription process remains unknown so far, but it seems that a filamentous stage is not required. The relation between the function of actin in transcription and in chromosome condensation is also not clear.

Injection of an antibody directed against both heavy and light chains of starfish egg myosin was found to effectively inhibit cytokinesis in blastomeres of starfish embryos or in fertilized eggs (Mabuchi and Okuno, 1977; Kiehart *et al.*, 1982). Since this antibody was not affinity purified but used as immune globulin fraction obtained from serum, stoichiometric calculations cannot be carried out. However, the same concentration of antibody that prohibited cytokinesis had no effect on movement of pronuclei or chromosomes. Thus, it seems unlikely that myosin contributes to nuclear movement or chromosome distribution during mitosis.

An affinity-purified antibody directed against the heavy chain of smooth

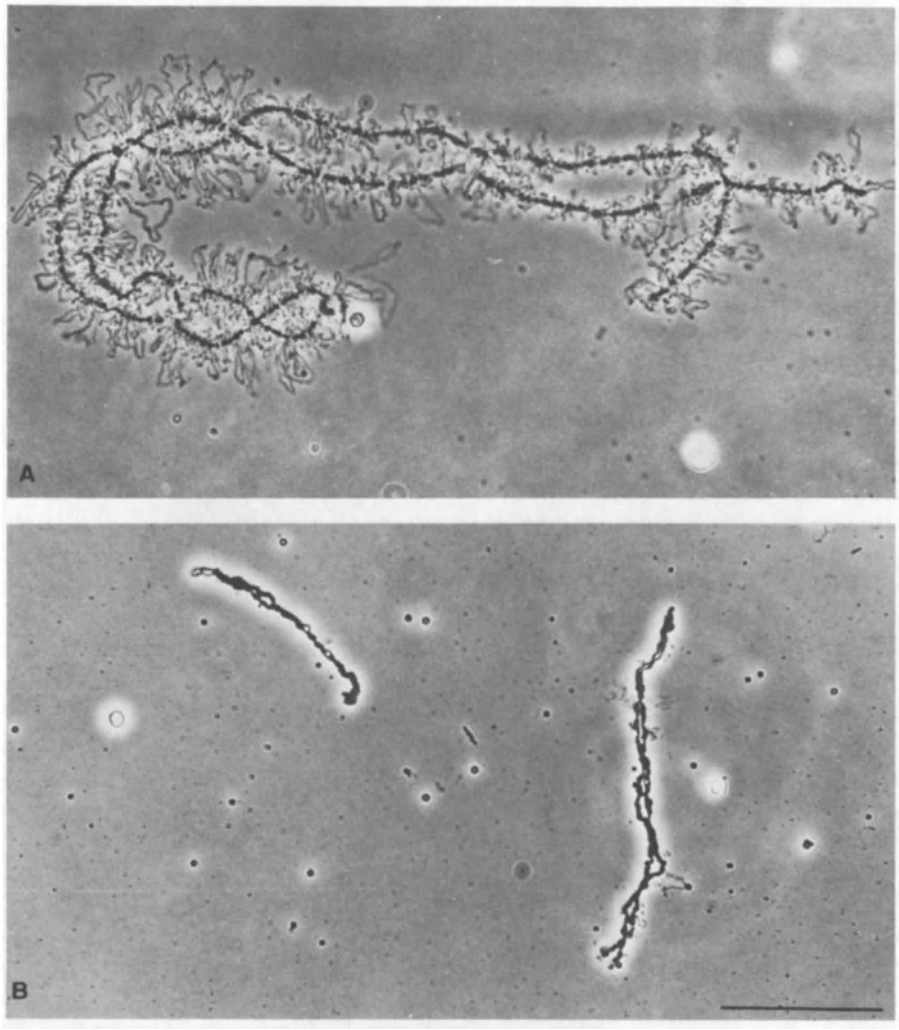


Figure 4. Effect of a polyclonal, affinity-purified antiactin on lampbrush chromosomes. Nuclei of newt oocytes were injected with 2 nl of either nonspecific rabbit IgG (1 mg/ml) (A), or antiactin (0.2 mg/ml) (B). The nuclear envelope was removed 4.5 hr after injection, and lampbrush chromosomes were examined by phase contrast microscopy. Note the complete retraction of lateral loops in B, indicating that transcription of protein genes has stopped (Scheer *et al.*, 1984). Bar: 50 μm.

muscle myosin but cross-reacting with nonmuscle vertebrate myosin, was found to remove myosin selectively from stress fibers in living tissue culture cells (Fig. 5; Höner *et al.*, 1985; Jockusch *et al.*, 1985a). At a calculated intracellular antibody:myosin ratio of approximately 1:1, myosin disappeared from the stress fibers within 20 min p.i., while actin filaments and focal con-

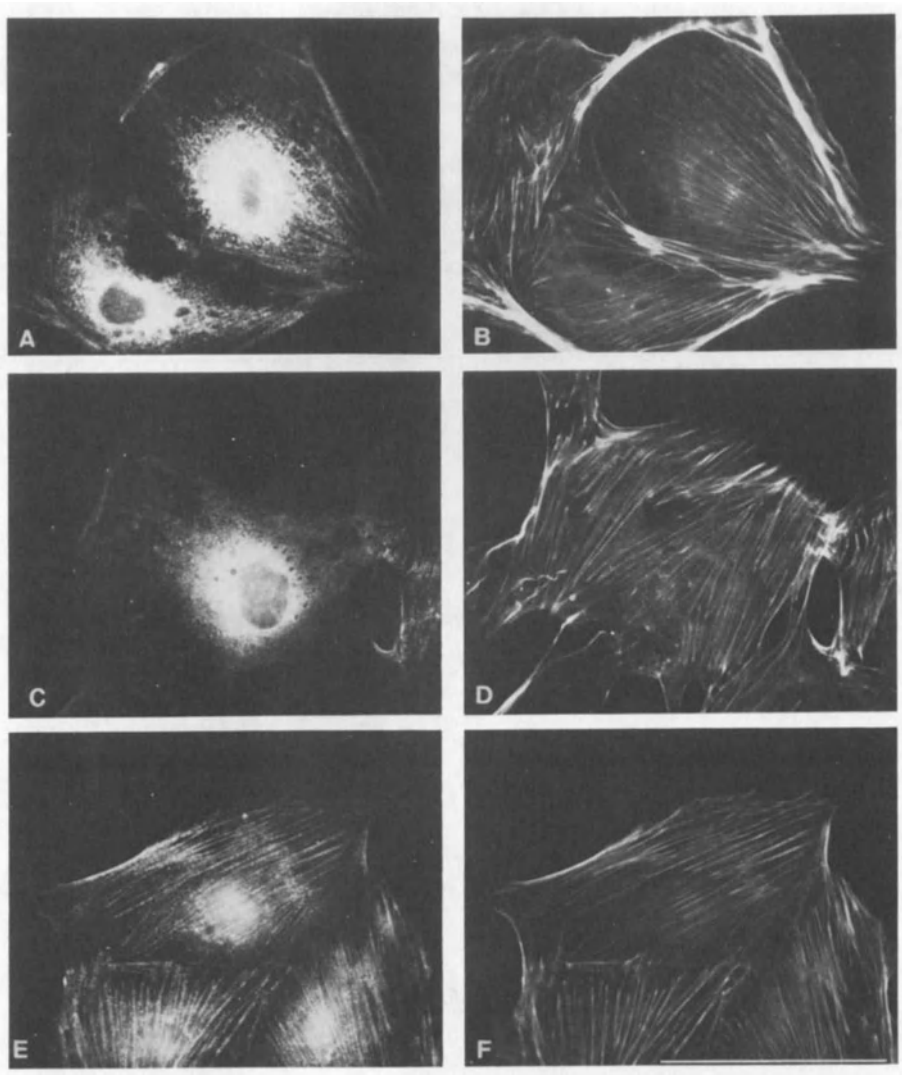


Figure 5. Effect of a polyclonal, affinity-purified rabbit antibody against chicken gizzard myosin on stress fibers in PtK_2 cells. Cells were injected with 1 mg/ml of antimyosin and fixed with formaldehyde at 5 min (A, B), 45 min (C, D), and 12 hr (E, F) after injection. They were double-stained with the same antibody and rhodamine-labeled sheep-anti-rabbit IgG for myosin (A, C, E) and with NDB-phallacidin for actin (B, D, F). At 5 min p.i., the injected antibody is seen accumulating in the perinuclear area. Stress fiber myosin is clearly visible in the marginal regions of the cell (A). At 45 min p.i., no myosin is visible as stress fiber component, while stress fiber actin seems unperturbed (C, D). At 12 hr p.i., myosin is again present in stress fibers (E), in a pattern indistinguishable from the one found in noninjected controls. Bar: 50 μm.

tacts remained intact. Within the next few hours, myosin reappeared again as a stress fiber component. It seems likely that this is not based on new synthesis but on recruitment of myosin from other compartments of the cell. Preliminary experiments (Höner and Füchtbauer, unpublished observations) indicate that this antibody also interferes reversibly with ruffling in the leading lamella of V2 rabbit carcinoma cells.

Two different antibodies against smooth muscle vinculin have been described to interfere with stress fiber organization and focal contacts in nonmuscle tissue culture cells. Antivinculin injected into human fibroblasts caused the disappearance of focal contacts (Birchmeier *et al.*, 1982; Kreis and Birchmeier, 1982). An affinity-purified antibody reacting with both the 130-kD vinculin (Geiger, 1979) and the larger "metavinculin" identified in smooth muscle (Siciliano and Craig, 1982; Feramisco *et al.*, 1982) led to disintegration of focal contacts in epithelial and fibroblastic cells of different species (Jockusch and Füchtbauer, 1983; Füchtbauer, 1984; Füchtbauer and Jockusch, 1984). The effect of this antibody on cellular morphology is drastic. Even with intracellular vinculin:antibody ratios as low as $1:10^{-4}$ (based on an intracellular vinculin concentration of 0.1 mg/ml), cells lost their focal contacts completely and irreversibly, stress fibers disintegrated slowly, and their terminal regions frayed out (Figs. 6 and 7). These effects, obtained with such low antibody concentrations, can only be explained by the assumption that this antibody effectively blocks epitopes at the vinculin molecule which are indispensable for the proper organization of the focal contact area. Such epitopes can be envisioned either for vinculin:actin (cf. Jockusch and Isenberg, 1981; Wilkins and Lin, 1982) or for vinculin:membrane (cf. Geiger *et al.*, 1984b) interactions. There is evidence that muscle and nonmuscle cells contain several isovinculins that differ in their intracellular distribution (Geiger, 1982): the most acidic form is predominantly associated with stress fibers. Therefore, it is tempting to speculate that the antibody preferentially reacts with this form, and that this vinculin isoform is limited in its intracellular concentration to the absolutely necessary level: any interference with even a few molecules of this sort results in complete disintegration of cellular contacts and stress fibers.

Microinjection of antibodies against another actin-binding protein, spectrin, was used to study a possible function of this protein in microfilament organization. Spectrins have been found in a wide variety of cells as actin-binding molecules with a submembraneous distribution (cf. Glenney *et al.*, 1982; Burridge *et al.*, 1982) and are therefore possible candidates to mediate actin plasma membrane attachment. Microinjection of a monoclonal IgM antibody against pig brain spectrin or of an affinity-purified antibody against bovine brain spectrin into several cell lines demonstrated that the intracellular spectrin was precipitated by the antibody. However, the microfilament organization was unaffected, leaving focal contacts, stress fibers, and overall cellular morphology undisturbed (Mangeat and Burridge, 1984). Thus, spectrin, in contrast to vinculin (see Section 3.2) does not seem to be directly involved in actin organization in nonerythrocyte cells. Of all the cytoskeletal

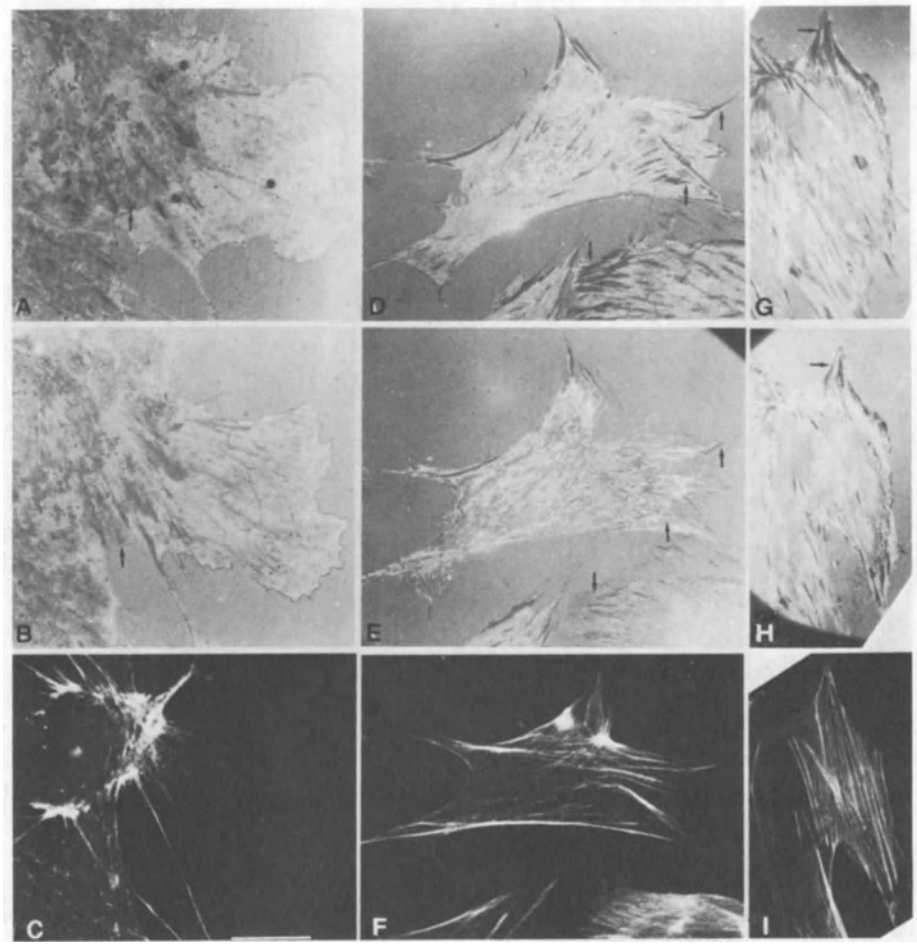


Figure 6. Effect of a polyclonal, affinity-purified rabbit antibody against chicken gizzard vinculin, on focal contacts and stress fibers in PtK_2 cells. Cells were injected with 2 mg/ml antivinculin in phosphate-buffered saline (PBS) (A–F) or with PBS (G–I). Cells were examined immediately before (A, D, G) and 15 min after (B, E, H) injection, using reflection contrast microscopy. At 45 min p.i., they were fixed with formaldehyde and stained with rhodamine–phalloidin. Note that with that concentration of antivinculin, focal contacts were severely reduced in size and number already 15 min p.i. (B, E, arrows). At 45 min p.i., stress fibers have partially disintegrated, and their terminal regions appear abnormal (C, F). Bar: 100 μm.

elements examined (microfilaments, microtubules, intermediate filaments, coated vesicles), only the intermediate filament system reacted to spectrin precipitation in the injected cells: vimentin filaments became distorted and formed aggregates which frequently colocalized with spectrin aggregates (Mangeat and Burridge, 1984). These results indicate an association between vimentin filaments, spectrin, and the plasma membrane.

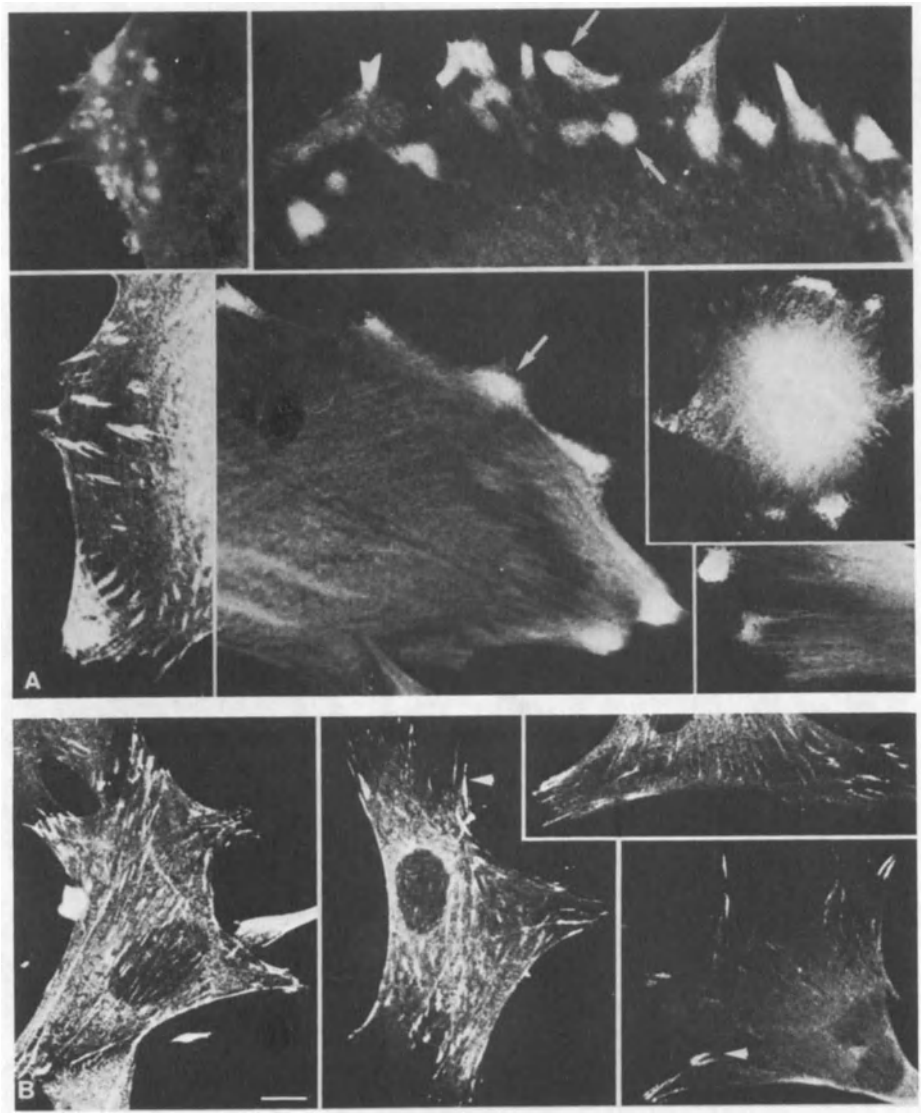


Figure 7. Effect of the same antivinculin described in Fig. 6 on the distribution of vinculin in chicken heart fibroblasts. Cells in A were injected with 0.9 mg/ml antivinculin. At 45 min p.i., they were fixed with formaldehyde and stained with the same antibody and rhodamine-coupled sheep-anti-rabbit IgG. Note the patchy, "fuzzy" distribution of vinculin at the plasma membrane of antivinculin-injected cells (arrows), as compared with the vinculin distribution in noninjected controls (B). Here, vinculin delineates sharply the terminal regions of stress fibers (arrowheads). Bar: 50 μm.

4. The Intermediate Filament System

4.1. Introducing Antibodies

Five classes of intermediate filaments can be discriminated in animal cells: neurofilaments in neural cells, filaments consisting of the "glial fibrillar acidic protein" in glia cells, vimentin filaments predominantly in mesenchymal, but also in other cells, desmin filaments in muscle cells, and cytokeratin filaments in epithelial cells. The latter class can be subdivided again, according to the various sets of cytokeratins which are expressed in different epithelial cells (cf. Lazarides, 1982). All intermediate filaments are chemically characterized by their extremely low solubility (cf. Franke *et al.*, 1982). Of the cytoskeletal elements known, they comprise the most stable structures; however, they are not completely resistant to endogenous or exogenous stimuli. For example, reorganization of cytokeratin filaments has been described during mitosis (Geiger *et al.*, 1984a), and certain epitopes on cytokeratin molecules seem to undergo a masking–unmasking cycle with the cell cycle (Lane and Klymkowsky, 1982; Geiger *et al.*, 1984a). Changes in the posttranslational modification of vimentin and of cytokeratin polypeptides during mitosis have been described (Bravo *et al.*, 1982). However, compared with cyclical alterations observed in microtubules and microfilaments, such changes are minor, and individual intermediate filaments, once assembled, are stable structures. Thus, injection of filament building units, which has revealed many interesting details on microtubule and microfilament dynamics, is meaningless in this case, but injection of specific antibodies can be expected to cause structural or positional changes.

The consequences of microinjection of certain antitubulins and antispectrin on vimentin filaments have been discussed in the sections on microtubules and microfilaments (see Sections 2.4 and 3.5).

The introduction of an affinity-purified antibody against denatured rabbit chondrocyte vimentin into rat fibroblasts led to collapse of the vimentin filament network into a perinuclear cap, at a calculated antibody:vimentin ratio of approximately 1:10 (Gawlitta *et al.*, 1981a). Microfilaments, cell shape, and locomotion were not affected. Injected cells underwent normal mitosis, distributing the caps to both or to one daughter cell. Within the caps, antibody-cross-linked vimentin filaments were localized. Quite similar results were obtained with monoclonal antibodies against vimentin (Klymkowsky, 1981) and against a minor protein component of vimentin filaments (Lin and Feramisco, 1981).

Microinjection of polyclonal antibodies against calf hoof keratin (Eckert and Daley, 1980a,b; Eckert *et al.*, 1982) and of several monoclonal antibodies against PtK_1 cytokeratins into PtK_2 cells (Lane and Klymkowsky, 1982; Klymkowsky *et al.*, 1983) resulted in gross distortion of the cytokeratin network. The polyclonal antibodies, introduced into cells by fusion with antibody-preloaded erythrocyte ghosts or by capillary injection, caused a collapse of cytokeratin filaments into a perinuclear cap (Eckert *et al.*, 1982), similar to

the vimentin caps. Capillary injection of two monoclonal antibodies specific for two different cytokeratin polypeptides of simple epithelia both disrupted keratin filaments in PtK_2 cells, but gave rise to different aggregates: while one antibody induced the formation of perinuclear caps or coils, combined with a "spliced" appearance of the remaining extended cytokeratin fibers, the other caused disruption of the cytokeratin network and formation of compact aggregates dispersed throughout the cell (Lane and Klymkowsky, 1982; Klymkowsky, 1982).

A number of different cell lines have been found to express two different classes of intermediate filament proteins, namely, vimentin in addition to the proteins specific for the particular celltype (for review see Lazarides, 1982). The question of whether such cells contain two different filament systems, or whether vimentin copolymerizes with other proteins into only one intermediate filament network, was addressed by several groups. Double immunofluorescence (Osborn *et al.,* 1980) and immuno electron microscopy (Henderson and Weber, 1981) suggested that PtK_2 cells coexpressed two different systems, that is, separate cytokeratin and vimentin filaments. However, microinjection experiments with an antibody against the T-lymphocytocyte surface antigen thy I as a vimentin-specific probe (Dulbecco *et al.,* 1981) and a monoclonal antibody against one cytokeratin polypeptide demonstrated that these two types of filaments were not completely independent from each other in PtK_2 cells: the collapse of vimentin filaments caused by antithy I also induced a partial perinuclear coiling of prekeratin filaments, and vice versa (Klymkowsky, 1982; Klymkowsky *et al.,* 1983). The collapsed structures were not completely superimposable, and the cytokeratin network recovered earlier, while vimentin caps, induced by antithy I, remained visible for days.

In contrast to these results with PtK cells, HeLa cells, which also express vimentin and cytokeratin polypeptides, seem to contain two completely independent filament systems: Injection of a monoclonal antivimentin or of monoclonal antibodies specific for individual cytokeratins found in this cell type caused the collapse of vimentin filaments, or, respectively, the disruption of the cytokeratin fibers, without affecting the corresponding filament network (Tölle *et al.,* 1985). In two other human cell lines, coexpressing either vimentin and glial fibrillar acidic protein or vimentin and desmin, disruption of one type of filaments by injection of the relevant antibodies also caused the collapse of the other system. Immuno electron microscopy suggested that indeed individual filaments are copolymers of both types of proteins (Tölle and Osborn, 1985). Thus, it seems that, depending on the cell type, both possibilities are verified in nature: cells can contain two independent sets of intermediate filaments, but they can also form copolymers from different classes of subunits.

Even more subtle analyses have been carried out with microinjection of antibodies reacting with individual cytokeratins and counterstaining the injected cells with antibodies against other cytokeratins. With such experiments, questions as to the protein composition of the cytokeratin network can be

addressed: do the cytokeratin polypeptides expressed in one cell participate in each cytokeratin filament, or do they build up different filaments? The evidence available so far suggests that the cytokeratin filaments in epithelial cells can contain more than two different cytokeratins (Tölle and Osborn, 1985; Tölle *et al.*, 1985).

When the literature on microinjection experiments with antibodies against intermediate filaments is reviewed, two general conclusions become evident: (1) The antibody concentration required to lead to disruption, collapse, or aggregation of intermediate filaments is relatively high; with monoclonal antibodies, concentrations of 6–7 mg/ml are required. (2) A disrupted intermediate filament organization has no effect on cellular activities. Thus, for life in cell culture, intermediate filaments seem dispensable.

4.2. Introducing mRNA

While the experiments described here indicate that cells do not need intermediate filaments when grown in culture, one might ask if an increase in intermediate filaments or the expression of the wrong type of intermediate filaments may be adverse to tissue culture cells. Experiments related to such questions have been carried out by microinjecting poly (A) RNA, highly enriched for cytokeratin mRNA, into cells that normally express only vimentin filaments (Kreis *et al.*, 1983; Geiger *et al.*, 1984a). The mRNA was faithfully transcribed, and cytokeratin filaments were assembled in injected bovine lens cells and also in rat fibroblasts, two cell types that normally do not express cytokeratins. The first cytokeratin-positive aggregates were seen as early as 1 hr after injection. Within 24–30 hr p.i., an extensive network of cytokeratin filaments developed which did not colocalize with vimentin filaments, or with microfilaments or microtubules. Growth and mitosis seemed unperturbed in the injected cells, and the induced cytokeratin filaments were distributed among the daughter cells (Kreis *et al.*, 1983).

5. The Coated Vesicle System

Coated vesicles are believed to be intimately involved in receptor-mediated endocytosis (cf. Goldstein *et al.*, 1979), that is, in the active uptake of exogenous molecules by the cell. There is also good evidence that coated vesicles participate in the reverse process, namely, in the transport of newly synthesized molecules toward the surface of the cell. For example, coated vesicles have been described as carriers of acetylcholine receptor molecules (Bursztajn and Fischbach, 1984), casein (Franke *et al.*, 1976), fibronectin (Birchmeier *et al.*, 1982), and viral proteins (Rothman *et al.*, 1980). The main structural protein of coated vesicles, clathrin, is thought to recycle and to associate reversibly with cellular membranes such as Golgi membranes and the plasma membrane (cf. Pearse, 1976, 1980). Close to the plasma mem-

brane, coated pits and coated vesicles can be identified in contact with microfilaments (cf. Salisbury *et al.*, 1982, and Fig. 8), which is indicative of a functional cooperativity of these two cytoskeletal systems in intracellular transport. Moreover, there is recent evidence that clathrin may also shuttle between a soluble and an insoluble, probably membrane-bound state (cf. Goud *et al.*, 1985). In analogy to the microtubule and the microfilament system, it is therefore an attractive idea to study the dynamics of these processes in living cells by microinjection.

Microinjection of antibodies against clathrin seemed a promising approach. An affinity-purified polyclonal antibody against bovine brain clathrin was used to study its effect on the uptake of α_2-macroglobulin and the export of viral proteins in mouse fibroblasts (Wehland *et al.*, 1981, 1982). The antibody, in concentrations up to 4 mg/ml in the injection solution, failed to inhibit α_2-macroglobulin uptake as well as export of vesicular stomatitis virus proteins in virus-infected cells. These results were interpreted by the authors as evidence against the existence of free coated vesicles and of dynamic coated pits. However, since nothing is known about the epitopes this antibody reacted with, or the topographic distribution and functional importance of those epitopes at the clathrin molecule, such conclusions seem premature.

6. *Conclusion*

In summary, analyzing the cytoskeleton of animal cells and protozoa by microinjection has resulted in a wealth of interesting data on the expression, organization, dynamics, and function of cytoskeletal elements. The construction of fluorochrome-labeled probes with properties close to or identical with those of the endogenous counterparts, the use of specific polyclonal or monoclonal antibodies, and the adaptation of video intensifying systems and of photobleaching techniques for cell biological questions have led to rapid progress within this field. Although, originally, studies on larger objects (sea urchin eggs, ameboid cells) were favored, microinjection with glass capillaries can now be done with almost all tissue culture cells that adhere to a substratum, and almost all cells tolerate the injection process quite well.

Naturally, probing the cytoskeleton by microinjection is limited to tissue culture cells or single cells. Thus, this approach cannot answer questions not relevant for such cells. One example is the problem of the functional importance of intermediate filaments for animal cells. Microinjection experiments did not solve this problem, since intermediate filaments are probably not needed for life of individual cells, but for cytoskeletal functions in tissues and organs.

Future research in this field will probably expand into combining microinjection with immuno electron microscopy and biochemical analysis of the injected cells. Injection of mRNAs is a new means of biotechnical manipulation of cells at the translational level.

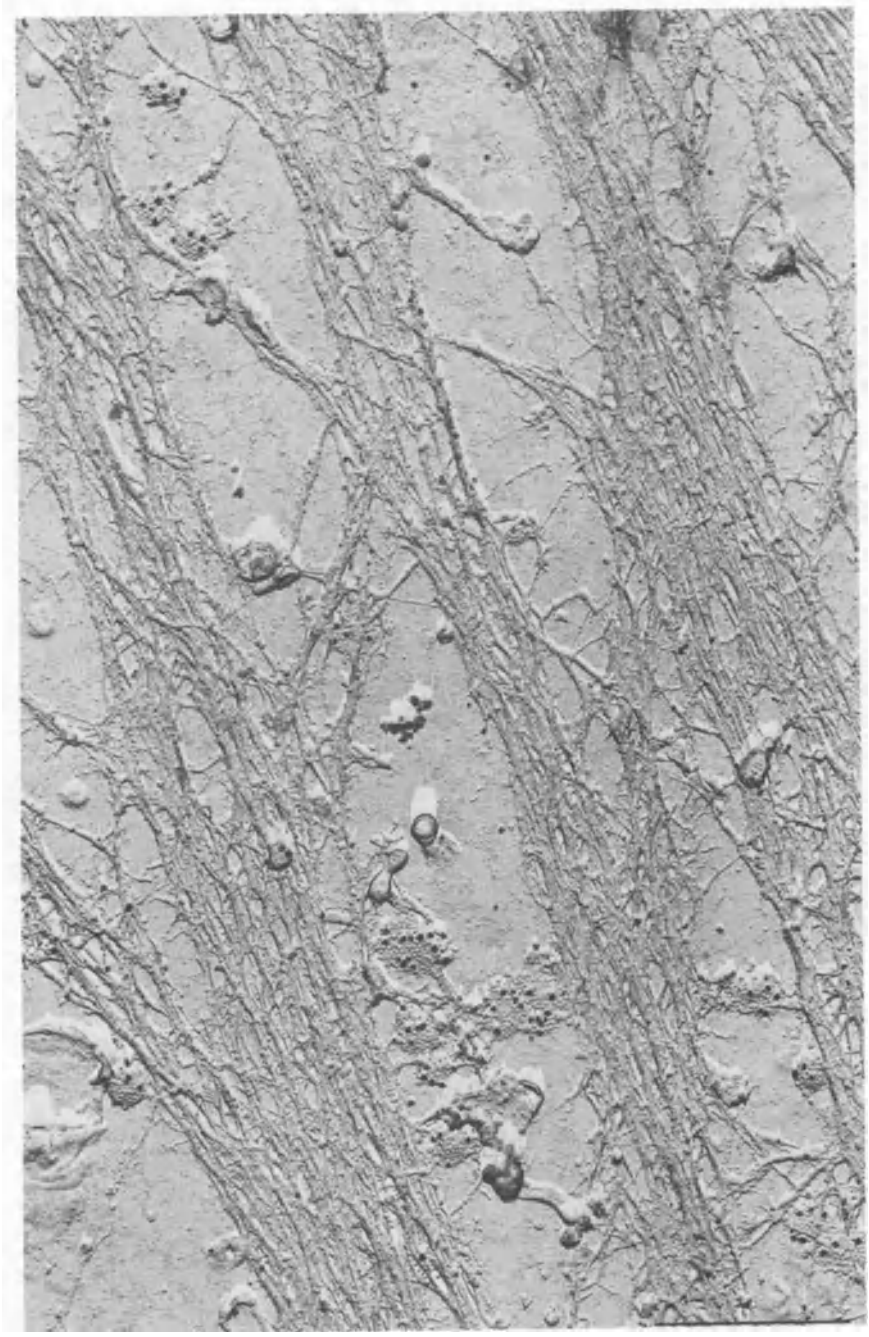


Figure 8. Platinum–carbon replica after rotary shadowing the inner face of the lower plasma membrane of a fibroblast. Stress fiber filaments are seen parallel to the plasma membrane. Coated vesicles and/or coated pits, labeled with antiserum to coated vesicles followed by protein-A-gold, are seen in close association with microfilaments (Nicol, Nermut, Doeinck, Robenek, Wiegand, and Jockusch, manuscript in preparation). Such preparations may become useful in future microinjection experiments, to examine functional relationships between microfilaments and intracellular transport. Bar: 1 μm.

ACKNOWLEDGMENTS. We thank Drs. De Brabander, Kreis, Osborn, and Wang for sending us their manuscripts prior to publication, Drs. Faulstich and Wieland for rhodamine-labeled phalloidin, and the Stiftung Volkswagenwerk and the Deutsche Forschungsgemeinschaft for generous financial support.

References

Adams, R. J., and Bray, D., 1983, Rapid transport of foreign particles microinjected into crab axons, *Nature* **303**:718.

Barak, L. S., Yocum, R. R., Nothnagel, E. A., and Webb, W. W., 1980, Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2oxa-1,3-diazole-phallacidin, *Proc. Natl. Acad. Sci. USA* **77**:980.

Beckerle, M. C., 1984, Microinjected fluorescent polystyrene beads exhibit saltatory motion in tissue culture cells, *J. Cell Biol.* **98**:2126

Beckerle, M. C., and Porter, K. R., 1982, Inhibitors of dynein activity block intracellular transport in erythrophores, *Nature* **295**:701.

Birchmeier, W., Libermann, T. A., Imhof, B. A., and Kreis, T. E., 1982, Intracellular and extracellular components involved in the formation of ventral surfaces of fibroblasts, *Cold Spring Harbor Symp. Quant. Biol.* **46**:755.

Blose, S. H., Meltzer, D. I., and Feramisco, J. R., 1984, 10 nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin, *J. Cell Biol.* **98**:847.

Bravo, R., Fey, S. J., Larsen, P. M., and Celis, J. E., 1982, Modification of vimentin polypeptides during mitosis, *Cold Spring Harbor Symp. Quant. Biol.* **46**:379.

Bretscher, A., and Weber, K., 1980, Villin is a major protein of the microvillus cytoskeleton which binds both G- and F-actin in a calcium-dependent manner, *Cell* **20**:839.

Bretscher, A., Osborn, M., Wehland, J., and Weber, K., 1981, Specific microfilamentous structures as seen by immunofluorescence microscopy on tissue sections and cells microinjected with villin, *Exp. Cell Res.* **135**:213.

Buckley, I., and Stewart, M., 1983, Ciliary but not saltatory movements are inhibited by vanadate microinjected into living cultured cells, *Cell Motil.* **3**:167.

Burridge, K., and Feramisco, J. R., 1980, Microinjection and localization of a 130 K protein in living fibroblasts: A relationship to actin and fibronectin, *Cell* **19**:587.

Burridge, K., Kelley, T., and Mangeat, P., 1982, Non-erythrocyte spectrins: actin membrane attachment proteins occurring in many cell types, *J. Cell Biol.* **95**:478.

Bursztajn, S., and Fischbach, G. D., 1984, Evidence that coated vesicles transport acetylcholine receptors to the surface membrane of chick myotubes, *J. Cell Biol.* **98**:498.

Chaponnier, Ch., Patebex, P., and Gabbiani, G., 1985, Human plasma actin-depolymerizing factor. Purification, biological activity and localization in leukozytes and platelets, *Eur. J. Biochem.* **146**:267.

Cleveland, D. W., and Kirschner, M. W., 1982, Autoregulatory control of the expression of α- and β-tubulins:implications for microtubule assembly, *Cold Spring Harbor Symp. Quant. Biol.* **44**:171.

Cleveland, D. W., Pittenger, M. F., and Feramisco, J. R., 1983, Elevation of tubulin levels by microinjection suppresses new tubulin synthesis, *Nature* **305**:738.

Clark, T. G., and Rosenbaum, J. C., 1979, An actin filament matrix in hand isolated nuclei of *X. laevis* oocytes, *Cell* **18**:1101.

Comings, D. E., and Harris, D. C., 1976, Nuclear proteins, II. Similarity of nonhistone proteins in nuclear sap and chromatin, and essential absence of contractile proteins from mouse liver nuclei, *J. Cell Biol.* **70**:440.

Craig, S. W., and Pollard, T. D., 1982, Actin binding proteins, *Trends Biochem. Sci.* **7**:88.

Daley, R. A., and Eckert, B. S., 1980, Microinjection of actin antibody into living PtK_1 cells and its effect on microfilaments, *J. Cell Biol.* **87**:221a.

De Brabander, M., De Mey, J., Geuens, R., Nuydens, F., Aerts, F., Willebrords, R., and Moeremans, R., 1985a, Microtubules in cell organization and motility, in: *Hormones and Cell Regulation* (J. E. Dumont and J. Nunez, eds.), Vol. 9, pp. 85–104, Elsevier Science, Amsterdam.

De Brabander, M., Geuens, G., Nuydens, R. Moeremans, M., and De Mey, J., 1985b, Probing microtubule-dependent intracellular motility with nanometer particle video ultra microscopy (Nanovid Ultramicroscopy), *Cytobios* **43**:273.

Dulbecco, R., Unger, M., Bologna, M., Battifora, A., Syka, P., and Okada, S., 1981, Crossreactivity between thy-1 and a component of intermediate filaments demonstrated using a monoclonal antibody, *Nature* **292**:772.

Dustin, P., 1985, *Microtubules,* 2nd ed. Springer, Berlin.

Eckert, B. S., and Daley, R. A., 1980a, Analysis of intermediate filament function in cultured mammalian cells by antibody microinjection, *Eur. J. Cell Biol.* **22**:379.

Eckert, B. S., and Daley, R. A., 1980b, Dissassembly of cytokeratin filaments in living PtK_1 cells by microinjection of antibody, *J. Cell Biol.* **87**:218a.

Eckert, B. S., Daley, R. A., and Parysek, L. M., 1982, *In vivo* disruption of the cytokeratin cytoskeleton in cultured epithelial cells by microinjection of antikeratin: Evidence for the presence of an intermediate-filament-organization center, *Cold Spring Harbor Symp. Quant. Biol.* **46**:403.

Egly, J. M., Miyamoto, N., Moncollin, V., and Chambon, P., 1984, Is actin a transcription initiation factor for RNA polymerase B? *EMBO J.* **3**:2363.

Feramisco, J. R. 1979, Microinjection of fluorescently labeled alpha actinin into living fibroblasts, *Proc. Natl. Acad. Sci. USA* **76**:3967.

Feramisco, J. R., and Blose, S. H., 1980, Distribution of fluorescently labeled alpha actinin in living and fixed fibroblasts, *J. Cell Biol.* **86**:608.

Feramisco, J. R., Smart, J. E., Burridge, K., Helfmann, D. M., and Thomas, G. P., 1982, Coexistence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle, *J. Biol. Chem.* **257**:11024.

Forman, D. S., 1982, Vanadate inhibits saltatory organelle movement in a permeabilized cell model, *Exp. Cell Res.* **141**:139.

Franke, W. W., Lüder, M. R., Kartenbeck, J., Zerban, H., and Keenan, T. W., 1976, Involvement of vesicle coat material in casein secretion and surface regeneration, *J. Cell Biol.* **69**:173.

Franke, W. W., Schmid, E., Schiller, D. L., Winter, S., Jarasch, E. D., Moll., R., Denk, H., Jackson, B. W., and Illmensee, K., 1982, Differentiation-related expression of proteins of intermediate-size filaments in tissues and cultured cells, *Cold Spring Harbor Symp. Quant. Biol.* **46**:431.

Füchtbauer, A., 1984, Analyse des Mikrofilamentsystems tierischer Zellen durch Mikroinjektion, Ph.D. thesis, University of Bielefeld, Bielefeld, Federal Republic of Germany.

Füchtbauer, A., and Jockusch, B. M., 1984, Vinculin as a functional component of focal contacts in tissue culture cells, *J. Submicrosc. Cytol.* **16**:109.

Füchtbauer, A., Jockusch, B. M., Maruta, H., Kilimann, M. W., and Isenberg, G., 1983, Disruption of microfilament organization after injection of F-actin capping proteins into living tissue culture cells, *Nature* **304**:361.

Füchtbauer, A., Herrmann, M., Mandelkow, E. M., and Jockusch, B. M., 1985, Disruption of microtubules in living cells and cell models by high affinity antibodies to beta-tubulin, *EMBO J.*, **4**:2807.

Fukui, Y., 1978, Intranuclear actin bundles induced by dimethyl sulfoxide in interphase nucleus of Dictyostelium, *J. Cell Biol.* **76**:146.

Fukui, Y., and Katsumaru, H., 1979, Nuclear actin bundles induced by dimethyl sulfoxide in Amoeba proteus and human HeLa cells, *Exp. Cell Res.* **120**:451.

Fukui, A., and Katsumaru, H., 1980, Dynamics of nuclear actin bundle formation by dimethyl sulfoxide and factors affecting its development, *J. Cell Biol.* **84**:131.

Gabbiani, G., Montesano, R., Tuchweber, B., Salas, M., and Orci, L., 1975, Phalloidin-induced hyperplasia of actin filaments in rat hepatocytes, *Lab. Invest.* **33**:562.

Gaskin, F., Cantor, C. R., and Shelanski, M. L., 1974, Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules, *J. Mol. Biol.* **89:**737.

Gawlitta, W., Stockem, W., Wehland, J., and Weber, K., 1980a, Pinocytosis and locomotion of amoebae. XV. Visualization of Ca^{++}-dynamics by chlorotetracycline (CTC) fluorescence during induced pinocytosis in living *A. proteus*, *Cell Tissue Res.* **213:**9.

Gawlitta, W., Stockem, W., Wehland, J., and Weber, K. 1980b, Organization and spatial arrangement of fluorescein-labeled native actin microinjected into normal locomoting and experimentally influenced *A. proteus*, *Cell Tissue Res.* **206:**181.

Gawlitta, W., Osborn, M., and Weber, K., 1981a, Coiling of intermediate filaments induced by microinjection of a vimentin-specific antibody does not interfere with locomotion and mitosis, *Eur. J. Cell Biol.* **26:**83.

Gawlitta, W., Stockem, W. and Weber, K. 1981b, Visualization of actin polymerization and depolymerization cycles during polyamine-induced cytokinesis in living *Amoeba proteus*, *Cell Tissue Res.* **215:**249.

Geiger, B., 1979, A 130 K protein from chicken gizzard: Its localization at the termini of microfilament bundles in cultured chicken cells, *Cell* **18:**193.

Geiger, B., 1982, Microheterogeneity of avian and mammalian vinculin. Distinctive subcellular distribution of different isovinculins, *J. Mol. Biol.* **159:**685.

Geiger, B., and Singer, S. J., 1980, Association of microtubules and intermediate filaments in chicken gizzard cells as detected by double immunofluorescence, *Proc. Natl. Acad. Sci. USA* **77:**476.

Geiger, B., Kreis, T. E., Gigi, O., Schmid, E., Mittnacht, S., Jorcano, J. L., von Bassewitz, D. B., and Franke, W. W., 1984a, Dynamic rearrangements of cytokeratins in living cells, in: *Cancer Cells: The Transformed Phenotye*, pp. 201–215. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Geiger, B., Avnur, Z., Kreis, T. E., and Schlessinger, J., 1984b, The dynamics of cytoskeletal organization in areas of cell contact, in: *Cell and Muscle Motility* (J. W. Shay, ed.), Vol. 5, pp. 195–234, Plenum Press, New York.

Glacy, S. D., 1983a, Pattern and time course of rhodamine-actin incorporation in cardiac myocytes, *J. Cell Biol.* **96:**1164.

Glacy, S. D., 1983b, Subcellular distribution of rhodamine-actin microinjected into living fibroblastic cells, *J. Cell Biol.* **97:**1207.

Glenney, J. R., Glenney, P., and Weber, K., 1982, Erythroid spectrin, brain fodrin and intestinal brush border proteins (TW 260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit, *Proc. Natl. Acad. Sci. USA* **79:**4002.

Goldberg, A. L., and Dice, J. F., 1974, Intracellular protein degradation in mammalian and bacterial cells, Part 1, *Annu. Rev. Biochem.* **43:**835.

Goldberg, A. L., and St. John, A. C., 1976, Intracellular protein degradation in mammalian and bacterial cells, Part 2., *Annu. Rev. Biochem.* **45:**747.

Goldstein, J. L., Anderson, R. G. W., and Brown, M. S., 1979, Coated pits, coated vesicles and receptor-mediated endocytosis, *Nature* **279:**679.

Goldstein, L. R., Rubin, R., and Ko, C., 1977, The presence of actin in nuclei: a critical appraisal, *Cell* **12:**601.

Götz von Olenhusen, K., and Wohlfarth-Bottermann, K. E., 1979, Evidence for actin transformation during the contraction–relaxation cycle of cytoplasmic acto-myosin: Cycle blockade by phalloidin injection, *Cell Tissue Res.* **196:**455.

Goud, B., Huet, Ch., and Louvard, D., 1985, Assembled and unassembled pools of clathrin: A quantitative study using an enzyme immunoassay, *J. Cell Biol.* **100:**521.

Gounon, P., and Karsenti, E., 1981, Involvement of contractile proteins in the changes in consistency of oocyte nucleoplasm of the newt *Pleurodeles waltlii*, *J. Cell Biol.* **88:**410.

Hamaguchi, Y., and Mabuchi, I., 1982, Effects of phalloidin microinjection and localization of fluorescein-labeled phalloidin in living sand dollar eggs, *Cell Motil.* **2:**103.

Hamaguchi, Y., Toriyma, M., Sakai, H., and Hiramoto, Y., 1985, Distribution of fluorescently labeled tubulin injected into sand dollar eggs from fertilization through cleavage, *J. Cell Biol.* **100**:1262.

Harris, H. E., and Gooch, J., 1981, An actin-depolymerizing protein from pig plasma, *FEBS Lett.* **123**:49.

Harris, H. E., Bamburg, J. R., and Weeds, A. G., 1980, Actin filament disassembly in blood plasma, *FEBS Lett.* **121**:175.

Henderson, D., and Weber, K., 1981, Immuno electron microscopical identification of the two types of intermediate filaments in established epithelial cells, *Exp. Cell Res.* **132**:297.

Hendricks, M., and Weintraub, H., 1981, Tropomyosin is decreased in transformed cells, *Proc. Natl. Acad. Sci. USA* **78**:5633.

Hesterberg, L. K., and Weber, K., 1983, Demonstration of three distinct calcium binding sites in villin, a modulator of actin assembly, *J. Biol. Chem.* **258**:365.

Hill, T., and Kirschner, M. W., 1982, Bioenergetics and kinetics of microtubule and actin filament assembly-disassembly, *Int. Rev. Cytol.* **78**:1.

Hiller, G., and Weber, K., 1978, Radioimmune assay for tubulin:a quantitative comparison of the tubulin content of different established tissue culture cells and tissues, *Cell* **14**:795.

Hirokawa, N., 1982, Cross-linker system between neurofilaments, microtubules and membraneous organelles in frog axons revealed by the quick-freeze, deep etching method, *J. Cell Biol.* **94**:129.

Hitchcock, S. E., Carlsson, L., and Lindberg, U., 1976, DNAase I-induced depolymerization of actin filaments, in: *Cell Motility* (R. Goldman, T. Pollard, and J. Rosenbaum, eds.), pp. 545–559, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hoffmann, H. U., Stockem, W., and Gruber, B., 1984, Dynamics of the cytoskeleton in *A. proteus:* II. Influence of different agents on the spatial organization of microinjected fluorescein-labeled actin, *Protoplasma* **119**:79.

Höner, B., Füchtbauer, A., and Jockusch, B. M., 1985, Effects of microinjected anti-myosin on microfilament organization and intracellular motility in tissue culture cells, *Eur. J. Cell Biol.* **36**:28.

Howe, C. L., Mooseker, M. S., and Graves, T. A., 1980, Brush-border calmodulin: A major component of the isolated microvillus core, *J. Cell Biol.* **85**:916.

Jockusch, B. M., 1983, Patterns microfilament organization in animal cells, *Mol. Cell. Endocrinol.* **29**:1.

Jockusch, B. M., and Füchtbauer, A., 1983, Organization and function of structural elements in focal contacts of tissue culture cells, *Cell Motil.* **3**:391.

Jockusch, B. M., and Füchtbauer, A., 1985, Funktionsanalyse zellulärer Strukturproteine durch Mikroinjektion, in: *Molekular und Zellbiologie, Aktuelle Themen* (N. Blin, M. F. Trendelenburg, and E. R. Schmidt, eds.), pp. 1–12, Springer Verlag, Berlin.

Jockusch, B. M., and Isenberg, G., 1981, Interaction of α-actinin and vinculin with actin:opposite effects on filament network formation, *Proc. Natl. Acad. Sci. USA* **78**:3005.

Jockusch, B. M., Becker, M., Hindennach, I., and Jockusch, H., 1974, Slime mould actin: Homology to vertebrate actin and presence in the nucleus, *Exp. Cell Res.* **89**:241.

Jockusch, B. M., Haemmerli, G., and In Albon, A., 1983, Cytoskeletal organization in locomoting cells of the V2 rabbit carcinoma, *Exp. Cell Res.* **144**:251.

Jockusch, B. M., Füchtbauer, A., Wiegand, Ch., and Höner, B., 1985a, Dynamics of microfilament organization and membrane anchorage, in: *Hormones and Cell Regulation* (J. E. Dumont, and J. Nunez, eds.), Volume 9, pp. 135–142, Elsevier, Amsterdam.

Jockusch, B. M., Huckriede, A., Wiegand, C., and Füchtbauer, A., 1985b, Structural proteins involved in cellular motility as studied by microinjection, *Exp. Biol. Med.* **10**:69–80.

Karsenti, E., Gounon, P., and Bornens, M., 1978, Immunocytochemical study of lampbrush chromosomes: Presence of tubulin and actin, *Biol. Cell.* **31**:219.

Keith, Ch. H., Feramisco, J. R., and Shelanski, M. 1981, Direct visualization of fluorescein-labeled microtubules *in vitro* and in microinjected fibroblasts, *J. Cell Biol.* **88**:234.

Kiehart, D. P., 1981, Studies on the *in vivo* sensibility of spindle microtubules to calcium ions and evidence for a vesicular calcium-sequestering system, *J. Cell Biol.* **88**:604.

Kiehart, D. P., Mabuchi, I., and Inoue, S., 1982, Evidence that myosin does not contribute to force production in chromosome movement, *J. Cell Biol.* **94**:165.

Kilimann, M. W., and Isenberg, G., 1982, Actin filament capping protein from mammalian brain, *EMBO J.* **1**:889.

Kilmartin, J. V., Wright, B., and Milstein, C., 1982, Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line, *J. Cell Biol.* **93**:576.

Kirschner, M. W., 1978, Microtubule assembly and nucleation. *Int. Rev. Cytol.* **58**:1.

Klymkowsky, M. W., 1981, Intermediate filaments in 3T3 cells collapse after intracellular injection of a monoclonal antiintermediate filament antibody, *Nature* **291**:249.

Klymkowsky, M. W., 1982, Vimentin and keratin intermediate filament systems in cultured PtK_2 epithelial cells are interrelated, *EMBO J.* **1**:161.

Klymkowsky, M. W., Miller, R. H., and Lane, E. B., 1983, Morphology, behaviour and interaction of cultured epithelial cells after the antibody-induced disruption of keratin filament organization, *J. Cell Biol.* **96**:494.

Korn, E. D., 1982, Actin polymerization and its regulation by proteins from nonmuscle cells, *Physiol. Rev.* **62**:672.

Kreis, T. E., and Birchmeier, W., 1982, Microinjection of fluorescently labeled protein into living cells with emphasis on cytoskeletal proteins, *Int. Rev. Cytol.* **75**:209.

Kreis, T. E., Winterhalter, K. H., and Birchmeier, W., 1979, *In vivo* distribution and turnover of fluorescently labelled actin microinjected into human fibroblasts, *Proc. Natl. Acad. Sci. USA* **76**:3814.

Kreis, T. E., Geiger, B., and Schlessinger, J., 1982, Mobility of microinjected rhodamine actin within living chicken gizzard cells determined by fluorescence photobleaching recovery, *Cell* **29**:835.

Kreis, T. E., Geiger, B., Schmid, E., Jorcano, J. C., and Franke, W. W., 1983, *De novo* synthesis and specific assembly of keratin filaments in non-epithelial cells after microinjection of mRNA for epidermal keratin, *Cell* **32**:1125.

Kreis, T. E., Avnur, Z., Schlessinger, J., and Geiger, B., 1984, Dynamic properties of cytoskeletal proteins in focal contacts, in: *Molecular Biology of the Cytoskeleton* (G. G. Borisy, D. W. Cleveland, and D. B. Murply, eds.), pp. 45–57, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Krohne, G., and Franke, W. W., 1980, A major soluble acidic protein located in nuclei of diverse vertebrate species, *Exp. Cell Res.* **129**:167.

Kukulies, J., and Stockem, W., 1985, Morphology and dynamics of the microfilament system in cell fragments of *Physarum polycephalum*, *Eur. J. Cell Biol.* **36**:39.

Lane, E. B., and Klymkowsky, M. W., 1982, Epithelial tonofilaments: investigating their form and function using monoclonal antibodies, *Cold Spring Harbor Symp. Quant. Biol.* **46**:387.

Lazarides, E., 1976a, Actin, alpha actinin and tropomyosin interaction in the structural organization of actin filaments in non-muscle cells, *J. Cell Biol.* **68**:202.

Lazarides, E., 1976b, Two general classes of cytoplasmic actin filaments in tissue culture cells: The role of tropomyosin, *J. Supramol. Struct.* **5**:531.

Lazarides, E., 1982, Intermediate filaments, a chemically heterogenous, developmentally regulated class of proteins, *Annu. Rev. Biochem.* **51**:219.

Lazarides, E., and Burridge, K. 1975, Alpha actinin, immunofluorescence localization of a muscle structural protein in nonmuscle cells, *Cell* **6**:289.

Lazarides, E., and Lindberg, U., 1974, Actin is the naturally occurring inhibitor of deoxyribonuclease I, *Proc. Natl. Acad. Sci. USA* **71**:4742.

Leonardi, C. L., Warren, R. H., and Rubin, R. W., 1982, Lack of tropomyosin correlates with the absence of stress fibers in transformed rat kidney cells, *Biochim. Biophys. Acta* **720**:154.

Leslie, R. J., Saxton, W. M., Neighburs, B., Salmon, E. D., and McIntosh, J. R., 1983, Biochemical characterization of fluorescein (DTAF) bovine brain tubulin, *J. Cell Biol.* **97**:192a.

LeStourgeon, W. M., 1978, The occurrence of contractile proteins in nuclei and their possible functions, in: *The Cell Nucleus*, Vol. VI, pp. 305–326, Academic Press, New York.

Lin, J. J., and Feramisco, J. R., 1981, Disruption of the *in vivo* distribution of the intermediate filaments in fibroblasts through the microinjection of a specific monoclonal antibody, *Cell* **24:**185.

Littauer, U. Z., and Ginzburg, I., 1984, Expression of microtubule proteins in brain, in: *Gene Expression in Brain* (C. Zomzely-Neurath, and W. A. Walker, eds.), John Wiley, New York.

Mabuchi, I., and Okuno, M., 1977, The effect of myosin antibody on the division of starfish blastomeres, *J. Cell Biol.* **74:**251.

Mandelkow, E. M., Herrmann, M., Füchtbauer, A., and Jockusch, B. M., 1983, Structural polymorphism and disassembly of microtubules induced by antibodies specific for alpha or beta tubulin, *Eur. J. Cell Biol.* **30:**25.

Mandelkow, E. M., Herrmann, M., and Rühl, U., 1985a, Tubulin domains probed by limited proteolysis and subunit-specific antibodies, *J. Mol. Biol.* **185:**311.

Mandelkow, E. M., Herrman, M., and Rühl, U., 1985b, The domain structure of tubulin probed by controlled proteolysis and antibody labeling, *Eur. J. Cell Biol.* **36:**42.

Mangeat, P., and Burridge, K., 1984, Immuno-precipitation of nonerythrocyte spectrin within live cells following microinjection of specific antibodies:relation to cytoskeletal structures, *J. Cell Biol.* **98:**1363.

Maruta, H., Isenberg, G., Schreckenbach, T., Hallmann, R. Risse, G., Schibayama, T., and Hesse, T., 1983, Ca-dependent actin-binding phosphoprotein in *Physarum polycephalum*, I. Ca/actin-dependent inhibition of its phosphorylation, *J. Biol. Chem.* **258:**10144.

Maruyama, K., and Sakai, H., 1981, Cell β-actinin, an accelerator of actin polymerization, isolated from rat kidney cytosol, *J. Biochem. (Tokyo)* **89:**1337.

Matsudaira, P., and Burgess, D. R., 1982, Partial reconstruction of the microvillus core bundle: characterization of villin as a Ca^{++}-dependent, actin-bundling/depolymerizing protein, *J. Cell Biol.* **92:**648.

McKenna, N. M., Wang, Y. L., and Meigs, J. B., 1984, Motility of fluorescently labeled alpha actinin in muscle and nonmuscle cells, *J. Cell Biol.* **99:**310a.

McKenna, N., Meigs, J. B., and Wang, Y. L., 1985, Identical distribution of fluorescently labeled brain and muscle actins in living cardiac fibroblasts and myocytes. *J. Cell Biol.* **100:**292.

Meeusen, R. L., Bennett, J., and Cande, Z., 1980, Effect of microinjected *N*-ethylmaleimide-modified heavy meromyosin on cell division in amphibian eggs, *J. Cell Biol.* **86:**858.

Neff, N. T., Bourrest, L., Miao, P., and Dice, F., 1981, Degradation of proteins microinjected into IMR-90 human diploid fibroblasts, *J. Cell Biol.* **91:**184.

Osborn, M., Franke, W. W., and Weber, K., 1980, Direct demonstration of the presence of two immunologically distinct intermediate-size filament systems in the same cell by double immunofluorescence microscopy. Vimentin and cytokeratin in cultured epithelial cells, *Exp. Cell Res.* **125:**37.

Payne, M. R., and Rudnick, S. E., 1984, Tropomyosin as a modulator of microfilaments, *Trends Biochem. Sci.* **9:**361.

Pearse, B. N. F., 1976, Clathrin, a unique protein associated with intracellular transfer of membrane by coated vesicles, *Proc. Natl. Acad. Sci. USA* **73:**1255.

Pearse, B. M. F., 1980, Coated vesicles, *Trends Biochem. Sci.* **5:**131.

Rothman, J., Bursztyn-Pettigrew, H., and Fine, R. E., 1980, Transport of the membrane protein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles, *J. Cell Biol.* **86:**162.

Rubenstein, N., Chi, J., and Holtzer, H., 1976, Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and nonmyogenic cells, *Exp. Cell Res.* **97:**387.

Rungger, D., Rungger-Brändle, E., Chaponnier, C., and Gabbiani, G., 1979, Intranuclear injection of anti-actin antibodies into Xenopus oocytes blocks chromosome condensation, *Nature* **282:**320.

Salisbury, J. L., Condeelis, J. S., Maihle, N. J., and Satir, P., 1982, Receptor-mediated endocytosis by clathrin-coated vesicles: evidence of a dynamic pathway, *Cold Spring Harbor Symp. Quant. Biol.* **46:**733.

Salmon, E. D., Saxton, W. M., Leslie, R., Karrow, M. L., and McIntosh, J. R., 1983, Measurements

of spindle microtubule dynamics by fluorescence redistribution after photobleaching, *J. Cell Biol.* **97**:253a.

Sanger, J. W., 1975, Changing patterns of actin localization during cell division, *Proc. Natl. Acad. Sci. USA* **72**:1913.

Sanger, J. W., Sanger, J. M., Kreis, T. E., and Jockusch, B. M., 1980a, Reversible translocation of cytoplasmic actin into the nucleus caused by dimethyl sulfoxide, *Proc. Natl. Acad. Sci. USA* **77**:5268.

Sanger, J. W., Gwinn, J., and Sanger, J. M., 1980b, Dissolution of cytoplasmic actin bundles and the induction of nuclear actin bundles by dimethyl sulfoxide, *J. Exp. Zool.* **213**:227.

Sanger, J. W., Sanger, J. M., and Jockusch, B. M., 1983a, Differences in stress fibers between fibroblastic and epithelial cells, *J. Cell Biol.* **96**:961.

Sanger, J. W., Sanger, J. M., and Jockusch, B. M., 1983b, Differential response of three types of actin filament bundles to depletion of cellular ATP levels, *Eur. J. Cell Biol.* **31**:197.

Sanger, J. W., Mittal, B., and Sanger, J. M., 1984a, Interaction of fluorescently-labeled contractile proteins with the cytoskeleton in cell models, *J. Cell Biol.* **99**:918.

Sanger, J. W., Mittal, B., and Sanger, J. M., 1984b, Analysis of myofibrillar structure and assembly using fluorescently labeled contractile proteins, *J. Cell Biol.* **98**:825.

Saxton, W. M., Leslie, R. J., Salmon, E. D., Zavortink, M., and McIntosh, J. R., 1983, Tubulin dynamics *in vivo*, *J. Cell Biol.* **97**:192a.

Scheer, U., Hinssen, H., Franke, W. W., and Jockusch, B. M., 1984, Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes, *Cell* **39**:111.

Scherson, T., Kreis, T. E., Schlessinger, J. Littauer, U. Z., Borisy, G. G., and Geiger, B., 1984, Dynamic interactions of fluorescently labeled microtubuli-associated protein in living cells, *J. Cell Biol.* **99**:425.

Schliwa, M., and Van Blerkom, J., 1981, Structural interaction of cytoskeletal components, *J. Cell Biol.* **90**:222.

Schroeder, T. E., 1975, Dynamics of the contractile ring, in: *Molecules and Cell Movement* (S. Inoue and R. E. Stephens, eds.), pp. 334–352, Raven press, New York.

Siciliano, J. D., and Craig, S. W., 1982, Metavinculin: A vinculin-related protein with solubility properties of a membrane protein, *Nature* **300**:533.

Smith, S. S., Kelley, K. H., and Jockusch, B. M., 1979, Actin copurifies with RNA polymerase II, *Biochem. Biophys. Res. Commun.* **86**:161.

Southwick, F. S., and Hartwig, J. H., 1982, Acumentin, a protein in macrophages which caps the pointed end of actin filaments, *Nature* **297**:303.

Stacey, D. W., and Allfrey, V. G., 1977, Evidence for the autophagy of microinjected proteins in HeLa cells, *J. Cell Biol.* **75**:807.

Stockem, W., and Gruber, B., 1983, Dynamics of the cytoskeleton in *Amoeba proteus:* I. Redistribution of microinjected fluorescein-labeled actin during locomotion, immobilization and phagocytosis, *Cell Tissue Res.* **232**:79.

Stockem, W., Weber, K., and Wehland, J., 1978, The influence of microinjected phalloidin on locomotion, protoplasmic streaming and cytoplasmic organization in *Amoeba proteus* and *Physarum polycephalum*, *Cytobiologie* **18**:114.

Taylor, D. L., and Wang, Y. L., 1978, Molecular cytochemistry: incorporation of fluorescently labeled actin into living cells, *Proc. Natl. Acad. Sci. USA* **75**:857.

Taylor, D. L., and Wang, Y. L., 1980, Fluorescently labeled molecules as probes of the structure and functions of living cells, *Nature* **284**:405.

Taylor, D. L., Wang, Y. L., and Heiple, J. M., 1980a, Contractile basis of amoeboid movement. VII. The distribution of fluorescently labeled actin in living amoebas, *J. Cell Biol.* **86**:590.

Taylor, D. L., Blinks, J. R., and Reynolds, G., 1980b, Contractile basis of amoeboid movement. VIII. Aequorin luminescence during amoeboid movement, endocytosis and capping, *J. Cell Biol.* **86**:599.

Thompson, W. C., 1982, The cyclic tyrosination/detyrosination of alpha-tubulin, *Methods Cell Biol.* **24**:235.

Tilney, L. G., Bonder, E. M., and DeRosier, D. J., 1981, Actin filaments elongate from their membrane associated ends, *J. Cell Biol.* **90:**485.

Timasheff, S. N., and Grisham, L. M., 1980, *In vitro* assembly of cytoplasmic microtubules, *Ann. Rev. Biochem.* **49:**565.

Tölle, H. G., and Osborn, M., 1985, Microinjection of monoclonal antibodies specific for a single IF polypeptide into living cells, *Eur. J. Cell Biol.* **36:**67.

Tölle, H. G., Weber, K., and Osborn, M., 1985, Microinjection of monoclonal antibodies specific for one intermediate filament protein in cells containing multiple keratins allow insight into the composition of particular 10 nm filaments, *Eur. J. Cell Biol.* **38:**234

Vandenbunder, B., and Borisy, G. G., 1983, Fluorescently labeled MAPs do not perturb mitosis when microinjected in PtK_1 cells, although they bind to microtubules, *J. Cell Biol.* **97:**192a.

Wadsworth, P., and Sloboda, R. D., 1983, Microinjection of fluorescent tubulin into dividing sea urchin cells, *J. Cell Biol.* **97:**1249.

Wang, Y. L., 1984, Reorganization of actin filament bundles in living fibroblasts, *J. Cell Biol.* **99:**1478.

Wang, Y. L., 1985, Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling, *J. Cell Biol.* **101:**597.

Wang, Y. L., and Taylor, D. L., 1979, Distribution of fluorescently labeled actin in living sea urchin eggs during early development, *J. Cell Biol.* **82:**672.

Wang, Y. L., and Taylor, D. L., 1980, Preparation and characterization of a new molecular cytochemical probe: 5-Iodoacetamido fluorescein labeled actin, *J. Histochem. Cytochem.* **28:**1198.

Wang, Y. L., McKenna, N. M., and Beigs, J. B., 1985, How actin filaments reorganize in living cells—Studies of microinjected structural components, *Eur. J. Cell Biol.* **36:**70.

Wanger, M., and Wegner, A., 1985, Equilibrium constant for binding of an actin filament binding protein to the barbed end of actin filaments, *Biochemistry* **24:**1035.

Warren, R. H., Azarnia, R., and Gordon, E., 1983, Microinjection of tropomyosin and anti-tropomyosin into normal and transformed cells, *J. Cell Biol.* **97:**277a.

Weeds, A., 1982, Actin-binding proteins, regulators of cell architecture and motility, *Nature* **296:**811.

Wegner, A., 1976, Head to tail polymerization of actin, *J. Mol. Biol.* **108:**139.

Wegner, A., 1982, Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments, *J. Mol. Biol.* **161:**217.

Wehland, J., and Weber, K., 1980, Distribution of fluorescently labeled actin and tropomyosin after microinjection in living tissue culture cells as observed with TV image intensification, *Exp. Cell Res.* **127:**397.

Wehland, J., and Weber, K. 1981, Actin rearrangement in living cells revealed by microinjection of a fluorescent phalloidin derivative, *Eur. J. Cell Biol.* **24:**176.

Wehland, J., and Willingham, M. C.,1983, A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. II. effects on cell movement, organization of microtubules and intermediate filaments, and arrangement of Golgi elements, *J. Cell Biol.* **97:**1476.

Wehland, J., Osborn, M., and Weber, K., 1977, Phalloidin-induced actin polymerization in the cytoplasm of cultured cells interferes with cell locomotion and growth, *Proc. Natl. Acad. Sci. USA* **74:**5613.

Wehland, J., Weber, K., Gawlitta, W., and Stockem, W., 1979, Effects of the actin-binding protein DNAase I on cytoplasmic streaming and ultrastructure of Amoeba proteus, *Cell Tissue Res.* **199:**353.

Wehland, J., Osborn, M., and Weber, K., 1980a, Phalloidin associates with microfilaments after microinjection into tissue culture cells, *Eur. J. Cell Biol.* **21:**188.

Wehland, J., Weber, K., and Osborn, M., 1980b, Translocation of actin from the cytoplasm into the nucleus in mammalian cells exposed to dimethyl sulfoxide, *Biol. Cellul.* **39:**109.

Wehland, J., Willingham, M. C., Dickson, R., and Pastan, I., 1981, Microinjection of anticlathrin antibodies into fibroblasts does not interfere with the receptor-mediated endocytosis of α_2-macroglobulin, *Cell* **25:**105.

Wehland, J., Willingham, M. C., Gallo, M. G., Rutherford, A. V., Rudnick, J., Dickson, R. B., and Pastan, I., 1982, Microinjection of anticlathrin antibodies into cultured fibroblasts: Clathrin-coated structures in receptor-mediated endocytosis and exocytosis, *Cold Spring Harbor Symp. Quant. Biol.* **46:**743.

Wehland, J., Willingham, M. C., and Sandoval, I. V., 1983, A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. I. Biochemical characterization, effects on microtubules polymerization *in vitro*, and microtubule polymerization and organization *in vivo*, *J. Cell Biol.* **97:**1467.

Weisenberg, R. C., 1972, Microtubule formation *in vitro* in solutions containing low calcium concentrations, *Science* **177:**1104.

Wieland, T., 1975, Phallotoxins and microfilaments, in: *Molecular Basis of Motility* (L. G. Heilmeyer, J. C. Rüegg, and T. Wieland, eds.), pp. 203–214, Springer Verlag, Berlin, Heidelberg, New York.

Wilkins, J. A., and Lin, S., 1982, High-affinity interaction of vinculin with actin filaments *in vitro*, *Cell* **28:**83.

Zavortink, M., Thacher, T., and Rechsteiner, M., 1979, Degradation of proteins microinjected into cultured mammalian cells, *J. Cell. Physiol.* **100:**175.

2

Structural Aspects of Intermediate Filaments

Norbert Geisler and Klaus Weber

1. Introduction

Microtubules, microfilaments, and intermediate filaments (IF) form the three filamentous organizations of the cytoplasm. Whereas the major structural components of the two former systems—actin and tubulin—are highly conserved in different cell types, the constituent proteins of IF can vary greatly in amino acid sequence and length (40–200 K). This peculiar property led originally to much confusion as to the similarity and divergence of IF proteins. By the late 1970s it was obvious that IF proteins could be subdivided by biochemical and particularly by immunological data in a histologically meaningful manner as their expression pattern coincided with known rules of embryonic differentiation (for review see Lazarides, 1982; Osborn *et al.*, 1982). Five subclasses were identified: epithelial keratins, neuronal neurofilaments, desmin filaments of most muscles, GFAP filaments of glia, and vimentin filaments present primarily in mesenchymal cells. Subsequent biochemical results documented around 20 different human keratins some of which were again markers of morphologically distinct epithelia (for review see Moll *et al.*, 1982).

Although the names used for different IF proteins emphasized a molecular distinction particularly useful for embryologists and pathologists interested in differential gene expression patterns that parallel histologically distinct cell types, it has always been obvious that the various IF are built around a common structural principle. They share similar biochemical prop-

Norbert Geisler and Klaus Weber • Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany.

erties (a high α-helix content and a relatively low solubility), an α-type X-ray diffraction pattern (Day and Gilbert, 1972; Steinert, 1978; Steinert *et al.*, 1978, 1980; Renner *et al.*, 1981), and a common electron microscopic appearance characterized by a diameter of 7–11 nm and a lateral periodicity of 21 nm, which is seen particularly well after exposure to glycerol and metal shadowing (Aebi *et al.*, 1983; Henderson *et al.*, 1982; Milam and Erickson, 1982). Biochemical properties as well as current sequence data distinguish the epithelial keratins from the nonepithelial IF proteins in line with their distinct appearances in different cell types. The former are built as obligatory heteropolymers containing at least two complementary keratins (Lee and Baden, 1976; Steinert *et al.*, 1976; Milstone, 1981; Moll *et al.*, 1982; Fuchs and Marchuk, 1983; Franke *et al.*, 1983), while the latter group seems primarily based on proteins able to form homopolymers (Rueger *et al.*, 1979; Geisler and Weber, 1981a; Liem and Hutchinson, 1982; Renner *et al.*, 1981; Steinert *et al.*, 1981).

Here we review the common structural organization of IF proteins, with particular emphasis on nonepithelial IF. The epithelial keratins are covered in detail in two other chapters in this book (Fuchs *et al.*, Chapter 4; Roop and Steinert, Chapter 3), as is the current state of the organization of the genes for the IF proteins (same reviews and Quax and Bloemendal, Chapter 5). We shall concentrate on the actual proteins and their measurable properties and summarize data that seem to allow an understanding of the transition from the linear sequence of IF proteins via the tetrameric protofilament to the 10-nm filament proper.

2. *Domain Organization of IF Proteins*

The general organization of IF proteins was first documented in detail by the proteolytic cleavage pattern of chicken desmin and the biochemical and protein chemical characterization of the resulting domains. Mild chymotryptic digestion defined three structurally distinct domains. The largest fragment, with a polypeptide molecular weight 40 K, can be purified in urea and reconstitutes into a tetrameric rodlike structure about 50 nm long and 2–3 nm thick, which has an experimentally determined α-helical content of about 80% (Geisler *et al.*, 1982a). As this fragment displayed sequences that showed extended regions of heptade repeats, it must be built from α-helices present in coiled-coil conformation, a finding also in agreement with the α-type X-ray diffraction pattern typical of IF (see, for instance, Steinert *et al.*, 1978; Renner *et al.*, 1981). By analogy to myosin and in agreement with its morphology in the electron microscope, we called this domain the rod (Geisler *et al.*, 1982a). Its tetrameric character (Geisler and Weber, 1982; Geisler *et al.*, 1985a) excluded earlier proposals of three-stranded coiled-coils (Steinert, 1978; Steinert *et al.*, 1980, 1981) and emphasized instead the presence of two normal double-stranded coiled-coils. Sequence data showed also that the coiled-coil arrays are interrupted by short non-α-helical spacers giving rise to two

major coiled-coil units (Geisler and Weber, 1982; see also Figs. 1 and 2). The rod spans residues 74–415 of the molecule and thus covers the 310 residues structurally conserved in all IF proteins (positions 97–407 in desmin). It is flanked by two non-α-helical terminal domains. The carboxyterminal tailpiece (residues 416–463) can be isolated because it displays a moderate stability against chymotrypsin (Geisler *et al.*, 1982a; Geisler and Weber, 1982) although it shows no ordered secondary structure in circular dichroism spectra (our own unpublished observation). As the aminoterminal headpiece has many β turns, it is highly sensitive to proteases and is digested by chymotrypsin into small fragments (Geisler *et al.*, 1982a). Isolated as a CNBr fragment (residues 1–82) it shows no measurable ordered secondary structure (our own unpublished observation). As the available partial sequences of porcine vimentin, bovine GFA, the neurofilament protein NF-L, the two α-keratins from sheep wool, and one human epidermal keratin (Geisler and Weber, 1981b; Hong and Davison, 1981; Geisler *et al.*, 1982b; Crewther *et al.*, 1980; Sparrow and Inglis, 1980; Hanukoglu and Fuchs, 1982) could be aligned along the complete desmin sequence, a general IF model was reached (Geisler and Weber, 1982). A highly α-helical rod domain able to form two-stranded coiled-coils is flanked by non-α-helical terminal domains. Whereas the rod of 310 residues is highly conserved in sequence principles and in length, the terminal domains are very variable in both properties. Thus all molecular

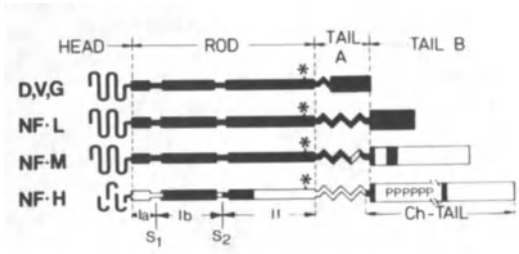


Figure 1. Schematic representation of the domain structure of nonepithelial IF proteins. The common α-helical rod domain is interrupted by the nonhelical spacers S_1 and S_2 situated between helices Ia and Ib, and Ib and II. Helices I (a + b) and II are approximately of equal size. A further nonhelical insertion could occur early in helix II (see text). The asterisk marks the approximate position of the epitope of a monoclonal antibody that recognizes all IF proteins (Pruss *et al.*, 1981; Geisler *et al.*, 1983a). The rod domains are flanked at the aminoterminal side by the nonhelical headpieces (head) and at the carboxyterminal side by the nonhelical tailpieces (tail). In the three nonneuronal proteins desmin (D), vimentin (V), and GFA (G), the chymotrypsin-resistant tailpieces (bar) are connected by short hinge regions to the rod domains. In the neurofilament triplet proteins (NF-L, NF-M, and NF-H), the chymotrypsin-resistant tailpiece extensions (tail B, Ch-tail) are connected by longer chymotrypsin-sensitive hinge regions (tail A) to the rods. The tailpieces (tail B) increase in size from NF-L to NF-M and NF-H. P in the NF-H tailpiece indicates the subdomain, which is highly phosphorylated. The tailpiece of NF-M is less heavily phosphorylated, and the phosphate sites have not yet been assigned to specific places within the domain (see text). The filled-in areas indicate established amino acid sequences (see Fig. 2 for references). Only fragments of the headpiece of NF-H are currently known. The location of the sequenced regions in the tailpiece extensions is tentative. For the short sequences from fragments of the tail domains of NF-M and NF-H and the headpiece of NF-H see Geisler *et al.* (1984, 1985b).

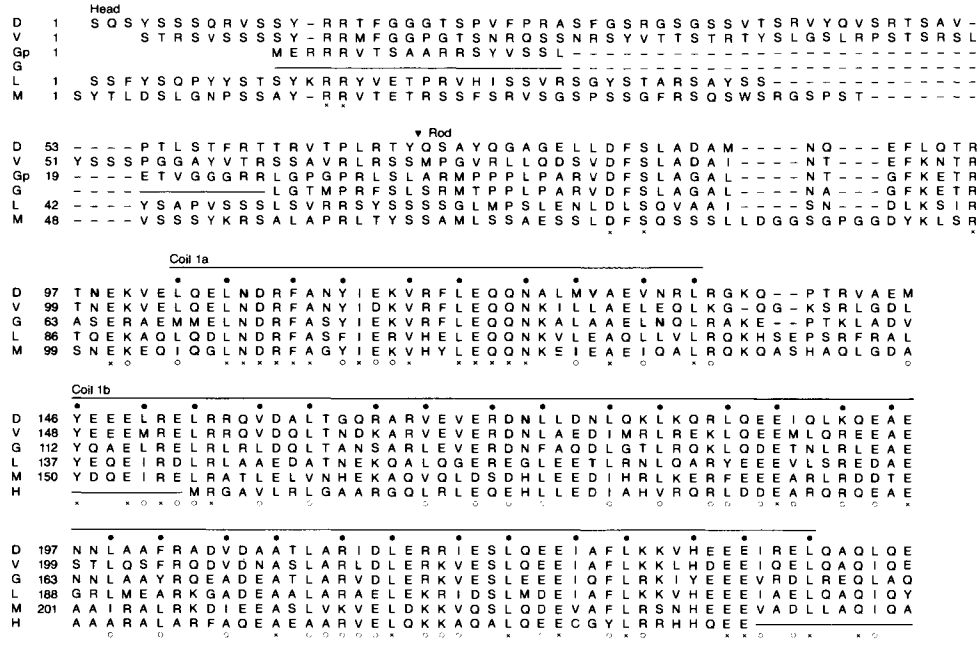
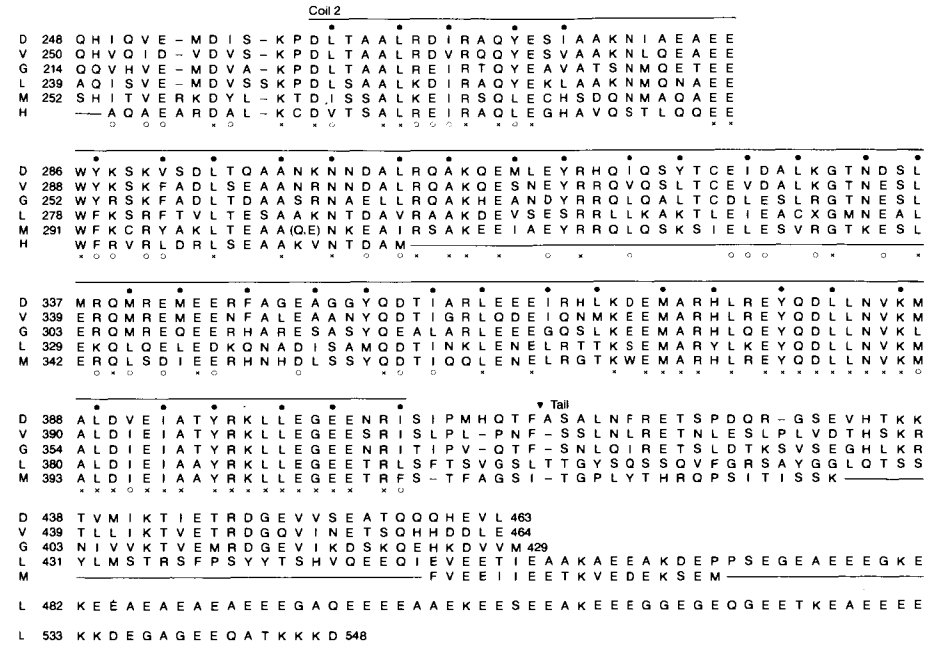


Figure 2. Sequence relationship among the six nonepithelial IF proteins. Alignment is based on previous arguments (see text). For primary sequence data see the following references (Geisler and Weber, 1982, 1983; Lewis *et al.*, 1984; Quax *et al.*, 1983; Geisler *et al.*, 1983a, 1984, 1985b,c). D, desmin (chicken); V, vimentin (hamster); G, GFA (mouse); L, M, and H, the neurofilament components (pig); G_p, the aminoterminal region of porcine GFA (Geisler and Weber, 1983). Together with the nearly complete murine DNA sequence (Lewis *et al.*, 1984) a prototype GFA sequence can be derived. Horizontal lines indicate as yet unestablished sequences. X is an arginine or lysine residue in L. The three structural domains are indicated (head, rod, tail) as are the hydrophobic *a* and *d* positions (dots) in the consecutive heptades of the presumptive coiled-coils (lines above the sequence blocks). Note some irregularities early in coil 2 and the reversal around desmin residue 342 also common to other proteins. Deletions (dashes) allow for a better

weight variability of IF proteins resides in the length of the head- and tail-pieces (see Section 8). A relatively similar view was developed independently from the nearly complete cDNA sequence of a human epidermal keratin, although the sequence comparison with α-keratins was rather limited and the coiled-coil problem was not approached directly (Hanukoglu and Fuchs, 1982).

The general model of IF protein structure that we proposed in 1982 (Geisler and Weber, 1982) has been confirmed by an avalanche of sequences developed since then. These studies also solved two of the remaining major structural problems. First, the high-molecular-weight neurofilament proteins were recognized as true IF proteins carrying particularly long tailpiece regions (Geisler *et al.*, 1983a, 1984, 1985b, 1985c; see also Fig. 1). Second, the high complexity of epithelial keratins was reduced to variations on two prototypes when the second partial cDNA sequence became available for a



alignment in the short spacer regions and to some extent in the hypervariable non-α-helical terminal domains. Along the rod length variability is very small and occurs exclusively in the short non-α-helical spacers. Between coil 1a and 1b (spacer 1) there are 13 residues in L and M and 11 in the three nonneuronal proteins. These show 21 residues between coil 1b and 2 (spacer 2) whereas L and M have 22. Crosses indicate an identical residue in all sequences. Circles mark highly homologous residues in all sequences. Note the consensus-type sequences early in coil 1 and at the carboxyl end of coil 2. For alignment of these sequences with those of epidermal keratins and α-keratins see previous references. Partial sequence data also exist for L (mouse) (Lewis and Cowan, 1985), V (pig) (Geisler and Weber, 1981b; Geisler *et al.*, 1982b, 1983b), GFA (pig) (Geisler and Weber, 1983), and D from pig (Geisler and Weber, 1981b; Geisler *et al.*, 1982b) and hamster (Quax *et al.*, 1984).

human epidermal keratin (Hanukoglu and Fuchs, 1983). This information, together with the earlier recognition of the two wool α-keratin sequences as specialized epithelial keratins (Weber and Geisler, 1982), has drawn the structural information accumulated on wool α-keratins into general models of IF structure.

The topographical model of IF proteins summarized here differs distinctly from earlier versions developed for either α-keratins (Crewther and Dowling, 1971; Fraser *et al.*, 1976; Crewther, 1976) or IF proteins in living cells (Steinert, 1978; Steinert *et al.*, 1980, 1981). α-Keratin models assumed only one coiled-coil array (coil 1b in the following discussion) but at least in later versions invoked double-stranded coiled-coils (Ahmadi and Speakman, 1978; Ahmadi *et al.*, 1979; Woods and Gruen, 1981). The model of Steinert based on various observations but devoid of supporting sequence data was upheld in one important point. Regardless of differing molecular weight, IF

proteins keep the same absolute sequence length in coiled-coil conformation, and this organization can be approximately described as two major units as had been earlier suggested for epidermal keratins (Skerrow *et al.*, 1973). However, because of a false location of the coiled-coils along the polypeptide, the non-α-helical headpiece and its size variability were missed, and instead a size variability of the spacer separating the two major coiled-coils was proposed. The reasons for these discrepancies remain unclear as does the reason for the difference for the strandedness of the coiled-coils, i.e., three rather than two. Nevertheless the interpretation of the complete sequences of two mouse epidermal keratins recently proposed by Steinert *et al.* (1983, 1984) is fully consistent with the general topographical IF model illustrated for desmin. As Steinert *et al.* (1984) no longer seem to believe their earlier proposal of three-stranded models, there seems to be general agreement that IF molecules are based on double-stranded coiled-coils. Remaining minor difficulties concern subtleties of interpreting linear sequences in structural terms.

3. Neurofilaments

Mammalian neurofilaments (NF) contain three components. The triplet proteins NF-L, NF-M, and NF-H have apparent molecular weights of 70 K, 160 K, and 200 K in gel electrophoresis (Geisler and Weber, 1981a; Liem and Hutchinson, 1982; Hoffman and Lasek, 1975). Because of their increased molecular weights in comparison to desmin, vimentin, and GFA, their relation to other IF proteins posed several problems, which were only solved in steps. NF-L was the first neurofilament component to be identified as a non-epithelial IF protein. It formed *in vitro* homopolymeric filaments (Geisler and Weber, 1981a; Liem and Hutchinson, 1982) with the characteristic 21-nm lateral periodicity (Henderson *et al.*, 1982) and contained a short segment easily aligned with the emerging sequences of desmin and vimentin (Geisler *et al.*, 1982b). Various observations, however, seemed to indicate that NF-M and NF-H acted as associated proteins bound to a filament wall built from NF-L. Immunoelectron microscopy, while showing continuous decoration for NF-L, emphasized a peripheral organization for NF-M and particularly for NF-H (Willard and Simon, 1981; Sharp *et al.*, 1982; Hirokawa *et al.*, 1984; Liem *et al.*, 1985). In reconstitution experiments neither of the two high-molecular-weight proteins showed self-assembly although both were readily incorporated into filaments formed by NF-L. The resulting "hairy" filaments obtained particularly with NF-H (Geisler and Weber, 1981a) seemed to emphasize again the character of an associated protein also invoked from some proteolytic studies (Chin *et al.*, 1983; Julien and Mushynski, 1983).

A combination of biochemical, immunological, and protein–chemical data led to a different model (Geisler *et al.*, 1983a) (Fig. 1). Mild proteolytic digestion of all three proteins provided a highly α-helical rod domain of about 40 K, which harbored the epitope for a monoclonal antibody able to recognize all IF proteins (Pruss *et al.*, 1981; Geisler *et al.*, 1983a). As this epitope is

situated around the consensus sequence at the end of coil 2, a new structural model was possible for NF-M and NF-H. These were recognized as hybrid molecules carrying, in addition to sequences typical of IF proteins, separate autonomous domains accounting for their increased molecular mass. As the partial sequence of NF-L documented a peculiar glutamic acid-rich domain at the carboxyl end (Fig. 2), it was proposed that triplet proteins differ by tailpiece extensions increasing in size. The amino acid composition (see Section 8) of these autonomous domains in NF-M and NF-H pointed, as in NF-L, to glutamic-acid-rich regions not found in any other IF protein type (Geisler *et al.*, 1983a, 1984, 1985b).

Subsequent sequence data fully confirmed this model. The complete sequence of NF-L (548 residues) (Geisler *et al.*, 1985c) shows an arginine-rich headpiece preceding the common rod region. This is in turn connected via a highly protease-sensitive hinge region (45 residues) to the carboxyterminal tailpiece extension, which has a highly unusual sequence. Within 106 residues it has 47 glutamic acids and 12 lysines (Fig. 2). The currently known aminoterminal 436 residues of NF-M (Geisler *et al.*, 1984) cover the arginine-rich headpiece, the entire rod, and the beginning of the hinge region. Partial sequence data exist for the aminoterminal part of the tailpiece extension and again reveal, in line with compositional data, the wealth of glutamic acid and lysine. In the case of NF-H, fragments of the aminoterminal headpiece are known and about half of the rod domain has been characterized by the sequence of a 17-K fragment (compare Figs. 1 and 2). The early and late part of the rod remain to be sequenced. Two additional short sequences of large fragments high in glutamic acid and lysine account for the carboxyterminal tailpiece extension (Geisler *et al.*, 1985b). Thus the extra mass of all three neurofilament proteins is located in their tailpieces (Fig. 1).

In line with the sequence data, it has recently become possible, under special conditions, to form homopolymeric IFs from purified NF-M (Gardner *et al.*, 1984). Although similar conditions lead only to short and curly filamentous fragments of NF-H, it seems possible that the particularly large and highly phosphorylated (Julien and Mushynski, 1982; Jones and Williams, 1982; Geisler *et al.*, 1985b) tailpiece extension of this protein interferes in the refolding process of the IF domain upon dialysis from urea into the reconstitution buffer. Alternatively, we cannot exclude the possibility that because of a yet unknown property, incorporation of NF-H into filaments is promoted by the presence of either NF-M or NF-L. The combined data leave little doubt as to the relative structural disposition of the three NF proteins. As in other IFs, they seem to interact with each other via their coiled-coil regions in a pattern related to that discussed in Section 6 for IF proteins in general. Since the tubular filament structure proposed for IFs cannot accommodate true IF proteins situated at the filament periphery without being present in the filament wall, the immunoelectron microscopic data obtained with various NF-H-specific antibodies (Willard and Simon, 1981; Sharp *et al.*, 1982; Hirokawa *et al.*, 1984; Liem *et al.*, 1985) required a reevaluation. Although they strongly emphasized that NF-H is involved in the whiskerlike crossbridges between

neighboring axonal neurofilaments, they have never excluded the possibility that only a part of NF-H is involved in this structure. As monoclonal antibodies recognizing an epitope in the tailpiece extension of NF-H (Geisler *et al.*, 1983a; Liem *et al.*, 1985) provide the same decoration pattern as originally observed with polyclonal antibodies, it seems likely that it is the tail domain which is involved in cross-bridge formation between neighboring filaments. We note, however, that it remains unknown whether the cross-bridge contains, in addition to the tailpiece of NF-H, the corresponding domains of the other two components. The neurofilament model developed here also explains the results of certain proteolytic studies (Julien and Mushynski, 1983; Chin *et al.*, 1983). While the shorter 40-K fragments of NF-H and NF-M retained in the insoluble material are related to the α-helical rod domains, the much larger fragments released into the supernatant are now recognized as the tailpieces of the two proteins.

4. Organization of the Sequences along the Rod Domain

α-Helices in coiled-coil formation are recognized by a particular organization. The sequences reveal a seven-residue or heptade $(a, b, c, d, e, f, g)_x$ repeat pattern, where positions *a* and *d* are usually occupied by hydrophobic residues (Sodek *et al.*, 1972; McLachlan and Stewart, 1975). When appropriately folded, these residues will form a hydrophobic seam indicating those positions where two (or possibly three) such α-helices can align. The resulting coiled-coil is then stabilized by hydrophobic bonds. As extended parts of the rod domains are organized in heptades (Fig. 2), it is relatively easy to determine the borders of the α-helical coiled-coil domains, and the various interpretations given in the literature differ only slightly from that originally offered for desmin (Geisler and Weber, 1982). A non-α-helical spacer of about 20 residues situated approximately in the middle of the rod regions separates two approximately equally sized α-helical regions, the aminoterminal coil 1 and the carboxyterminal coil 2. A further nonhelical spacer of about 15 residues divides coil 1 into the shorter and aminoterminally located coil 1a and the longer coil 1b. The number of heptades for 1a, 1b, and 2 is about 4–5, 14–15, and 21, respectively. Alternatively, there could be an additional spacer of about 10 residues early in coil 2. There is disagreement as to the precise location of this spacer. One laboratory puts it just prior to the centrally located tryptophan residue (desmin position 286) (Steinert *et al.*, 1984), whereas another locates its start some 12 residues past the tryptophan (Hanukoglu and Fuchs, 1983; Marchuk *et al.*, 1984). Although we noted earlier (Geisler and Weber, 1982) some irregularities or breaks in the heptades early in coil 2, we have avoided invoking a spacer in coil 2 as no IF protein currently known shows any length variability in this region whereas subtle length variability is documented in the other two spacers discussed. Another irregularity occurs some 60 residues prior to the carboxyl end of coil 2. Here an *a*-to-*d* reversal occurs in the repeat pattern probably resulting in a

shift of the pitch in the coiled-coil. These irregularities, as well as a number of *a* and *d* positions carrying a charged rather than a hydrophobic residue, seem general to all IF proteins including the keratins. All analyses have confirmed the existence of the consensus-type sequences we had located early in coil 1a and at the carboxyl end of coil 2 (Geisler and Weber, 1982; Geisler *et al.*, 1983a).

In addition to the heptade organization, the coiled-coil domains have a further structural regularity as the charged residues reveal a periodic pattern, which was analyzed in detail for the carboxyterminal 80 residues of coil 2 in desmin (McLachlan and Stewart, 1982). Each of the two types of charged residues shows significant periods of 28/3 and 28/10 residues, suggesting that the charge pattern has a fundamental period of 28 residues. The 28/3-residue period results in alternating positively and negatively charged bands present on the surface of the postulated coiled-coil. These bands are further divided by the 28/10-residue period into slanting patches of charge. Similar charge periods have been described for an epidermal keratin and α-keratin (Steinert *et al.*, 1984; Parry *et al.*, 1977; McLachlan, 1978). Interestingly, the 28/3-residue period is found not only in IF proteins but also along the myosin rod (Parry, 1981; McLachlan and Karn, 1982). As it is absent in the simple double-stranded coiled-coil of tropomyosin, the charge pattern must be involved in the proper fit between polymerizing IF molecules and thus should contribute to the forces that stabilize the filament. The 28/10-residue period seems absent in the myosin rod and therefore possibly related to a structural feature distinguishing the coiled-coils of myosin from those of the IF proteins.

Keratins are obligatory heteropolymers, and the complexity of some 20 different human keratins (Moll *et al.*, 1982) is now understood as a variation around two complementary prototypes: keratin I and keratin II (Fuchs and Marchuk, 1983; Hanukoglu and Fuchs, 1983; Franke *et al.*, 1983; Weber and Geisler, 1984). This complementarity is present on the level of the tetrameric protofilament, and it is generally assumed to reflect the presence of both prototypes within a double-stranded coiled-coil (Quinlan *et al.*, 1984). We note, however, that this point is not rigorously proven, and the possibility remains that each prototype can form a double-stranded coiled-coil and the subsequent process of the tetramer formation requires both prototypes (see also Steinert *et al.*, 1984).

5. *Comparison of the Six Nonepithelial Proteins along the Rod Domain*

Three of the six nonepithelial IF proteins are known by full sequence: desmin (chicken) (Geisler and Weber, 1982), vimentin (hamster) (Quax *et al.*, 1983), and NF-L (pig) (Geisler *et al.*, 1985c). DNA and protein data on murine and porcine GFA together (Geisler and Weber, 1983; Lewis *et al.*, 1984) establish a fourth protein in full length. For porcine NF-M (Geisler *et al.*, 1984) the aminoterminal 436 residues are known and the sequence of a 17-kD

fragment from the rod domain is available for porcine NF-H (Geisler *et al.*, 1985b). Therefore, the structurally conserved rod domains of five of the six nonepithelial IF proteins can be compared for sequence identity over the entire length (Table 1). As already seen in earlier studies (Geisler *et al.*, 1984), desmin and vimentin are the closest pair. They reveal an identity of 73%, although an avian and a mammalian protein are compared. GFA is somewhat less related as it shares only 63% identity with desmin or vimentin. NF-L and NF-M are the most remote members, displaying only ~50% identity with desmin and vimentin. Interestingly, these two neurofilament proteins have strongly diverged from each other, as they share only 54% identity. When the comparison is restricted to the rod segment currently established for NF-H, essentially similar results are obtained, although the absolute values are lower as the consensus-type sequences at the ends of the rod are not covered (compare Fig. 1 and Fig. 2). Again, the three nonneuronal proteins are much closer to each other (60–70% identity) than the three neurofilament proteins, which have noticeably diverged (L/M 46%, M/H 44%, L/H 37% identity). Despite these gradual differences, the six proteins are recognized as members of one subgroup of IF, i.e., the nonepithelial proteins, since the two complementary keratin types are more remote. Regions of extreme sequence homology are clearly delineated at the two ends of the coiled-coil array covering as consensus sequences both the keratins and the nonepithelial IF proteins (Geisler and Weber, 1982; Geisler *et al.*, 1984). In the latter case we notice particularly the carboxyterminal 37 residues of coil 2, which display 30 identically positioned residues (Fig. 2).

Partial data on the rod exist also for porcine (Geisler and Weber, 1981b) and hamster desmin (Quax *et al.*, 1984), murine NF-L (Lewis and Cowan, 1985), and porcine GFA (Geisler and Weber, 1983). Usually a few conservative exchanges are noted between the corresponding porcine and rodent proteins. The carboxyterminal 122 residues of the desmin rod are known for

Table 1. Sequence Identities (in %) along the Rod Domains of Five of the Six Nonepithelial IF Proteins[a]

	V	D[b]	G	L	M
V	—	73	63	53	50
D[b]	(68)	—	63	53	49
G	(61)	(60)	—	48	48
L	(51)	(54)	(45)	—	54
M	(39)	(39)	(37)	(46)	—
H	(37)	(37)	(37)	(37)	(44)

[a] Values in parentheses arise when comparison is made for all six proteins using only the segments established for NF-H (see text and Figs. 1 and 2). V, vimentin; D, desmin; G, GFA; L, NF-L; M, NF-M; H, NF-H (for references to the sequences see legend to Fig. 2) (From Geisler *et al.*, 1985c).

[b] Residues 97–407 (see Fig. 1).

three species. Seven of the eight replacements that distinguish porcine and chicken desmin are present also in hamster desmin; thus, the two mammalian proteins are much closer than the avian protein (99.2% identity versus 93.4%). This species-specific drift together with the presence of separate genes for desmin and vimentin in all classes of vertebrates (Quax *et al.*, 1984) favors a model where the corresponding genes arose by gene duplication from a common precursor present at or prior to the evolution of vertebrates. In such a model GFA would have separated earlier from desmin/vimentin and the NF proteins or their precursors diverged even earlier from a putative tree of IF protein precursors. As argued elsewhere (Geisler *et al.*, 1985c), such models require a detailed study of IF proteins in invertebrates, which is currently lacking as there is only occasional documentation of neurofilaments in some species (Eagles *et al.*, 1981; Lasek *et al.*, 1983). We also note that when the same IF protein is compared from different species, the headpiece region seems the most variable part (Geisler and Weber, 1983).

6. *Structural Characterization of the Rod*

As the rod domains are highly conserved in sequence principles and length, it is believed that they provide the essential structural building block of IF. The variable head- and tailpieces may act in consolidating the structure and/or in mediating cellular interactions with other components (see Section 8).

The rod is obtained by mild chymotryptic digestion of the soluble form of desmin, the protofilaments (Geisler *et al.*, 1982a). It has a polypeptide molecular weight of 40,000 and spans residues 70–415 (Geisler and Weber, 1982). It thus fully covers the conserved α-helical domain (residues 97–407 in desmin) with 26 and eight additional residues protruding at the two ends. As the rod is unable to polymerize into filaments, it is a suitable tool to study the structure of the protofilament. Electron micrographs reveal a thin and long particle with a length of about 50 (±5) nm and a diameter of 2–3 nm (Geisler *et al.*, 1982a). Chemical cross-linking with dimethylsuberimidate suggested a tetrameric state in line with the presence of two double-stranded coiled-coils (Geisler and Weber, 1982). Sedimentation equilibrium centrifugation has consolidated the tetrameric unit in various buffer systems including the one used for the cross-linking experiments (Geisler *et al.*, 1985a). The measured molecular weight of 165,000 divided by the polypeptide molecular weight of 40,000 given by the sequence provides 4.05 chains per rod molecule (Fig. 3). Further confirmation of the tetrameric state has meanwhile been reported in other laboratories using cross-linking and molecular weight determinations on protofilaments of desmin, vimentin, and liver keratin (Pang *et al.*, 1983; Quinlan *et al.*, 1984). This information, together with the heptade repeat pattern, suggested the presence of two double-stranded coiled-coils rather than the triple-stranded coiled-coils previously proposed (Steinert *et al.*, 1980). The interacting α-helices of the well-analyzed double-stranded coiled-

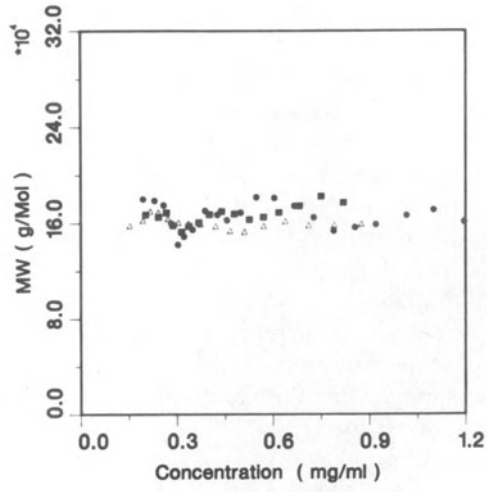


Figure 3. Molecular weight determination of the chicken desmin rod domain by high-speed equilibrium sedimentation centrifugation.

Original protein concentrations were △ = 0.3 mg/ml; ■ = 0.6 mg/ml; ● = 0.9 mg/ml. The column height was about 4 mm, and the centrifugations were performed at 20°C in 0.15 M NaCl, 0.05 M Tris-HCl, pH 8.3, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. The molecular weight was calculated using a partial specific volume of 0.723 ml/g. Given the polypeptide molecular weight of the chymotryptic rod monomer of 40,000, the native rod domain is a tetramer (molecular weight 163,000) (Geisler *et al.*, 1985a).

coils of tropomyosin and myosin are arranged in parallel and in register (McLachlan and Stewart, 1976; McLachlan and Karn, 1982). If a similar arrangement is taken as the most likely possibility for the two-stranded coiled-coils of IF proteins, two questions arise for the tetrameric desmin rod. First, are the two presumptive coiled-coils arranged antiparallel or parallel to each other? Second, are they in register or are they staggered?

In order to decide between parallel and antiparallel arrangements we have used a desmin-specific mouse monoclonal IgG_1 antibody whose epitope is located between residues 324 and 415 of the molecule, i.e., in the carboxyterminal part of the rod (Debus *et al.*, 1983). Fab fragments were mixed with the rod. The complexes were purified by gel filtration and visualized by electron microscopy after metal shadowing (Geisler *et al.*, 1985a). The micrographs of Fig. 4 document dumbbell-shaped structures in which both ends of the rod are labeled by a globular Fab molecule. The mean distance of the two Fab fragments (center to center) was about 45 nm, in agreement with the length of the rod. The structure obtained is best explained if the two neighboring coiled-coils of the tetramer are antiparallel to each other. If one accepts that about 280 residues of the rod can be involved in coiled-coil formation, this would lead to a minimal length of 42 nm provided the coiled-coils are stretched out to full length and backfolding does not occur. We have reported the length of the desmin rod to be about 50 (±5) nm (Geisler *et al.*, 1982a). Conformation for such a length comes from work on tetrameric protofilaments of liver keratin and protofilaments of desmin and vimentin reported by others (Pang *et al.*, 1983; Quinlan *et al.*, 1984). Values of 48–50 nm make a long stagger between the two neighboring coiled-coils very unlikely although they cannot exclude a relatively small stagger. There are presently only a few clues, as to the exact alignment of the two coiled-coils. One possible arrangement without any stagger is shown in Fig. 5A.

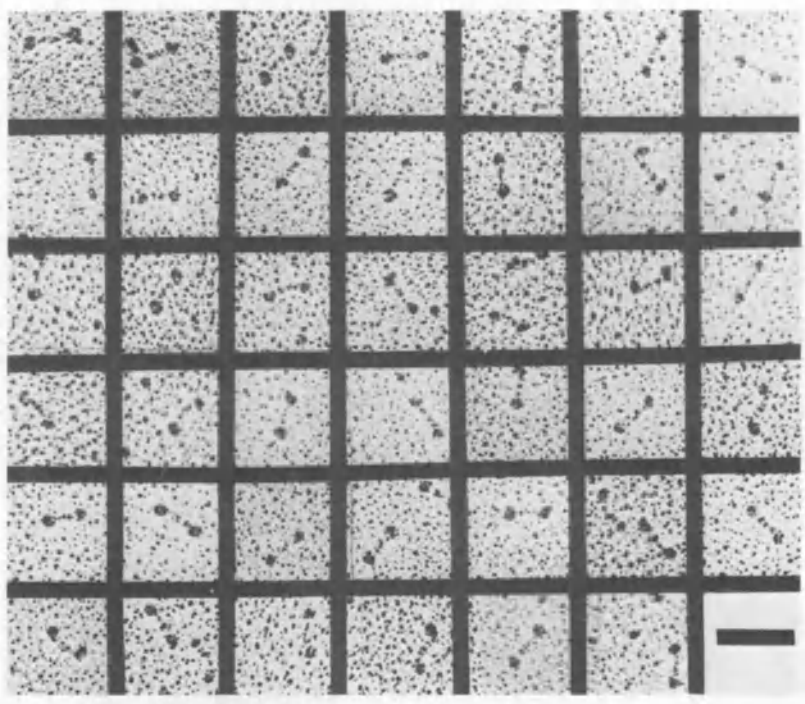


Figure 4. Desmin rods decorated with Fab fragments of the monoclonal antibody DE-U-10. Chymotryptic desmin rods and papain-cleaved DE-U-10 antibodies were mixed in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM phenylmethylsulfonylfluoride, and 0.1 mM dithiothreitol. The complex was purified by gel filtration and rotary-shadowed with tantalum/tungsten at an angle of 10° after mixing with glycerol. The bar is 10 nm. Note the dumbbell-shaped complexes implying that both ends of the rod are tagged by Fab fragments (Geisler *et al.*, 1985a).

7. From the Rodlike Protofilament to the 10-nm Filament

The structure suggested here for the desmin rod differs from a smaller complex isolated from carboxymethylated and reconstituted wool α-keratins by chymotryptic digestion (Ahmadi and Speakman, 1978; Woods and Gruen, 1981). This α-helical particle consists of four chains of coil 1b, as all other parts of the rod have been digested. The tetrameric state of this particle has led to the postulation of a tetrameric keratin protofilament unit of 60- to 66-nm length, where the two coiled-coils are antiparallel and staggered by about 20 nm (Fraser and McRae, 1983; Crewther *et al.*, 1983). In this particle (Fig. 5B) the coils 2 protrude essentially without binding partner while the coils 1 are essentially in a tetramer form. However, a full α-keratin rod or protofilament unit of the postulated length (≥60 nm) has neither been visualized by electron microscopy nor isolated directly. In addition, the length seems incompatible with the values found for the tetrameric liver cytokeratin protofilaments, i.e., 48 nm as for desmin (Quinlan *et al.*, 1984; Pang *et al.*, 1983).

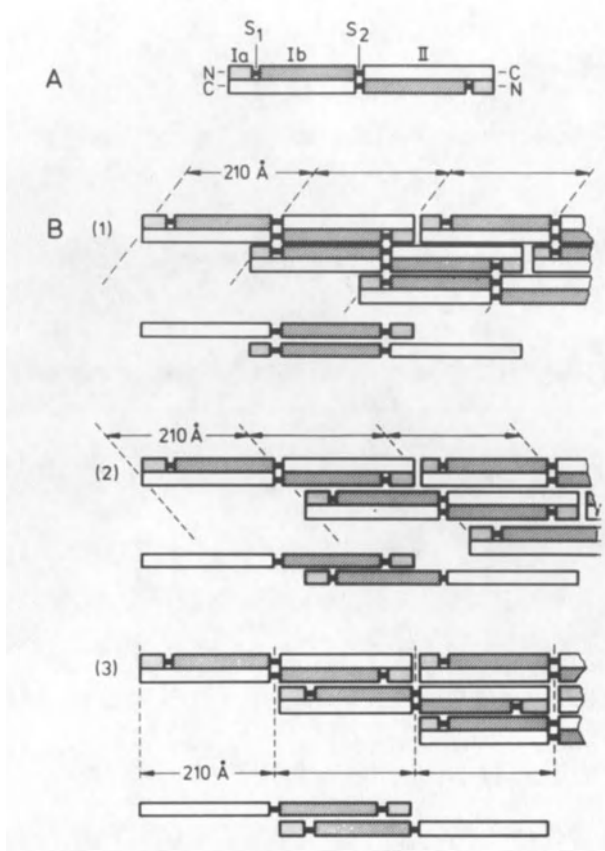


Figure 5. Diagrammatic representation of the rod complex (protofilament) and possible ways of its assembly into filaments (Geisler *et al.*, 1985a). Bars represent double-stranded coiled-coils. For better orientation coiled-coils I are hatched. (A) The protofilament is built from two double-stranded coiled-coils that are antiparallel to each other and in register. The nonhelical insertions S_2 (see Figs. 1 and 2) divide the complex into two approximately equally sized halves. Considering the length of the tetrameric rod of about 48 nm (Geisler *et al.*, 1982a; Pang *et al.*, 1983; Quinlan *et al.*, 1984) and the minimal length of 42 nm for one coiled-coil (see text), there could also be a small stagger of up to about 6 nm between the coiled-coils. (B) Two different ways of assembly for the protofilaments that lead to IF. The rods are approximately parallel to the axis of the filament. In all models the packing involves staggering of neighboring protofilaments by about half a protofilament length. Such packing would give rise to the 21-nm cross-striations seen on native filaments (see Fig. 6 and Henderson *et al.*, 1982; Milam and Erickson, 1982) as the head- and tailpieces are present at defined places separated by 21 nm. In addition, the postulated tetrameric α-keratin complexes (Fraser and McRae, 1983; Crewther *et al.*, 1983) could be explained as arising from two neighboring protofilaments (see text). For convenience the α-keratin-type complexes are extracted from the three model structures and shown below. The excess length of the rod of approximately 48 nm versus 2 × 21 nm could probably be accommodated by small overlaps of consecutive protofilaments due to small staggers between the coiled-coils as discussed in A. Alternatively, this excess length could be irrelevant, for instance, if the residues exceeding the actual rod domain at both sides (see text) would be responsible for the excess length but would not actually occupy space in the filament framework. Using the approximate spatial relations of neighboring protofilaments depicted in (1) and (2) the structure could close into a tubelike

Nevertheless, model building shows that the postulated α-keratin complex could arise from higher orders of protofilament aggregates (Fig. 5B). If the filament wall were built from the 48-nm-long "desmin-type" protofilaments staggered by approximately half a length to each other, the resulting brick wall arrangement given in Fig. 5B shows that the postulated α-keratin complex would be present as a structure formed between two desmin-type rods. Such a filament arrangement could give rise either to the desmin rods observed or to the postulated 60- to 66-nm-long α-keratin rods.

Until now minimal protofilamentous complexes covering the full length of the α-helices in IF proteins provided measured values of only 48–50 nm (Geisler *et al.*, 1982a; Quinlan *et al.*, 1984; Pang *et al.*, 1983), while the 60- to 66-nm-long α-keratin-type complex has only been evoked from the properties of a particle retaining only coil 1b. However, we note that differences in experimental conditions used in renaturation experiments may influence the molecular nature of the particles obtained and that currently we cannot even exclude the possibility that distinct IF could show a different behavior in this respect. If the true protofilament in a general IF structure were the 60-nm "α-keratin" unit rather than the 50-nm "desmin" unit, the exact spatial relationship of nearest neighbors would differ distinctly. Nevertheless, certain general features are kept in both cases: antiparallelity of the coiled-coils in the tetramers, the brickwall arrangement of the coiled-coils, and the number of tetramers per filament diameter.

The brickwall arrangement based on close to a half-unit-length repeat also seems to fit the 21-nm lateral periodicity reported by us and others in various IF preparations exposed to glycerol and then metal-shadowed (Aebi *et al.*, 1983; Henderson *et al.*, 1982; Milam and Erickson, 1982; and see Fig. 6). If the protofilaments were approximately parallel to the axis of the filament, the excess length of the rod, i.e., 48 nm versus twice 21 nm, could probably be accommodated by slight overlaps at the ends of consecutive rods due to a small stagger between the coiled-coils.

From our micrographs of shadowed filaments (Henderson *et al.*, 1982; see also Fig. 6) we cannot decide whether the 21-nm repeat reflects a true helical arrangement of half rods on the filament surface in agreement with interpretations of X-ray data on α-keratin (Fraser and McRae, 1983; Crewther *et al.*, 1983) or indicates a straight disklike arrangement as some specimens seem to imply. However, the basic arrangement would be rather similar in both cases with small differences in stagger resulting in either the helical or the disk forms.

---

filament with seven protofilaments side by side. The 21-nm repeat would then represent the pitch length of a helix around the filament as in recent interpretations of X-ray reflections obtained on wool α-keratins (Fraser and McRae, 1983; Crewther *et al.*, 1983). In (3) filament closure would require eight protofilaments also aligned side by side. The 21-nm repeat would reflect the length of straight disks in agreement with the often straight appearance of this repeat in recent electron micrographs of desmin (see Fig. 6) and the interpretation of minimal-length filaments obtained in vimentin assembly studies (see text and Ip *et al.*, 1985).

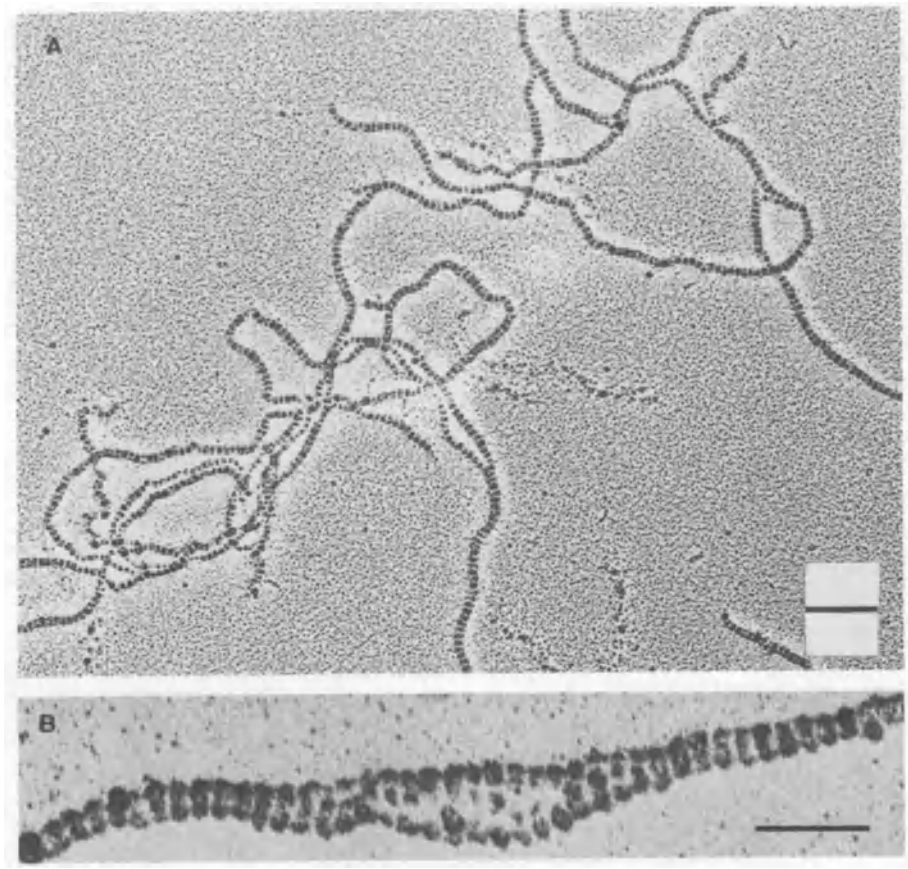


Figure 6. Electron micrographs of desmin filaments. Filaments were mixed with glycerol, sprayed onto freshly cleaved mica, and shadowed with tantalum/tungsten at an angle of 10°. While negatively stained filaments usually appear smooth without any repeat structure, the rotary-shadowed filaments reveal a pronounced 21-nm cross-striation (see also Henderson *et al.*, 1982; Milam and Erickson, 1982). It is conceivable that the repeat arises from the head- and possibly the tailpieces present at these distances on the filament surface according to the models discussed in the text (see Fig. 5). The absence of deposited metal between the "pearls" could be caused by constrictions of the filament due to the glycerol. The glycerol either "tightens the grip" of the headpieces onto the neighboring rods or loosens the cohesion of protofilaments between the headpieces thus causing a swelling between the intersections. Considering the different models of IF structure (see Fig. 5), the repeat would reflect either the pitch height of a helix around the filament or the length of straight disks. (A) Filaments polymerized from desmin lacking the carboxyterminal 27 residues (see Fig. 7 and Kaufmann *et al.*, 1985). Note the normal appearance of the filaments. The bar is 200 nm. (B) A desmin filament at higher magnification. The 21-nm sections at both ends appear straight. The broadening in the middle could have arisen from an opening of the tubular filament at one or more sides parallel to the axis of the filament. In line with the models discussed, the 21-nm repeat is, however, retained (see Fig. 5). The bar is 100 nm.

Improved X-ray data and a chemical understanding of nearest neighbors could be very helpful in the future. As far as the latter point is concerned, current evidence is disappointing. The induced dimer formation by oxidation of the sole cysteine in desmin, vimentin, GFA, and their hybrid polymers (Quinlan and Franke, 1982, 1983) is difficult to interpret in structural terms and does not yet favor a particular model. This cysteine residue occurs at the same relative position in coil 2 (see Geisler and Weber, 1981b, 1983) and is in all sequence presentations assigned an *f* location in the heptade, i.e., a place where disulfide formation between the two helices of the same double-stranded coiled-coil is impossible as long as the coiled-coil is stable. Thus, a disulfide within a coiled-coil could only occur due to a local weakness of the structure around the cysteine (see also the charge analysis by McLachlan and Stewart, 1982). If, however, the coiled-coils were undisturbed around the cysteine residues, S–S bridge formation would have to occur between coiled-coils of neighboring protofilaments. Since the cysteine residue is, by approximately 30 residues, closer to the aminoterminal end than to the carboxyterminal end of coil 2, the spatial possibility of forming such links is provided by either of the two variations leading to a helical model (Fig. 5B, 1, 2), if the coiled-coils were approximately positioned by their twist. In the disk arrangement (Fig. 5B, 3) an inter-coiled-coil S–S bridge would necessitate the introduction of further small staggers. Alternatively, if the tetramers were to contain double-stranded coiled-coils of antiparallel α-helices or parallel double-stranded coiled-coils, disulfide formation within the tetramer would be possible. However, these options are currently not very attractive in view of the structure of other coiled-coils and the indications for antiparallelity between coiled-coils discussed in all models.

The filament structure discussed (see Fig. 5B) could close into a tube with seven tetrameric protofilaments in the helical arrangement (1 and 2), while eight protofilaments is the more likely value for a straight disk organization (3). The corresponding linear mass densities of 35,000 M_r/nm (helical structure) or 40,000 M_r/nm (disk structure) are in fair agreement with experimental values of around 37,000 M_r/nm reported by Steinert and co-workers for vimentin (Steven *et al.*, 1982), which is particularly closely related to desmin. Owing to some flexibility of the envisioned structure, filament closure could also occur either with two additional protofilaments or when two protofilaments are lacking. This property would account for the two minor species of increased and decreased linear mass densities found in various IF preparations (Steven *et al.*, 1983). This point is discussed elsewhere in detail (Geisler *et al.*, 1985a).

Although the proposed arrangements of rods seem to lead to a tubular filament, various electron micrographs of unraveled filaments seem to suggest a more ropelike organization containing three to four subfibers (see for instance Aebi *et al.*, 1983; Stromer *et al.*, 1981). The molecular basis of these subfibers is currently not clear, and the images do not necessarily argue against a primarily tubelike structure formed by seven or eight protofilaments. If, in unraveling filaments, some of the neighboring protofilamentous

fibers would for yet unknown reasons stick closer together, an impression of subfibers could easily arise.

8. Structure and Function of the Terminal Domains in IF Proteins

8.1. The Involvement of Headpieces in Filament Formation

Complete headpiece sequences are available for five of the six nonepithelial IF proteins (Quax *et al.*, 1983; Geisler and Weber, 1982, 1983; Geisler *et al.*, 1983a, 1984, 1985b). Although they are difficult to align, certain similarities in composition and structure are recognized (Weber and Geisler, 1984) (Fig. 2). The headpieces are very basic because of a wealth of arginine residues. Negatively charged residues and lysine, although present occasionally, are rare. Headpieces have a high content of serine and glycine and display several prolines. Secondary structural prediction rules indicate several β-turns (Geisler *et al.*, 1982a) and the measured CD spectrum of the desmin headpiece (residues 1 to 82) (our own unpublished observation) reveals absence of α-helix and β-sheet. The β-turns postulated are in line with the extreme protease sensitivity of this domain (Geisler *et al.*, 1982a). The staircase degradation pattern of decreasing molecular weight and isoelectric point obtained in two-dimensional gels (see, for instance, Nelson and Traub, 1983) conforms with the gradual proteolytic loss of basic headpiece regions from molecules carrying an overall acidic rod domain. At their carboxyterminal side the headpieces are connected via a leader-type sequence with the common rod domain. This stretch of some 20 residues already contains acidic residues and has α-helical potential in desmin, vimentin, GFA, and NF-L. As it is perturbed by additional glycine and proline residues in NF-M (Geisler *et al.*, 1984) and so far lacking in epidermal keratins (compare sequence alignment in Geisler *et al.*, 1984), its structural importance is unknown. Thus, the actual headpiece domain covers in nonepithelial IF proteins some 8–12 arginines present in 45–76 residues, with GFA displaying the shortest domain.

That either one or both terminal domains of IF proteins are involved in filament formation was first indicated by the inability of the chymotryptically exised desmin rod to form filaments under physiological conditions (Geisler *et al.*, 1982a). Subsequent work in two different laboratories led to contradictory results. Traub and co-workers (Nelson and Traub, 1983; Traub and Vorgias, 1983, 1984) assembled various arguments for a direct participation of the head domain in filament formation whereas Lu and Johnson (1983) postulated that the removal of the first 97 residues of chicken desmin by thrombin did not abolish polymerization ability. In order to overcome this discrepancy we have recently studied two derivatives of chicken desmin lacking either only the aminoterminal 67 residues or only the carboxyterminal 27 residues. These were obtained by digestion with thrombin (T desmin) or lysine-specific protease (L desmin), respectively (Fig. 7). Whereas L desmin is fully polymerization-competent and can be quantitatively obtained from normal des-

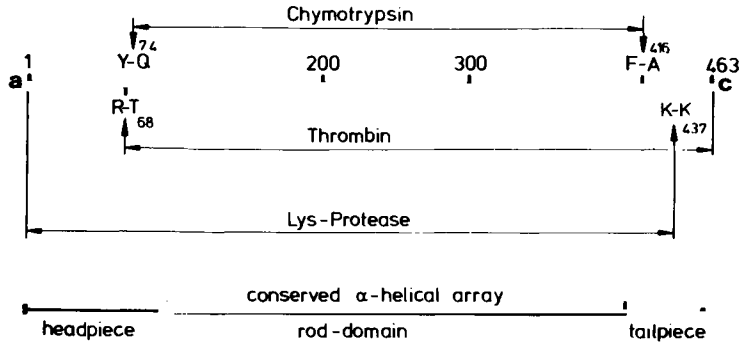


Figure 7. Diagrammatic presentation of desmin fragments used to assess the function of the terminal domains. Digestion of protofilaments with chymotrypsin results in the loss of the headpiece and the tailpiece (Geisler *et al.*, 1982a), whereas a similar digestion with thrombin results in the low of the headpiece only (T desmin; residues 68–463). Lysine-specific protease acting on filaments removes only the carboxyterminal 27 residues (L desmin; residues 1–436) and thus about half of the tailpiece (Kaufmann *et al.*, 1985). The α-helical coiled-coils start at residue 103 and end at residue 407 (see Fig. 2).

min filaments, the T desmin remains by electron microscopic and hydrodynamic criteria at the protofilamentous level of the tetramer and cannot form filaments on its own when physiological buffer and salt conditions are used with normal protein concentrations (Kaufmann *et al.*, 1985). The reason for the discrepancy between our results on T desmin and those of Lu and Johnson (1983) is unknown. However, there are now several arguments for a direct participation of the headpiece in filament formation and a structural involvement of the arginine residues in this process. First, the removal of the aminoterminal 67 residues, which include all 10 arginines of the desmin head (T desmin), provides, at least in our hands, a derivative frozen at the protofilament stage (Kaufmann *et al.*, 1985). Second, brief digestion of desmin and vimentin filaments by a Ca^{2+}-dependent protease leads to protofilamentous units rather than filaments (Nelson and Traub, 1981, 1983). The coordinates of the resulting fragments in two-dimensional gels argue that they have lost the headpiece region to various extents. One of these vimentin derivatives purified to homogeneity was directly shown to be polymerization-incompetent (Traub and Vorgias, 1983). Third, a rather basic vimentin fragment of 11K most likely covering the headpiece acts as an inhibitor of vimentin assembly (Traub and Vorgias, 1983), and we have found similar effects of the desmin head (residues 1–82). Fourth, filament formation of vimentin and desmin is inhibited by 150 mM arginine or guanidine but not by lysine (Traub and Vorgias, 1983). Future experiments have to explore a putative binding partner of the basic headpiece along the acidic rod domain and to decide whether the arginine residues of the head act by specific electrostatic interactions or by the formation of highly specific bidentated hydrogen bonds that cannot be provided by lysine. Alternatively, we cannot rule out an additional hydrophobic effect of the headpiece and note the decreased number of corre-

sponding residues in the case of GFA (Geisler and Weber, 1983), which depolymerizes particularly easily (Rueger *et al.*, 1979; Steinert *et al.*, 1981). We also note that the DNA sequences of epidermal keratins indicate so far an aminoterminal region devoid of acidic residues and displaying at least some arginine residues in addition to many hydrophobic residues and a wealth of glycine (Steinert *et al.*, 1983, 1984; Marchuk *et al.*, 1984). In the case of wool α-keratins several cysteine residues point to a permanently frozen state connected to multiple disulfide linkages, which could occur either between neighboring keratins or between these and the peculiar and cysteine-rich matrix proteins (Weber and Geisler, 1982; Dowling *et al.*, 1983). For a more detailed discussion of the possibility that the headpieces act as enhancers of filament formation rather than as obligatory elements see Kaufmann *et al.* (1985).

While T desmin lacking the aminoterminal 67 residues is unable to form filaments on its own, the derivative is accepted into filaments formed when desmin and T desmin are mixed in 7 M urea prior to dialysis (Kaufmann *et al.*, 1985). As this copolymerization does not occur when the two separately formed protofilaments are mixed, we have speculated that certain tetrameric protofilaments containing less than four headpieces can be accommodated in the filament. These recent findings may indicate a gradual rather than total destruction of IFs within the cell. Not every removal of a headpiece by the Ca^{2+}-dependent protease described by Nelson and Traub (1981) must result in a filament break.

In a series of papers Traub and co-workers proposed that IF proteins are DNA-binding proteins and proteolytic fragments may act as intracellular hormones in the nucleus (reviewed in Traub and Vorgias, 1984). Although the latter point is so far without direct support, we have successfully exploited the reported affinity of IF protein fragments retaining the headpiece for single-stranded DNA (Nelson *et al.*, 1982). This affinity is present in 6 M urea and clearly somehow related to the wealth of arginines in the headpiece. An additional, although much weaker, DNA binding reported to be independent of the arginine residues (Traub and Vorgias, 1984) failed to show up in our experiments, which, however, were restricted to a commercially available affinity matrix and did not use the preparations of Traub and his colleagues.

The IF structure proposed here has certain implications as to end-on attachment of filaments to other organelles may they be plasma membrane, membrane specializations like desmosomes, or the nuclear membrane—all of which can be envisioned to have IF anchoring proteins. Each end of the filament structure discussed has "surplus" headpieces, which are in first approximation not involved in filament stability and integrity and may therefore act as binding partners for the anchoring proteins.

8.2. Structural Characterization of the Tailpieces

The tailpieces of desmin, vimentin, and GFA reveal "normal" sequences, which are clearly related and probably form globular structures connected via a short protease-sensitive hinge to the carboxyl end of the rod (Fig. 2). About half

of the total 55 residues following the rod can be removed without interfering with filament structure (L desmin; see Section 8.1). Interestingly, the three NF proteins reveal a remarkably distinct organization at the carboxyl end.

The tailpieces of the NF proteins show certain similarities among each other but differ in other aspects including their length. They are unusually highly charged owing to wealth of glutamic acid and lysine. The complete sequence of NF-L reveals 46 glutamic acids and 12 lysines within the carboxyterminal 106 residues (Geisler *et al.*, 1983a). For NF-M and NF-H the relative content of glutamic acid is decreased whereas lysine is increased (Geisler *et al.*, 1983a, 1984, 1985b) (Table 2). Arginine is either completely absent (NF-L) or very rare. As hydrophobic residues are also rare, long regions of peculiar sequences are predicted for NF-M and NF-H. In addition, these regions are highly phosphorylated. Between 20 and 100 phosphoserines are concentrated in more aminoterminally located subdomain for NF-H. While the NF-M tailpiece contains some 9–25 phosphate residues, the corresponding region of

Table 2. Amino Acid Composition of the Tail Domains of the Neurofilament Proteins[a]

	Tail B domains of[b]		
	NF-L[c]	NF-M[d]	NF-H[d]
Asx	3	3.2	1.8
Thr	4	1.6	2.2
Ser	3	5.5	8.5
Glx	52[e]	30.3[f]	27.3[f]
Pro	2	7.4	12.6
Gly	9	8.6	0.9
Ala	16	11.8	15.2
Val	2	8.3	4.7
Met	—	0.5	0.2
Ile	2	1.4	0.3
Leu	—	2.9	0.8
Tyr	—	0.4	—
Phe	—	0.3	—
Lys	12	16.7	24.9
His	1	0.3	—
Arg	—	1.2	0.3
	106	~100	~100

[a]Note the wealth of glutamic acid and lysine residues which together account for about 50% of the residues in all three tailpieces. From Geisler *et al.*, 1983a, 1984, 1985b.
[b]See Fig. 1.
[c]Numbers are taken from the sequence (Geisler *et al.*, 1983a) of the tailpiece domain of NL-L and are not leveled to 100.
[d]Values are given in mol %.
[e]The 52 Glx residues comprise 46 glutamic acids and six glutamines.
[f]Between 85 and 95% of the Glx residues are present as glutamic acid.

NF-L has not been analyzed in this respect. If there are, however, serine phosphates, their number is low (Julien and Mushynski, 1982, 1983; Jones and Williams, 1982; Geisler *et al.*, 1985b).

Some short sequences exist for the tailpieces of NF-M (Geisler *et al.*, 1984) and NF-H (Geisler *et al.*, 1985b). These are rather similar in pattern to the sequences found for NF-L. Circular dichroism spectra of the isolated domains detect no ordered structure (Geisler *et al.*, 1983a), but several findings indicate that these domains have some structural organization and do not act as random coils. The domain of NF-L behaves as a dimer, and its last 82 residues account for the small, highly glutamic-acid-rich proteins isolated from brain in several laboratories (Weber and Geisler, 1983). The regions of NF-H and NF-M have some resistance to proteolytic digestion and survive even brief exposure to trypsin although lysine is a major constituent amino acid (Chin *et al.*, 1983; our unpublished results). In addition, the tailpiece of NF-H participates in the cross-bridge structure present between neighboring axonal filaments (Sharp *et al.*, 1982; Hirokawa *et al.*, 1984; Liem *et al.*, 1985). In line with these predictions, the large tailpieces behave as elongated structures in preliminary hydrodynamic studies (our own unpublished results). The tailpieces seem connected via a shorter hinge region of about 45 residues to the carboxyl end of the rod. This highly protease-sensitive region is documented by sequence for NF-L, and partial data also exist in the case of NF-M (Geisler *et al.*, 1983a, 1984).

The unusual sequences of the tailpieces account for the overestimation of molecular weights in SDS gel electrophoresis found for all three NF proteins (Kaufmann *et al.*, 1984). Gel filtration and sedimentation equilibrium centrifugation in 6 M guanidine point to values of 63, 107, and 110 to 140,000 for L, M, and H, respectively. In the case of NF-L the complete sequence of 548 residues provides a molecular weight of 61,900 in line with the direct physical–chemical measurements (Geisler *et al.*, 1985c). As M and H have much longer tailpieces, the overestimate in gel electrophoresis seems more severe. Chymotryptically derived tailpieces of H and M have apparent molecular weights of 150–160,000 and 100–120,000 in gel electrophoresis, while the direct physical–chemical measurements point to values around 70,000 an 63,000, respectively (Kaufmann *et al.*, 1984).

8.3. Are the Carboxyterminal Tailpieces Appendages Communicating between Stiff Filaments and the Cytoplasm?

Several observations allow us to propose this hypothesis at least for nonepithelial IF proteins. First, desmin, vimentin, and GFA have similar tailpieces (see Fig. 2). Treatment of desmin filments with lysine-specific protease removes the carboxyterminal 27 residues without interfering with filament integrity (Kaufmann *et al.*, 1985). Although one cannot yet exclude a possible filament stabilizing site within the remaining half of the tailpiece, at least the more carboxyterminal half must be situated outside the filament wall. Second,

under carefully controlled proteolytic conditions large fragments of NF proteins can be solubilized without loss of filament structure (Julien and Mushynski, 1983; Chin *et al.*, 1983). These fragments are now identified as the glutamic-acid- and lysine-rich tailpiece extension. Their high charge density together with their phosphate content (Julien and Mushynski, 1983) make it very unlikely that such domains are not on the outside of the filament. Third, in the case of NF-H several monoclonal and polyclonal antibodies recognizing the tailpiece extension are related in immunoelectron micrographs with the fine cross-bridges connecting neighboring filaments in axons and neurites (see Section 3). Thus, it seems that most, if not all, of the tailpiece region and its extension is extrafilamentous material, and at least in desmin we can put a lower size limit to a putative filament stabilizing site. If this site really would occur, it should be less than 30 residues long (compare Fig. 7), and we note that there is as yet no evidence for such a site. Therefore, nearly the entire tailpiece seems free to communicate with the cytoplasm. It could act as a binding site for IF-associated proteins or be involved in anchoring other structures such as microtubules. In the case of neurofilaments, the unique tailpiece extensions offer for the first time a cell type specificity of IF proteins. No other currently known IF proteins display these peculiar glutamic-acid- and lysine-rich long regions. Thus, if the gene programs governing IF expression are related in any way with functional cellular differences, the tailpiece extensions of the neurofilament proteins are the obvious candidates. In addition to acting as cross-bridges, as seen for NF-H, they may provide scaffolds for permanent or transient interactions with other components of the neuronal cytoplasm.

Much less is known about the structural organization of the non-α-helical domains of the keratins. In analogy to the results on nonepithelial IF, it seems likely that the glycine- and serine-rich tailpieces of certain epidermal keratins (Hanukoglu and Fuchs, 1983; Steinert *et al.*, 1983, 1984) form extrafilamentous material, as at least one of the human epidermal keratins has a tailpiece somewhat related to those of desmin, vimentin, and GFA (Hanukoglu and Fuchs, 1982). The sequences of the headpieces of several epidermal keratins tentatively indicate two domains. The aminoterminal region carries all the charged residues (arginine and lysine; see above) whereas the following region which precedes the rod lacks charged residues and displays curious repeats of glycine flanked by hydrophobic amino acids (Marchuk *et al.*, 1984; Steinert *et al.*, 1983, 1984). It may well be possible that the latter region is also situated at the outside of the filament. Future studies must address the contribution of these putative extrafilamentous regions of keratins for filament packing and possible interaction with other cytoplasmic structures. The hypervariability of the terminal domains seems connected with different IF proteins of different cell types rather than the same IF protein of different species. Therefore, the hypervariable regions may indicate a cell-type-specific function, which, with the exception of neurofilaments, needs to be understood in functional terms.

9. Addendum

Recently Ip *et al.* (1985) followed the formation of vimentin filaments by electron microscopy. Similar to our desmin rod, they describe a 48-nm-long tetrameric protofilament, which is present at low ionic strength and high pH. With increasing ionic strength these tetramers seem first to dimerize using a half-length stagger. Then four such dimers seem to form tiny filaments about 66-nm long. These structures which already have the full width can then form long IF. This model is in full agreement with our proposal on filament structure (Geisler *et al.*, 1985a). The 66-nm minifilaments seem to favor the disk arrangement of the filament surface (see p. 53). In addition, Fraser and McRae (1985) also discuss a similar basic arrangement of coiled-coils in the filament framework. This paper offers also the possibility that a disc arrangement could fit the X-ray data available for α-keratin. Ip *et al.* do not address parallelity or antiparallelity of the coiled-coils within the protofilament, while Fraser and McRae suggest antiparallelity similar to our proposal. However, although the available data seem to point into the same direction, we realize that more experimental data are necessary to decide between a disk and a helical arrangement. In addition, the problem of parallel or antiparallel arrangement of the coiled-coils within the protofilament unit needs further experimentation as we do not rigorously exclude the possibility that the dumbbell structures observed could correspond to some specific or even unspecific complex of the coiled-coils which differs from the true protofilament. Furthermore, the chemical nature of the subfibrils (e.g., Aebi *et al.*, 1983) must still be solved.

References

Aebi, U., Fowler, W. E., Rew, P., and Sun, T. T., 1983, The fibrillar substructure of keratin filaments unraveled, *J. Cell Biol.* **97:**1131.

Ahmadi, B., and Speakman, P. T., 1978, Suberimidate crosslinking shows that a rod shaped, low cystin, high helix protein prepared by limited proteolysis of reduced wool has four protein chains, *FEBS Lett.* **94:**365.

Ahmadi, B., Boston, N. M., Dobb, M. G., and Speakman, P. T., 1979, Possible four-chain repeating unit in the microfibril of wool, in: *Fibrous Proteins* (D. A. D. Parry, ed.), Vol. 2, pp. 161–166, Academic Press, New York.

Chin, T. K., Eagles, P. A. M., and Maggs, A., 1983, The proteolytic digestion of ox-neurofilaments with trypsin and α-chymotrypsin, *Biochem. J.* **215:**239.

Crewther, W. G., 1976, Primary structure and chemical properties of wool, in: *Proceedings of the 5th International Wool Textile Research Conference,* Aachen, 1975, Vol. 1, pp. 1–101.

Crewther, W. G., and Dowling, L. M., 1971, The preparation and properties of large peptides from the helical regions of low-sulphur proteins of wool, *Appl. Polymer Symp.* **18:**1–20.

Crewther, W. G., Dowling, L. M., and Inglis, A. S., 1980, Amino acid sequence data from a microfibrillar protein of α-keratin, in: *Proceedings of the 6th Quinquennial International Wool Textile Research Conference,* Pretoria, Vol. 2, pp. 79–91.

Crewther, W. G., Dowling, L. M., Steinert, P. M., and Parry, D. A. D., 1983, The structure of intermediate filaments, *Int. J. Biol. Macromol.* **5:**267.

Day, W. A., and Gilbert, D. S., 1972, X-ray diffraction pattern of axoplasm, *Biochem. Biophys. Acta* **285**:506.

Debus, E., Weber, K., and Osborn, M., 1983, Monoclonal antibodies to desmin, the muscle-specific intermediate filament protein, *EMBO J.* **2**:2305.

Dowling, L. M., Parry, D. A. D., and Sparrow, L. G., 1983, Structural homology between α-keratin and the intermediate filament proteins desmin and vimentin, *Biosci. Rep.* **3**:73.

Eagles, P. A. M., Gilbert, D. S., and Maggs, A., 1981, The polypeptide composition of axoplasm and of neurofilaments from the marine worm *Myxicola infundibulum*, *Biochem. J.* **199**:89.

Franke, W. W., Schiller, D. L., Hatzfeld, R., and Winter, S., 1983, Protein complexes of intermediate-sized filaments: Melting of cytokeratin complexes in urea reveals different polypeptide separation characteristics, *Proc. Natl. Acad. Sci. USA* **80**:7113.

Fraser, R. D. B., and McRae, T. P., 1983, The structure of the α-keratin microfibril, *Biosci. Rep.* **3**:517.

Fraser, R. D. B., and McRae, T. P., 1985, *Biosci. Rep.* **5**:573–579.

Fraser, R. D. B., McRae, T. P., and Suzuki, E., 1976, Structure of the α-keratin microfibril, *J. Mol. Biol.* **108**:435.

Fuchs, E., and Marchuk, D., 1983, Type I and type II keratins have evolved from lower eukaryotes to form the epidermal intermediate filaments in mammalian skin, *Proc. Natl. Acad. Sci. USA* **80**:5857.

Gardner, E. E., Dahl, D., and Bignami, A., 1984, Formation of 10-nanometer filaments from the 150 K-Dalton neurofilament protein *in vitro*, *J. Neurosci. Res.* **11**:145.

Geisler, N., and Weber, K., 1981a, Selfassembly *in vitro* of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate-sized filaments, *J. Mol. Biol.* **151**:565.

Geisler, N., and Weber, K., 1981b, Comparison of the proteins of two immunologically distinct intermediate-sized filaments by amino acid sequence analysis: desmin and vimentin, *Proc. Nat. Acad. Sci. USA* **78**:4120.

Geisler, N., and Weber, K., 1982, The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins including the wool α-keratins, *EMBO J.* **1**:1649.

Geisler, N., and Weber, K., 1983, Amino acid sequence data on glial fibrillary acidic protein (GFA); implications for the subdivision of intermediate filaments into epithelial and non-epithelial members, *EMBO J.* **2**:2059.

Geisler, N., Kaufmann, E., and Weber, K., 1982a, Proteinchemical characterization of three structurally distinct domains along the protofilament unit of desmin 10 nm filaments, *Cell* **30**:277.

Geisler, N., Plessmann, U., and Weber, K., 1982b, Related amino acid sequences in neurofilaments and non neuronal intermediate filaments, *Nature* **296**:448.

Geisler, N., Kaufmann, E., Fischer, S., Plessmann, U., and Weber, K., 1983a, Neurofilament architecture combines structural principles of intermediate filaments with carboxy-terminal extensions increasing in size between triplet proteins, *EMBO J.* **2**: 1295.

Geisler, N., Plessmann, U., and Weber, K., 1983b, Amino acid sequence characterization of mammalian vimentin, the mesenchymal intermediate filament protein, *FEBS Lett.* **163**:22.

Geisler, N., Fischer, S., Vandekerckhove, J., Plessmann, U., and Weber, K., 1984, Hybrid character of a large neurofilament protein (NF-M): Intermediate filament type sequence followed by a long and acidic carboxy-terminal extension, *EMBO J.* **3**:2701.

Geisler, N., Kaufmann, E., and Weber, K., 1985a, Antiparallel orientation of the two double-stranded coiled-coils in the tetrameric protofilament unit of intermediate filaments, *J. Mol. Biol.* **182**:173.

Geisler, N., Fischer, S., Vandekerckhove, J., Van Damme, J., Plessmann, U., and Weber, K., 1985b, Protein-chemical characterization of NF-H, the largest mammalian neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension, *EMBO J.* **4**:57.

Geisler, N., Plessmann, U., and Weber, K., 1985c, The complete amino acid sequence of the major mammalian neurofilament protein (NF-L), *FEBS Lett.* **182**:475.

Hanukoglu, I, and Fuchs, E., 1982, The CDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins, *Cell* **31:**243.

Hanukoglu, I., and Fuchs, E., 1983, The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratins, *Cell* **33:**915.

Henderson, D., Geisler, N., and Weber, K., 1982, A periodic ultrastructure in intermediate filaments, *J. Mol. Biol.* **155:**173.

Hirokawa, N., Glicksman, M. A., and Willard, M. B., 1984, Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton, *J. Cell Biol.* **98:**1523.

Hoffman, P. N., and Lasek, R. J., 1975, The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons, *J. Cell Biol.* **66:**351.

Hong, B., and Davison, P. F., 1981, Isolation and characterization of a soluble, immunoreactive peptide of glial fibrillary acidic protein, *Biochim. Biophys. Acta* **670:**139.

Ip, W., Hartzer, M. K. Y-Y., Pang, S., and Robson, R. M., 1985, *In vitro* assembly of vimentin and its implications on the structure of intermediate filaments, *J. Mol. Biol.* **183:**365.

Jones, S. M., and Williams, C., 1982, Phosphate content of mammalian neurofilaments, *J. Biol. Chem.* **257:**9902.

Julien, J. P., and Mushynski, W. E., 1982, Multiple phosphorylation sites in mammalian neurofilament polypeptides, *J. Biol. Chem.* **257:**10467.

Julien, J. P., and Mushynski, W. E., 1983, The distribution of phosphorylation sites among identified proteolytic fragments of mammalian neurofilaments, *J. Biol. Chem.* **258:**4019.

Kaufmann, E., Geisler, N., and Weber, K., 1984, SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins, *FEBS Lett.* **170:**81.

Kaufmann, E., Weber, K., and Geisler, N., 1985, Intermediate filament forming ability of desmin derivatives lacking either the aminoterminal 67 or carboxyterminal 27 residues, *J. Mol. Biol.* **185:**733.

Lasek, R. J., Oblinger, M. M., and Drake, P. F., 1983, Molecular biology of neuronal geometry: Expression of neurofilament genes influences axonal diameter, *Cold Spring Harbor Symp. Quant. Biol.* **48:**731.

Lazarides, E., 1982, Intermediate filaments: a chemically heterogenous developmentally regulated class of proteins, *Ann. Rev. Biochem.* **51:**219.

Lee, L. D., and Baden, H. P., 1976, Organization of the polypeptide chains in mammalian keratin, *Nature* **264:**377.

Lewis, A. S., and Cowan, N. J., 1985, Genetics, evolution and expression of the 68 Kd neurofilament protein: isolation of a cloned cDNA probe, *J. Cell Biol.* **100:**843.

Lewis, S. A., Balcarek, J. M., Krek, V., Shelanski, M., and Cowan, N. J., 1984, Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: Structural conservation of intermediate filaments, *Proc. Natl. Acad. Sci. USA* **81:**2743.

Liem, R. K. H., and Hutchinson, S. B., 1982, Purification of individual components of the neurofilament triplet: filament assembly from the 70,000-dalton subunit, *Biochemistry* **21:**3221.

Liem, R. K. H., Chin, S. S. M., Moraru, E., and Wang, E., 1985, Monoclonal antibodies to epitopes on different regions of the 200,000 Dalton neurofilament protein, *Exp. Cell Res.* **156:**419.

Lu, Y., and Johnson, P., 1983, The *N*-terminal domain of desmin is not involved in intermediate filament formation: evidence from thrombic digestion studies, *Int. J. Biol. Macromol.* **5:**347.

Marchuk, D., McCrohon, S., and Fuchs, E., 1984, Remarkable conservation of structure among intermediate filament genes, *Cell* **39:**491.

McLachlan, A. D., 1978, Coiled coil formation and sequence regularities in the helical regions of α-keratin, *J. Mol. Biol.* **124:**297.

McLachlan, A. D., and Karn,J., 1982, Periodic charge distributions in the myosin rod amino acid sequence match cross-bridge spacings in muscle, *Nature* **299:**226.

McLachlan, A. D., and Stewart, M., 1975, Tropomyosin coiled-coil interactions: Evidence for an unstaggered structure, *J. Mol. Biol.* **98:**293.

McLachlan, A. D., and Stewart, M., 1976, The 14-fold periodicity in α-tropomyosin and the interaction with actin, *J. Mol. Biol.* **103**:271.

McLachlan, A. D., and Stewart, M., 1982, Periodic charge distribution in the intermediate filament proteins desmin and vimentin, *J. Mol. Biol.* **162**:693.

Milam, L., and Erickson, H. P., 1982, Visualization of a 21-nm axial periodicity in shadowed keratin filaments and neurofilaments, *J. Cell Biol.* **94**:592.

Milstone, L. M., 1981, Isolation and characterization of two polypeptides that form intermediate filaments in bovine esophageal epithelium, *J. Cell Biol.* **88**:317.

Moll, R., Franke, W. W., Schiller, D., Geiger, B., and Krepler, R., 1982, The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors, and cultured cells, *Cell* **31**:11.

Nelson, W. J., and Traub, P., 1981, Properties of a Ca^{2+}-activated protease specific for the intermediate-sized filament protein vimentin in Ehrlich ascitis tumour cells, *Eur. J. Biochem.* **116**:51.

Nelson, W. J., and Traub, P., 1983, Proteolysis of vimentin and desmin by the Ca^{2+}-activated proteinase specific for these intermediate filament proteins, *Mol. Cell Biol.* **3**:1146.

Nelson, W. J., Vorgias, C. E., and Traub, P., 1982, A rapid method for the large scale purification of the intermediate filament protein vimentin by single-stranded DNA-cellulose affinity chromatography, *Biochem. Biophys. Res. Commun.* **106**:1145.

Osborn, M., Geisler, N., Shaw, G., Sharp, G., and Weber, K., 1982, Intermediate filaments, *Cold Spring Harbor Symp. Quant. Biol.* **46**:413.

Pang, Y. Y-S., Robson, R. M., Hartzer, M. K., and Stromer, M. H., 1983, Subunit structure of the desmin and vimentin protofilament units, *J. Cell Biol.* **97**:226a.

Parry, D. A. D., 1981, Structure of rabbit skeletal muscle. Analysis of the amino acid sequences of two fragments from the rod region, *J. Mol. Biol.* **153**:459.

Parry, D. A. D., Crewther, W. G., Fraser, R. D. B., and McRae, T. P., 1977, Structure of α-keratin: structural implication of the amino acid sequences of the type I and type II chain segments, *J. Mol. Biol.* **113**:449.

Pruss, R. M., Mirsky, R., Raff, M. C., Thorpe, R., Dowding, A. J., and Anderton, B. H., 1981, Cell classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody, *Cell* **37**:419.

Quax, W. J., Egberts, W. V., Hendriks, W., Quax-Jeuken, Y., and Bloemendal, H., 1983, The structure of the vimentin gene, *Cell* **35**:215.

Quax, W., Van Den Heuvel, R., Egberts, W. V., Quax-Jeuken, Y., and Bloemendal, H., 1984, Intermediate filament cDNAs from BHK-21 cells: Demonstration of distinct genes for desmin and vimentin in all vertebrate classes, *Proc. Natl. Acad. Sci. USA* **81**:5970.

Quinlan, R. A., and Franke, W. W., 1982, Heteropolymer filaments of vimentin and desmin in vascular smooth muscle tissue and cultured hamster kidney cells demonstrated by chemical crosslinking, *Proc. Natl. Acad. Sci. USA* **79**:3452.

Quinlan, R. A., and Franke, W. W., 1983, Molecular interactions in intermediate-sized filaments revealed by chemical crosslinking, *Eur. J. Biochem.* **132**:477.

Quinlan, R. A., Cohlberg, J. A., Schiller, D. L., Hatzfeld, M., and Franke, W. W., 1984, Heterotypic tetramer (A_2D_2) complex of non-epidermal keratins isolated from the cytoskeletons of rat hepatocytes and hepatoma cells, *J. Mol. Biol.* **178**:365.

Renner, W., Franke, W. W., Schmid, E., Geisler, N., Weber, K., and Mandelkow, E., 1981, Reconstitution of intermediate-sized filaments from denatured monomeric vimentin, *J. Mol. Biol.* **149**:285.

Rueger, D-C., Huston, J. S., Dahl, D., and Bignami, A., 1979, Formation of 100 Å filaments from purified glial fibrillary acidic protein *in vitro*, *J. Mol. Biol.* **137**:53.

Sharp, G., Shaw, G., and Weber, K., 1982, Immunoelectronmicroscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurones, *Exp. Cell Res.* **137**:403.

Skerrow, D., Matoltsy, A. G., and Matoltsy, M. N., 1973, Isolation and characterization of the α-helical regions of epidermal prekeratin, *J. Biol. Chem.* **248**:4820.

Sodek, J., Hodges, R. S., Smillie, L. B., and Jurasek, L., 1972, Amino-acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure, *Proc. Natl. Acad. Sci. USA* **69**:3800.

Sparrow, L. G., and Inglis, A. S., 1980, Characterization of the cyanogen bromide peptides of component 7c, a major microfibrillar protein from wool in: *Proceedings of the 6th Quinquennial International Wool Textile Research Conference*, Pretoria, Vol. 2, pp. 237–246.

Steinert, P. M., 1978, Structure of the three-chain unit of the bovine epidermal keratin filament, *J. Mol. Biol.* **123**:49.

Steinert, P. M., Idler, W. W., and Zimmermann, S. B., 1976, Self-assembly of bovine epidermal keratin filaments *in vitro*, *J. Mol. Biol.* **108**:547.

Steinert, P. M., Zimmermann, S. B., Starger, J. M., and Goldman, R. D., 1978, Ten-nanometer filaments of hamster BHK-21 cells and epidermal keratin filaments have similar structures, *Proc. Natl. Acad. Sci. USA* **75**:6098.

Steinert, P. M., Idler, W. W., and Goldman, R. D., 1980, Intermediate filaments of baby hamster kidney (BHK-21) cells and bovine epidermal keratinocytes have similar ultrastructures and subunit structures, *Proc. Natl. Acad. Sci. USA* **77**:4534.

Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. D., 1981, *In vitro* assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells, *Proc. Natl. Acad. Sci. USA* **78**:3692.

Steinert, P. M., Rice, R. H., Roop, D. R., Trus, B. L., and Steven, A. C., 1983, Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments, *Nature* **302**:794.

Steinert, P. M., Parry, D. A. D., Racoosin, E. L., Joller, W. W., Steven, A. C., Trus, B. L., and Roop, D. R., 1984, The complete cDNA and deduced amino acid sequences of a type II mouse epidermal keratin of 60,000 Da: Analysis of the sequence differences between type I and type II keratins, *Proc. Natl. Acad. Sci. USA* **81**:5709.

Steven, A. C., Wall, J., Hainfield, J. F., and Steinert, P. M., 1982, Structure of fibroblastic intermediate filaments: analysis by scanning transmission electron microscopy, *Proc. Natl. Acad. Sci. USA* **79**:3101.

Steven, A. C., Hainfield, J. F., Trus, B. L., Wall, J. S., and Steinert, P. M., 1983, The distribution of mass in heteropolymer intermediate filaments assembled *in vitro*, *J. Cell Biol.* **97**:1939.

Stromer, M. H., Huiatt, T. W., Richardson, F. L., and Robson, R. M., 1981, Disassembly of synthetic 10-nm filaments from smooth muscle into protofilaments, *Eur. J. Cell Biol.* **25**:136.

Traub, P., and Vorgias, C. E., 1983, Involvement of the *N*-terminal polypeptide of vimentin in the formation of intermediate filaments, *J. Cell Sci.* **63**:43.

Traub, P., and Vorgias, C. E., 1984, Differential effect of arginine modification with 1,2-cyclohexanedione on the capacity of vimentin and desmin to assemble into intermediate filaments and to bind to nucleic acids, *J. Cell Sci.* **65**:1.

Weber, K., and Geisler, N., 1982, The structural relation between intermediate filament proteins in living cells and the α-keratins of sheep wool, *EMBO J.* **1**:1155.

Weber, K., and Geisler, N., 1983, Proteolysis of the neurofilament 68 kDa protein explains several previously described brain proteins of unique composition and high acidity, *FEBS Lett.* **164**:129.

Weber, K., and Geisler, N., 1984, Intermediate filaments—From wool α-keratins to neurofilaments: a structural overview, in: *Cancer Cells*, Vol. 1, *The Transformed Phenotype*, pp. 153–159, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Willard, M. B., and Simon, C., 1981, Antibody decoration of neurofilaments, *J. Cell Biol.* **89**:198.

Woods, E. F., and Gruen, L. C., 1981, Structural studies on the microfibrillar proteins of wool: characterization of the α-helix-rich particle produced by chymotryptic digestion, *Austr. J. Biol. Sci.* **34**:515.

3

The Structure and Evolution of Intermediate Filament Genes

Dennis R. Roop and Peter M. Steinert

1. Introduction

Intermediate filaments (IF) are major cytoskeletal components of most eukaryotic cells (Lazarides, 1980; Steinert *et al.*, 1984a). They have been classified into at least five distinct subclasses, each of which contains from 1 to 30 subunits encoded by separate genes (Lazarides, 1980; Fuchs and Hanakoglu, 1983). These genes are differentially expressed in different tissues and during different stages of differentiation (Lazarides, 1980; Steinert *et al.*, 1984a; Fuchs and Hanukoglu, 1983; Moll *et al.*, 1982; Eichner *et al.*, 1984; Roop *et al.*, 1984b). We have isolated and characterized cDNA clones corresponding to the major keratins synthesized in mouse epidermis (Roop *et al.*, 1983, 1985b). Several lines of evidence are presented which suggest that the expression of subsets of keratin genes is coordinately regulated and dependent on the state of differentiation. Analysis of amino acid sequence data deduced for these keratin subunits (Steinert *et al.*, 1983, 1984b, 1985a) has revealed fundamental differences in the primary sequences of keratin subunits that are expressed at different states of differentiation that may alter the properties and function of filaments containing these subunits (Steinert *et al.*, 1985a). In addition, a comparison of these amino acid sequences with those of other IF subunits (Hanakoglu and Fuchs, 1982, 1983; Quax *et al.*, 1983; Geisler and Weber, 1982; Lewis *et al.*, 1984) has shown that all IF subunits possess a common secondary structure, consisting of a conserved central α-helical rod domain

Dennis R. Roop • Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. ***Peter M. Steinert*** • Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205.

and non-α-helical end domains of variable size and sequence. The rod domain sequences have been divided into three distinct sequence types (Steinert *et al.*, 1985b): the acidic keratins form type I, the neutral–basic keratins form type II; and desmin, vimentin, and glial fibrillary acidic protein form type III. Insufficient sequence data precludes a similar classification of the neurofilament IF subunits. To explore the evolutionary relationship of these conserved and variable domains of IF, we compared the genomic structure of the mouse 59-kD keratin (Krieg *et al.*, 1985), a type I IF gene; the human 52-kD keratin (Marchuk *et al.*, 1984), a type I IF gene; the human 67-kD keratin (Johnson *et al.*, 1985), a type II IF gene; and hamster vimentin (Quax *et al.*, 1983), a type III IF gene. On the basis of these data, we propose a model for the evolution of IF genes.

1.1. Correlation of Keratin Gene Expression with Differentiation State

Of all the IF subclasses, the keratins of epithelia are by far the most complex. Perhaps as many as 30 different keratin subunits are expressed in different epithilial tissues of higher vertebrates.

The major keratins synthesized by newborn mouse epidermis are 67-, 60-, 59-, and 55-kD (Roop *et al.*, 1983). Placing newborn mouse epidermal cells in culture in medium containing low Ca^{2+} results in growth as a monolayer without stratification. These cells proliferate rapidly and display many characteristics associated with basal cells found in intact epidermis (Yuspa *et al.*, 1980). The major keratins synthesized by these cells are 60-, 59.5-, 55-, and 50-kD. In order to isolate cDNA clones for all the major keratins synthesized by epidermal cells, we prepared cDNA libraries from poly (A) RNA isolated from newborn mouse epidermis and primary cultures of mouse epidermal cells. We were able to identify and characterize cDNA clones corresponding to the 67- and 59-kD keratins synthesized *in vivo* and the 60-, 55-, and 50-kD keratins synthesized *in vitro* (Roop *et al.*, 1983, 1985b). Clones for the 59.5-kD keratin have not been characterized.

Although mRNAs coding for these keratin proteins share some sequence homology owing to common structural features (summarized in Section 1.2), it is possible to prepare very specific probes for each keratin mRNA by subcloning regions of the cDNAs corresponding to the 3′ noncoding region of each mRNA. When these cDNA clones are labeled by nick translation with ^{32}P and hybridized to RNA that was isolated from newborn mouse epidermis or primary cultures of mouse epidermal cells and blotted onto nitrocellulose paper, it is quite evident that the 59- and 67-kD keratin genes are not expressed at all or only at very low levels in primary epidermal cell cultures compared to newborn epidermis. Transcripts complementary to the cDNA clones isolated from the *in vitro* library, the 50-, 55-, and 60-kD keratins, are present in RNA isolated from intact epidermis but at reduced concentrations compared to those found in cell culture (Roop *et al.*, 1985b). These results presumably reflect the relative contribution of RNA in basal cells (which

consists of a single cell layer) to that of total epidermis (which consists of many cell layers at different stages of differentiation) and indicate that the 50-, 55-, and 60-kD keratin genes are predominantly expressed in proliferating basal cells and that the 59- and 67-kD keratins are predominantly expressed in differentiated cells.

Recently, we have obtained two lines of evidence to support this hypothesis. First, we have been able to produce antisera that are monospecific for the 55-, 60-, 59-, and 67-kD keratin subunits using synthetic peptides corresponding to unique sequences located at their C-termini (Roop *et al.*, 1984a, 1985a). These antisera have been used to localize these subunits within newborn mouse epidermis by indirect immunofluorescence. The 59- and 67-kD subunits were only present within the differentiated cells of the epidermis (the suprabasal layers) and not in the basal layer. The 55- and 60-kD subunits, however, were detected in both the basal and suprabasal layers. Second, we have recently been able to detect mRNA coding for different keratin subunits within cells in different layers of the epidermis by *in situ* hybridization to histological sections of newborn mouse skin (D. R. Roop, unpublished data). We have subcloned the keratin cDNAs into newly developed vectors that permit the synthesis of RNA transcripts that can be labeled with ^{35}S using [^{35}S]uridine 5′-(α-thio)-triphosphate. Insertion of the cDNAs into the vector in the correct orientation and subsequent transcription results in the synthesis of transcripts that are complementary to mRNA. In our initial experiments, we have been able to localize the majority of the mRNAs coding for the 55- and 60-kD keratins to the basal layer of the epidermis. Very little of these mRNAs is found in the differentiated cell layers, suggesting that expression of these keratin genes is "suppressed" as cells differentiate and migrate into the suprabasal layer. Just the opposite was observed for mRNAs for the 59- and 67-kD keratins; i.e., these mRNAs were predominantly localized within the differentiated suprabasal layers and not the basal layer.

On the basis of these combined data, we believe that the expression of specific subsets of keratin genes is correlated with the state of differentiation, and we consider expression of the 50-, 55-, and 60-kD keratin genes to be associated with proliferation and expression of the 59- and 67-kD keratin genes to be associated with differentiation.

1.2. Correlation of Keratin Subunit Structure with Differential Expression

The determination of the primary sequence of keratin proteins by classical protein sequencing techniques has not been feasible because of their insolubility. The availability of keratin cDNA clones provided the first opportunity to determine the amino acid sequence of these proteins. We have deduced the complete amino acid sequence of the 59- and 60-kD keratins and approximately two-thirds of the sequence of the 50-, 55-, and 67-kD keratins from the nucleotide sequence of their cloned cDNAs (Steinert *et al.*, 1983, 1984b, 1985a). This sequence information has enabled us to make several fundamen-

tal observations concerning the subunit structure of keratin proteins and to formulate a hypothesis explaining the requirement for expression of a subset of keratin genes during terminal differentiation (Steinert *et al.*, 1985a).

Comparison of these amino acid sequences with those of other IF subunits (Hanakoglu and Fuchs, 1982, 1983; Quax *et al.*, 1983; Geisler and Weber, 1982; Lewis *et al.*, 1984) has allowed us to propose the following model for IF subunit structure (Fig. 1). All IF subunits contain a central α-helical rod domain of highly conserved size and secondary structure, containing four distinct tracts (1A, 1B, 2A, and 2B) that can form a coiled-coil α-helical structure, because they contain a characteristic 7-residue (heptad) repeating sequence (Steinert *et al.*, 1985a). These four tracts are interrupted by three non-coiled-coil regions. Comparisons of the exact amino acid sequences of the rod domain of different IF subunits show, however, that they are three distinct sequence types: acidic keratin subunits form type I, neutral–basic keratin subunits form type II, and desmin, vimentin, and glial fibrillary acidic protein form type III. The presence of several more basic charges in the

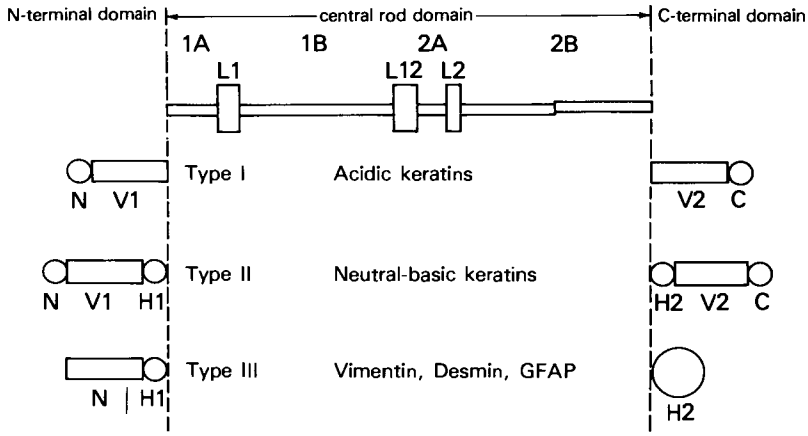


Figure 1. IF subunit structure. All IF subunits possess a central α-helical rod domain flanked by end domains. The rod domain in all cases consists of four segments of invariant size. These are composed of a seven-residue (heptad) quasirepeat, which can form a coiled-coil structure. Segment 1A is 35 amino acids long, 1B is 101, 2A is 19, and 2B is 121. These are separated by non-coiled-coil linkers: L1 varies from 8 to 14 residues in different subunits; L12 is 16 or 17, and L2 is always 8. At the middle of segment 2B, the polarity of the progression of heptads is abruptly reversed, a feature that will impose a "stutter" in the regularity of the coiled-coil at this point. Despite these conserved features, the exact sequences of the heptads in the segments differ and permit classification into three distinct sequence types. Each sequence type of rod domain is coupled with a specific set of end domains, which, as shown, may be further subdivided into subdomains based on homologous (H) sequences, variable (V) sequences, and basic terminal (N or C) sequences. In types I and II keratin IF subunits, the very basic N and C subdomains are 15–30 residues long, the highly variable V1 and V2 subdomains may vary from 0 to 130 residues. The homologous sequences of the H1 and H2 subdomains of type II keratins are 36 and 20 residues long, respectively. In type III IF subunits, H1 and H2 are 20 and 55 residues long; V1 varies from 60 to 75 residues.

coiled-coil tract 2B of type II keratins probably accounts for the overall basic charge of type II keratins.

In terms of this classification system, terminally differentiating epidermal cells express the 67 (type II) and 59 (type I) pair of keratins. Basal epidermal cells proliferating in culture express the 60 (type II) and 55 (type I) pair and the 59.5 (type II) and 50 (type I) pair. The structural and functional rationale for the coexpression of these characteristic pairs of keratins is not yet known. Clearly, however, both type I and type II keratins are required for IF formation *in vivo*, as was earlier suggested from *in vitro* assembly experiments (Steinert *et al.*, 1985b).

The central rod domain is flanked bilaterally by end domains, which can be divided into subdomains. Type II but not type I keratins contain short globular sequences, termed H1 and H2, immediately adjacent to the rod domain. These have been conserved in size and sequence. The absence of the H1 and H2 subdomains from type I keratins is the primary reason for the difference in mass of about 6–9 kD between type I and type II keratins of a coexpressed pair (e.g., the 59- and 67-kD keratins coexpressed in differentiated epidermal cells). Type III IF also contain a short H1 but a larger H2 subdomain, which comprises the entire C-terminal end domain, but these differ in sequence from those of type II IF. The keratins contain subdomains V1 and V2 that are highly variable in both length and amino acid sequence. The variability in mass among keratins of a given type (e.g., the 60- and 67-kD keratins are both type II) appears to reside in the size of their V1 and V2 subdomains. V1 and V2 subdomains often contain tandem peptide inexact repeats that are conspicuously rich in glycines and/or serines and conform to XY_n, where X = an apolar amino acid; Y = serine, glycine, and cysteine; and n = 1–9 (Steinert *et al.*, 1985a). Even though these subdomains tend to vary in sequence, pairs of type I and type II keratins that are coexpressed generally have similar V1 and V2 sequences (e.g., the type I and type II keratins expressed in basal cells contain peptide repeats enriched in serines, and the type I and type II keratins expressed in differentiated cells contain peptide repeats enriched in glycine). Variations in these end domain sequences in keratins that are expressed at different states of differentiation implicate their involvement in changes in keratin filament properties and function during differentiation.

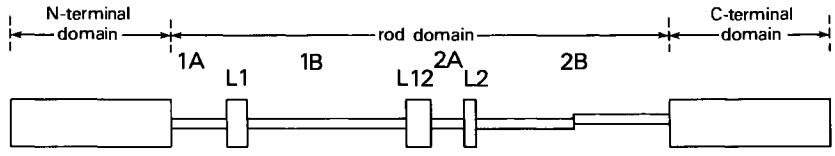
At the termini are strongly basic subdomains (N and C, respectively) that are variable in sequence. The unique sequence of the C-terminal residues and their probable accessible location on the periphery of keratin filaments (Steinert *et al.*, 1983) have been exploited to produce antisera that were monospecific for individual keratin subunits (Roop *et al.*, 1984a, 1985a).

2. *Structure of Intermediate Filament Genes*

The complete structure of four different IF genes has been determined. Initially the sequence of the hamster vimentin gene (type III) was reported

(Quax *et al.*, 1983). Marchuk *et al.* (1984) have recently sequenced the human 52-kD keratin gene (type I), and we have determined the structure of the human 67-kD keratin gene (type II) (Johnson *et al.*, 1985) and the mouse 59-kD keratin gene (type I) (Krieg *et al.*, 1985). A comparison of the organization of these IF genes is shown in Fig. 2. There are seven or eight introns within each gene, and most of these occur within the region encoding sequences predicted to form the coiled-coil rod domain. For clarity, we have indicated the location of introns with respect to our model for IF subunit structure (Fig. 2). Three of the introns occur in identical locations in all four genes. The location of two other introns, one near the beginning and the other at the end of the 2B segment, is highly conserved (Fig. 2). Each IF gene type also possesses one or two unique introns in the rod domain and one or two other introns in regions that encode the C-terminal but not the N-terminal domain.

It has previously been hypothesized that the intron/exon structure of eukaryotic genes reveals their evolutionary history and suggests that the genes evolved by recruitment and combination of small segments encoding distinct functional domains (Gilbert, 1978). In the case of IF genes, some of the introns within all three IF types are found at or near positions defining functional domains of these proteins. One is located near the end of the coiled-coil tract 1B, one near the beginning of the 2B tract, and the third at the end of the 2B tract. Another intron, unique to the mouse 59-kD keratin gene, occurs between the non-α-helical carboxyl terminal end domain and the 3′ noncoding sequence. The other introns located within the coiled-coil tracts do not appear to delineate obvious functional domains. However, further examination reveals that many of these introns are located at the beginning of the characteristic seven-residue heptad repeat that defines the coiled-coil



Mouse keratin 59kD (I)	–	14	42	–	94	4	-	46	119	–	–	113
Human keratin 52kD (I)	–	14	42	–	94	4	-	46	120	15	–	–
Human keratin 67kD (II)	17	–	42	62	94	-	7	46	119	12	–	–
Hamster vimentin 53kD (III)	–	–	42	62	94	4	-	46	120	14	42	–

Figure 2. Location of introns in three types of IF genes. Using the universal subunit domain model, arrows indicate the positions of introns in the four genes for which information is available. The numbers refer to the residue number along a coiled-coil segment. In many cases, the introns splice within the codon for the amino acid so numbered. Arrows with closed circles mark those introns which splice at the first residue of the heptad repeat. The smallest exon encoding continguous coiled-coil sequences occurs in segment 1B in type I keratins. —, No intron. References for data are given in text. Redrawn with permission from Krieg *et al.* (1985).

tracts. All these data suggest that the genes of these three IF types arose from a common ancestor and that the primordial IF gene was assembled from exons containing multiple heptad repeats (Krieg *et al.*, 1985).

3. A Model for the Evolution of Intermediate Filament Genes

3.1. Evolution of the Rod Domains of Intermediate Filament Genes

3.1.1. Evolution of the Primordial Gene

The nonrandom occurrence of introns between heptad repeats within the coiled-coil tracts, e.g., four of six in type I IF genes, three of six in the type II IF gene, and three of six in the type III IF gene (Fig. 2), may provide clues for the origin of the rod domain of intermediate filament genes. The smallest exon observed in IF genes to date that encodes exact heptad repeats is 84 base pairs (bp) in length and is located between positions 14 and 42 within the 1B tract of the type I IF genes. We suggest that the sequences encoding multiple heptads were generated by amplification of a simple DNA sequence. The length of the original sequence is uncertain, but it may have encoded only a single heptad (21 bp). The 84-bp exon, which is the smallest remnant of an amplified unit encoding multiple heptads, could have been generated by tandem amplification of a shorter sequence. The amplification of simple DNA sequences has previously been suggested to play an important role in the evolution of the genes encoding collagens (Yamada *et al.*, 1980), immunoglobulins (Ohno *et al.*, 1982), and albumin:α-fetoprotein (Alexander *et al.*, 1984).

Several models are possible for the evolution of the coiled-coil domain of IF genes. For simplicity we propose that the promordial IF gene encoded a polypeptide consisting only of sequences capable of forming a coiled-coil and was assembled from 84-bp exons, which encoded four heptad repeats (Fig. 3). The choice of this size of exon for the original building block is attractive, not only because this is consistent with the smallest exon-encoding heptad repeats identified to date, but also because a 28-residue repeat had been detected in the rod domain sequences of all IF subunits (Parry *et al.*, 1977; Crewther *et al.*, 1983). In addition, physical structural experiments employing synthetic peptides have indicated that a minimum of four heptads (28 residues) is required to form a stable two-chain coiled-coil *in vitro* (Lau *et al.*, 1984).

The presence of intervening sequences early during the formation of the primordial gene is implicit with this model. We suggested previously that the 84-bp exon could have arisen by tandem amplification of a shorter sequence. Numerous other amplification events would have been required to generate the entire coiled-coil domain; however, this model suggests that these were not tandem duplication events. Nontandem duplication of the 84-bp repeat initially and perhaps at later steps could explain the early inclusion of intervening sequences within the rod domain. It is unlikely that these introns were

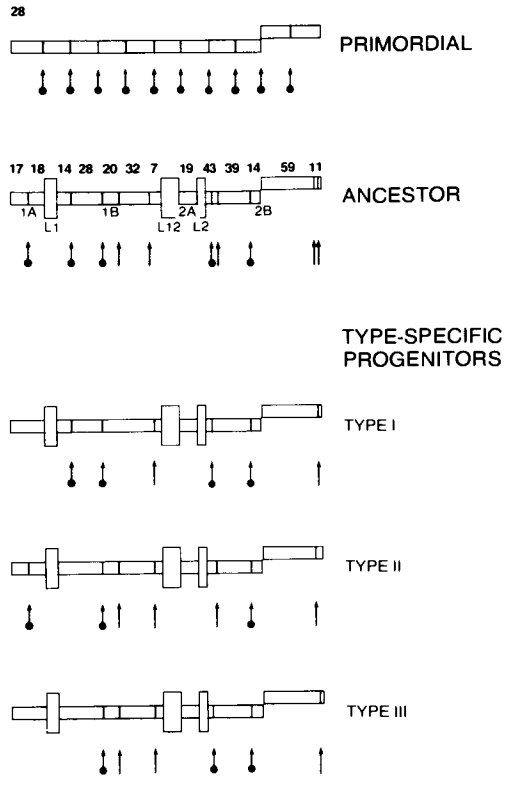


Figure 3. A model for the evolution of the rod domain of IF genes. We propose that the primordial IF gene was assembled from multiple heptad repeats, consisting of four heptads or 28 amino acid residues. The heptad polarity discontinuity may have been introduced at this step. Each 28-residue repeat was separated by an intron. Subsequent events, possibly sliding of intron–exon junctions, generated an ancestral gene that contained three interruptions in the regularity in the heptads (L1, L12, L2) and gave rise to four discrete segments (1A, 1B, 2A, 2B) that can form a coiled-coil rod domain. Duplication and divergence of this ancestral gene formed the type-specific progenitors.

inserted into the coding region of the rod domain after the formation of the primordial gene since their nonrandom locations, i.e., between heptad repeats, argues against an insertional model for their origin (Lonberg and Gilbert, 1985). The presence of intervening sequences within the coiled-coil region may function to stabilize the precise length of this domain, which is highly conserved in all IF subunits sequenced to date (Steinert *et al.*, 1985b), by inhibiting further amplification. A stabilization function for introns within genes encoding repetitive polypeptides, which cannot accommodate size variation, was first suggested by Seidman *et al.* (1978). The reversal of the heptad polarity (see Fig. 1), which has been observed in the 2B domain of all IF subunits sequenced to date (Steinert *et al.*, 1985b), probably was generated by a recombination event during the formation of the primordial gene.

3.1.2. Evolution of the Ancestral Gene

As discussed in Section 1.2, all IF subunits sequenced to date contain three nonhelical interruptions at identical locations within their rod domains (Steinert *et al.*, 1985b). We proposed that these disruptions in the rod domain occurred in an ancestral gene prior to duplication events to form the various

IF type-specific progenitors (Fig. 3). The rigid conservation of the location and size of these non-coiled-coil interruptions (Steinert *et al.*,1985b) throughout further evolution indicates that they provided some important evolutionary advantage such as filament formation and stability. The mechanism generating these disruptions is obviously not known. A possible explanation enabling sequences encoding residues incapable of forming coiled-coils to be included into the rod domain would involve sliding intron–exon junctions. A mutation at a splice junction resulting in the loss of a splice site and subsequent splicing at an alternate or cryptic site within the intron would result in an extension in the length of an exon with sequences disrupting the coiled-coil. Craik *et al.* (1983) originally proposed the sliding intron–exon junction model as a mechanism for generating length polymorphisms and divergent sequences in protein families.

From this model for the evolution of the primordial gene we predict that the ancestral gene lost some of the introns originally present in the primordial gene since introns are missing from these positions in all IF genes sequenced to date; e.g., introns proposed to exist in the primordial gene in the 2A and 2B coiled-coil tracts (Fig. 3) are absent from the IF genes shown in Fig. 2. Mechanisms apparently exist for the precise excision of intervening sequences without disturbing coding sequences since such deletions have been reported for the rat insulin gene (Lomedico *et al.*, 1979) and for the chicken proα 1 (I) collagen gene (Yamada *et al.*, 1980).

3.1.3. Evolution of the Type-Specific Progenitors

At least two early duplication events would have been required to generate three progenitor genes (Fig. 3). The subsequent divergence of the exons of these three genes would have resulted in variations at the amino acid sequence level to give the IF subunits encoded by these genes their type-specific properties (see Section 1.2). The selective loss of certain introns by the deletion mechanism discussed in Section 3.1.2 could have produced the type-specific intron patterns shown in Fig. 2. The variability in the location of the intron at the end of the 2B coiled-coil tract suggests that this splice junction can shift slightly. Close examination of the sequences at this junction for the mouse 59-kD keratin gene, the human 67-kD keratin gene, and the hamster vimentin gene indicate that this splice site has been arbitrarily placed at either position 119 or 120 to provide introns with consensus donor and acceptor sequences (Mount, 1982).

Additional duplications of the type-specific progenitors would have been required to produce the various members within each IF type. If this model is correct, one would predict that the genes encoding subunits of the same type would be more highly conserved and possibly linked. Interestingly, in the two type I keratin genes that have been sequenced (Marchuk *et al.*, 1984; Krieg *et al.*, 1985) both contain six introns within the rod domain; five are located in identical positions (Fig. 2); and the sixth intron is located at the end of the 2B coiled-coil tract and has been positioned at 119 in the mouse gene and 120 in

the human gene. The sequence of only one type II IF gene (the human 67-kD keratin) has been reported (Johnson *et al.*, 1985). However, an estimate of the organization of several bovine type II keratin genes by R-loop analysis (Lehnert *et al.*, 1984) indicates that each has 7 introns within the rod domain and these are in approximately the same positions as those of the human 67-kD keratin gene (Fig. 2).

Little is known concerning the linkage of genes of the same IF type. Recent data, obtained by restriction endonuclease polymorphism analysis, have shown that at least two mouse type II keratin genes are located on chromosome 15 and that they are linked (C. Blatt and D. Roop, unpublished data).

3.2. Evolution of the End Domains

To this point, we have only discussed a mechanism for the evolution of the highly conserved rod domain of IF. We must now consider a mechanism for the evolution of the highly variable end domains of IF. This discussion will be limited to the end domains of the keratin subunits (type I and type II IF) since more information is available for consideration. As discussed in Section 1.2, the amino and carboxyl end domains can be divided into the following subdomains: N, V1, H1, H2, V2, C (see Fig. 1). All keratin subunits possess N and C subdomains. The H1 and H2 subdomain are present only in type II keratin subunits. The size of the V1 and V2 subdomains is directly proportional to the size of the keratin subunits containing them (Table 1).

In the keratin genes examined so far, there are no introns in the N-terminal domain and those that do occur in the C-terminal domain do not delineate apparent subdomains. However, on the basis of existing information, it seems reasonable to propose that the following events occurred prior to duplication of the type-specific progenitors to form the additional mem-

Table 1. Numbers of Amino Acids in End-Terminal Subdomains of Keratin IF Subunits[a]

	Aminoterminal				Carboxylterminal		
	N	V1	H1	Rod domain	H2	V2	C
Wool 8c 46 kd (1)	32	—	—	311	—	—	28
Mouse 50 kd (1)	n.k.[b]	n.k.	—	311	—	19	27
Human 52 kd (1)	15	100	—	311	—	19	27
Mouse 55 kd (1)	n.k.	n.k.	—	311	—	25	27
Mouse 59 kd (1)	19	123	—	314	—	104	9
Human 56 kd (11)	n.k.	n.k.	n.k.	313	20	48	20
Mouse 60 kd (11)	26	89	36	313	20	48	20
Human 67 kd (11)	37	106	36	313	20	109	22
Mouse 67 kd (11)	n.k.	n.k.	36	313	20	121	21

[a]Data from Steinert *et al.*, 1985b.
[b]n.k. = not known.

bers of each type. First, recruitment of the H1 and H2 subdomains for the type II progenitor. These subdomains are present on either side of the rod domain of all type II keratin subunits sequenced thus far, and they have been highly conserved in size and sequence throughout further evolution, implicating an important role in filament assembly and/or stability. Second, recruitment of the V1 and V2 subdomains for both type I and type II progenitors. The initial V subdomains were presumably short and may have been extended in length during evolution by amplification events (see below). Third, recruitment of the N and C subdomains for both type I and type II progenitors. These subdomains have been allowed to diverge extensively and are highly variable in both size and sequence.

In Section 1.1 we suggested that the requirement for the expression of a new subset of keratin genes during terminal differentiation may be correlated with a specialized function for these new keratin subunits. As the complexity of an epithelium increases from simple to stratified-squamous to terminally differentiated, there is an increase in the size of the keratin subunits expressed (Sun *et al.*, 1984). We now know that this is due to corresponding increases in the size of the V subdomains (Steinert *et al.*, 1985a). Accordingly, we propose that as more complex epithelia evolved, the requirement for IF to perform more specialized functions was achieved by progressively enlarging the V subdomains and allowing these to diverge (Fig. 4).

It seems most likely that sequences within the V subdomains were generated by amplification of a simple sequence, as suggested for the rod domain, since there are numerous tandem peptide repeats within these subdomains (Steinert *et al.*, 1983, 1985a). Periodic amplification of these sequences during evolution could have allowed these subdomains to enlarge. Smaller keratin subunits contain V1 subdomains that are longer than their corresponding V2 subdomains (Steinert *et al.*, 1985a). This suggests that the V1 subdomain may have increased in size first. There is considerable variation in these sequences

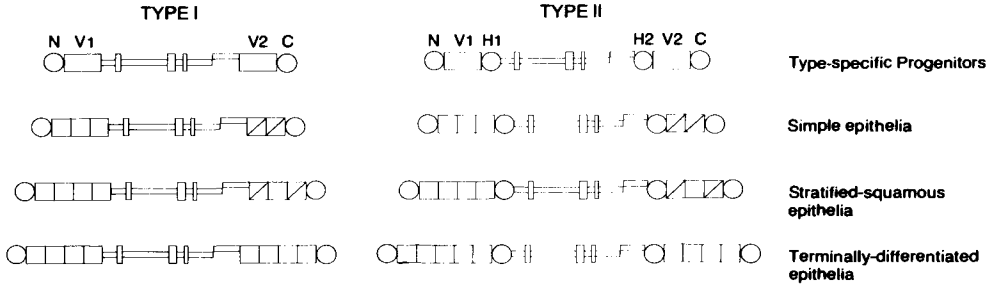


Figure 4. A model for the evolution of end domains of IF genes. We propose that the various subdomains (H1, H2, V1, V2, N, and C) were recruited onto type-specific progenitors. First, the H1 and H2 subdomains were recruited onto the type II progenitor. Second, the V subdomains were recruited onto both the type I and type II progenitors. Finally, the N and C subdomains were recruited onto both the type I and type II progenitors. The V subdomains evolved in size and sequence with the complexity of the epithelium. Open boxes are enriched in glycines and boxes with diagional lines are enriched in serines.

in different subunits, presumably resulting from selective pressure to perform more specialized functions. However, a consensus sequence for the original repeat is evident (Table 2). The repeats conform to XY_n where X = apolar (most commonly, F, Y, I), Y = G, S, or C, and n = 1–9. It is of interest to note that many of the changes in X and Y could easily be made by a single base change in the most commonly used codons (Table 2).

We have proposed that variations in the sequences within the V subdomains of subunits expressed in different epithelia are consistent with the properties of the filaments that they form (Steinert *et al.*, 1983, 1985a). For example, the keratin subunits expressed in simple epithelia contain fewer glycines in their V1 and no glycines in their V2 subdomains, which are enriched in serines instead. This suggests that the Y position of the XY_n repeat may have been serine in the type-specific progenitor keratin genes. The keratin IF formed by these subunits are presumably more flexible and soluble than those formed in more differentiated epithelia. As the complexity of epithelia increases, the V subdomains of the keratin subunits expressed in them not only increase in size but also change in sequence. In general, the serine content decreases and the glycine content increases. This contributes to a progressively more insoluble IF network in the more differentiated epithelial cells. In the case of terminally differentiated epidermis, we have proposed that these unusual V1 and V2 sequences interact with an epidermal matrix protein, filaggrin, to form an insoluble, yet flexible, structure that constitutes a major portion of the protective barrier of the outer layer of the skin (Steinert *et al.*, 1985a). The end domains of the keratins of hair, nail, etc.

Table 2. Peptide Repeats in V Subdomains of Keratin IF Subunits[a]

Simple epithelia		
V1	F/Y/I (G or S)$_{2-9}$	Common
V2	Apolar (S)$_{1-5}$	Common
	Apolar (G or S)$_{3-9}$	Rare
	where apolar = A, V, I, L, M, F, or Y	
Terminally Differentiating Epidermis		
V1	F (G)$_{1-8}$	Common
	F/Y (G or S)$_{1-6}$	Common
	Y (G)$_{1-6}$	Rare
	F/Y/I (G or S)$_{1-4}$	Rare
V2	Y (G/S/C)$_{3-9}$	Common
Conclusion		
Repeats conform to XY_n		
where X = apolar; Y = G or S or C; n = 1–9		
Notes		
Most commonly used codons:	F TTT/C	
	Y TAT/C	
	I ATT/C	
	G GGC	
	S AGC	
	C TGC	

[a]Data from Steinert *et al.*, 1985b.

differ considerably from the end domains of the epidermal keratins in that they contain highly folded cysteine-rich sequences of a flexible nature. It has been suggested that these form extensive disulfide bond cross-links with the cysteine-rich matrix (high-sulfur) proteins (Gillespie, 1983), and this would contribute to an insoluble and more rigid structure (Steinert *et al.*, 1985a). It should be reemphasized that the changes from serine to glycine or cysteine could easily be achieved by a single base change in the first position of their most commonly used codons (Table 2).

As discussed previously, we have reported that type I and type II keratin subunits that are coexpressed during differentiation possess end domains that have very similar V subdomains (Steinert *et al.*, 1985a). How did this recruitment of similar V1 and V2 subdomains onto type I and type II progenitor IF genes occur? We suggest two possible mechanisms. In the first, identical simple V subdomains were initially recruited onto both type I and type II-specific progenitors (Fig. 4) and subsequently evolved along with the type-specific rod domain. Alternatively, the V subdomains may have evolved in a separate location in the genome and were later recruited into coexpressed type I and type II IF genes. Both mechanisms seem plausible, and an analysis of additional sequence information for other IF genes will be required to determine which is correct.

4. Conclusion

The recent expansion of our knowledge of the expression and structure of IF genes, and in particular those of the keratins, has enabled us to propose a straightforward mechanism for the evolution of IF genes. The central rod domain may have formed by the amplification of a simple DNA sequence encoding multiple heptad repeats. Duplication and subsequent divergence of this primordial gene generated at least three type-specific progenitor genes and each of their various members. The various end domains, which define the function of the IF in which they are present, may also have evolved by amplification of simple sequences either in a separate location of the genome, and were later inserted, or concomitantly with individual type-specific genes. The isolation and characterization of further IF genes will be required to test and refine this model.

ACKNOWLEDGMENTS. We thank Dr. Stuart Yuspa for his critical review of this manuscript. We are especially grateful for the collaborative support of our colleagues Drs. Leslye Johnson, Thomas Krieg, Rune Toftgard, and our assistants, Christina Cheng and Will Idler.

References

Alexander, F., Young, P. R., and Tilghman, S. M., 1984, Evolution of the albumin:α-fetoprotein ancestral gene from the amplification of a 27 nucleotide sequence, *J. Mol. Biol.* **173:**159–176.

Craik, C. S., Rutter, W. J., and Fletterick, R., 1983, Splice junctions: Association with variation in protein structure, *Science* **220:**1125–1129.

Crewther, W. G., Dowling, L. M., Steinert, P. M., and Parry, D. A. D., 1983, The structure of intermediate filaments, *Int. J. Biol. Macromol.* **5:**267–274.

Eichner, R., Bonitz, P., and Sun, T.-T., 1984, Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression, *J. Cell Biol.* **98:**1388–1396.

Fuchs, E., and Hanakoglu, I., 1983, Unraveling the structure of the intermediate filaments, *Cell* **34:**332–334.

Geisler, N., and Weber, K., 1982, The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins, *EMBO J.* **1:**1649–1656.

Gilbert, W., 1978, Why genes in pieces? *Nature* **271:**501.

Gillespie, J. M., 1983, The structural proteins of hair: Isolation, characterization and regulation of biosynthesis, in: *Biochemistry and Physiology of the Skin* (L. A. Goldsmith, ed.), pp. 475–510, Oxford University Press, New York.

Hanukoglu, I., and Fuchs, E., 1982, The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins, *Cell* **31:**243–252.

Hanukoglu, I., and Fuchs, E., 1983, The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratins, *Cell* **33:**915–924.

Johnson, L. D., Idler, W. W., Zhou, X-M., Roop, D. R., and Steinert, P. M., 1985, The structure of the gene for the human epidermal keratin of 67,000 molecular weight, *Proc. Natl. Acad. Sci. USA* **82:**1896–1900.

Krieg, T. M., Schafer, M. P., Cheng, C. K., Filpula, D., Flaherty, P., Steinert, P. M., and Roop, D. R., 1985, Organization of a type I keratin gene: Evidence for evolution of intermediate filaments from a common ancestral gene, *J. Biol. Chem.* **260:**5867–5870.

Lau, S. Y. M., Taneja, A. K., and Hodges, R. S., 1984, Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded α-helical coiled-coils, *J. Biol. Chem.* **259:**13253–13261.

Lazarides, E., 1980, Intermediate filaments as mechanical integrators of cellular space, *Nature* **282:**249–256.

Lehnert, M. E., Jorcano, J. L., Hanswalter, Z., Blessing, M., Franz, J. K., and Franke, W. W., 1984, Characterization of bovine epidermal keratin genes: Similarities of exon patterns in genes coding for different keratins, *EMBO J.* **3:**3279–3287.

Lewis, S. A., Balcarek, J. M., Krek, V., Shelanski, M. L., and Cowan, N. J., 1984, Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: Structural conservation of intermediate filaments, *Proc. Natl. Acad. Sci. U.S.A.* **81:**2743–2746.

Lomedico, P., Rosenthal, N., Efstratiadis, Gilbert, W., Kolodner, R., and Tizard, R., 1979, The structure and evolution of the two nonallelic rat preproinsulin genes, *Cell* **18:**545–558.

Lonberg, N., and Gilbert, W., 1985, Intron/exon structure of the chicken pyruvate kinase gene, *Cell* **40:**81–90.

Marchuk, D., McCrohon, S., and Fuchs, E., 1984, Remarkable conservation of structure among intermediate filament genes, *Cell* **39:**491–498.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R., 1982, The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells, *Cell* **31:**11–21.

Mount, S. M., 1982, A catalogue of splice junction sequence, *Nucl. Acid Res.* **10:**459–472.

Ohno, S., Matsunaga, T., and Wallace, R. B., 1982, Identification of the 48-base-long primordial building block sequence of mouse immunoglobulin variable region genes, *Proc. Natl. Acad. Sci. U.S.A.* **77:**1999–2002.

Parry, D. A. D., Crewther, W. G., Fraser, R. D. B., and MacRae, T. P., 1977, Structure of alpha-keratin: structural implications of the amino acid sequences of the type I and type II chain segments, *J. Mol. Biol.* **113:**448–454.

Quax, W., Egberts, W. V., Hendricks, W., Quax-Jeuken, Y., and Bloemendal, H., 1983, The structure of the vimentin gene, *Cell*, **35:**215–223.

Roop, D. R., Hawley-Nelson, P., Cheng, C. K., and Yuspa, S. H., 1983, Keratin gene expression in mouse epidermis and cultured epidermal cells, *Proc. Natl. Acad. Sci. U.S.A.* **80:**716–720.

Roop, D. R., Cheng, C. K., Titterington, L., Meyers, C. A., Stanley, J. R., Steinert, P. M., and Yuspa, S. H., 1984a, Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies, *J. Biol. Chem.* **259:**8037–8040.

Roop, D. R., Toftgard, R., Yuspa, S. H., Kronenberg, M. S., and Clark, J. H., 1984b, Changes in mouse keratin gene expression during differentiation, in: *The Molecular Biology of the Cytoskeleton* (G. G. Borisy, D. W. Cleveland, and D. B. Murphy, eds.), pp. 409–414, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Roop, D. R., Cheng, C. K., Toftgard, R., Stanley, J. R., Steinert, P. M., and Yuspa, S. H., 1985a, The use of cDNA-cones and monospecific antibodies as probes to monitor keratin gene expression, *Ann. NY Acad. Sci.* **455:**426–435.

Roop, D. R., Cheng, C. K., Toftgard, R., and Yuspa, S. H., 1985b, The isolation of cDNA clones corresponding to keratin expressed in basal epidermal cells, (submitted for publication).

Seidman, J. G., Leder, A., Nau, M., Norman, B., and Leder, P., 1978, Antibody diversity: The structure of cloned immunoglobulin genes suggests a mechanism for generating new sequences, *Science* **202:**11–16.

Steinert, P. M., Rice, R. H., Roop, D. R., Trus, B. L., and Steven, A. C., 1983, Complete amino acid sequence of a mouse epidermal keratin subunit: Implications for the structure of intermediate filaments, *Nature* **302:**794–800.

Steinert, P. M., Jones, J. C. R., and Goldman, R. D., 1984a, Intermediate filaments, *J. Cell Biol.* **99:**22s–27s.

Steinert, P. M., Parry, D. A. D., Racoosin, E. L., Idler, W. W., Steven, A. C., Trus, B. L., and Roop, D. R., 1984b, The complete cDNA and deduced amino acid sequence of a Type II mouse epidermal keratin of 60,000 molecular weight: Analysis of sequence differences between Type I and Type II keratins, *Proc. Natl. Acad. Sci. U.S.A.* **81:**5709–5713.

Steinert, P. M., Parry, D. A. D., Idler, W. W., Johnson, L. J., Stevens, A. C., and Roop, D. R., 1985a, Amino acid sequences of mouse and human epidermal type II keratins of M_r 67,000 provide a systematic basis for the structural and functional diversity of the end domains of keratin intermediate filament subunits, *J. Biol. Chem.* **260:**7142–7149.

Steinert, P. M., Steven, A. C., and Roop, D. R., 1985b, The molecular biology of intermediate filaments, *Cell* **42:**411–419.

Sun, T.-T., Eichner, R., Schermer, A., Cooper, D., Nelson, W. G., and Weiss, R. A., 1984, Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model, in: *Cancer Cells 1, The Transformed Phenotype*, pp. 169–176, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Yamada, K., Avvedimento, V. E., Mudryji, M., Ohkubo, H., Vogeli, G., Irani, M., Pastan, I., and de Crombrugghe, B., 1980, The collagen gene: Evidence for its evolutionary assembly by amplification of a DNA segment containing an exon of 54 bp, *Cell* **22:**887–892.

Yuspa, S. H., Hawley-Nelson, P., Stanley, J. R., and Hennings, H., 1980, Epidermal cell culture, *Transpl. Proc.* **12 (suppl 1):**114–122.

4

Differential Expression of the Genes Encoding the Keratins of Cultured Human Epidermal Cells

Elaine Fuchs, Douglas Marchuk, and Angela Tyner

1. Introduction

The epidermis is a stratified squamous epithelium, where the innermost (basal) epidermal layer is the only layer that has the ability to proliferate. Under an as yet unidentified trigger of differentiation, a basal cell will cease to divide, and subsequently, it begins to migrate toward the skin surface. The cells enter the spinous layer and increase steadily in size (Rowden, 1975; Yardley and Goldstein, 1976) as they continue to be active biosynthetically (Fukuyama *et al.*, 1965; Fukuyama and Epstein, 1968). The spinous cells accelerate their synthesis of keratins, the proteins that comprise a major part of the protective cytoskeletal architecture of epidermal cells. These filaments assume a more orderly structure as they loop in and out of the numerous desmosomes that keep the spinous cells in close contact with one another. In the granular layer, the cells are only briefly anabolic (Hoober and Berstein, 1966) and soon enter a destructive phase in which the cytoplasmic organelles, including the nucleus, are lost (Lavker and Matoltsy, 1970). With the aid of a glycine-serine rich basic protein, fillagrin, the keratin filaments aggregate into macrofibrils and are hence resistant to the massive proteolysis that takes place in the granular layer (Dale *et al.*, 1978). As the cells reach the outer (stratum corneum) layer, a proteinaceous envelope cross-linked by γ-glutamyl lysine

Elaine Fuchs, Douglas Marchuk, and Angela Tyner • Departments of Molecular Genetics and Cell Biology, and Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637.

bonds is deposited beneath the plasma membrane, creating a sacculus to contain the bundles of keratin filaments (Rice and Green, 1977; Watt and Green, 1981; Simon and Green, 1984). By the time the cells are sloughed from the surface of the skin, they are merely tough, cellular skeletons with no metabolic activity.

Several laboratories have reported differences in size between the keratins of stratum corneum and those of the living layers of epidermis (Dale and Stern, 1975; Dale *et al.*, 1976; Baden and Lee, 1978; Skerrow and Hunter, 1978). There are also size differences between the keratins of stratum corneum and those of cultured epidermal cells (Sun and Green, 1978). Fuchs and Green (1980) showed that these differences were due to changes in keratin patterns that take place as the cells move from the basal layer outward toward the skin surface (Fig. 1). Cells of the basal epidermal layer contain small keratins (46–58 K), whereas the terminally differentiating cells of the epidermis contain large keratins (55–67 K) (Fuchs and Green, 1980; Bowden and Cunliffe, 1981; Jorcano *et al.*, 1984c; Skerrow and Skerrow, 1983). Plantar and palmar epidermis contain an unusual keratin of 63 K not present in other parts of the skin (Fuchs and Green, 1980). The presence of these large keratins in terminally differentiating epidermal cells was confirmed by indirect immunofluorescence using an antibody specific for the 67-K keratin (Viac *et al.*, 1980; Warhol *et al.*, 1983). These early differences in keratin pattern are due to changes in mRNA synthesis that occur when a basal cell begins to migrate toward the skin surface (Fig. 2, lanes 1–4; Fuchs and Green, 1979; 1980; Bladon *et al.*, 1982; Roop *et al.*, 1983; Jorcano *et al.*, 1984c). As the differentiating cell reaches the granular layer, proteolysis leads to a slight reduction in the size of these keratins (Fig. 2, lanes 5 and 6; Fuchs and Green, 1980; Bowden *et al.*, 1984).

The differentiative processes of the epidermis also take place in human epidermal cells grown in tissue culture: rapidly growing epidermal cells cultured in the presence of epidermal growth factor (Rheinwald and Green, 1977), cAMP-inducing agents (Green, 1978), vitamin A (Fuchs and Green, 1981), and a fibroblast feeder layer (Rheinwald and Green, 1975) simulate basal epidermal cells and synthesize mRNAs encoding the keratins expressed in these cells. When vitamin A is removed from the culture medium, the cells begin to stratify, and terminal differentiation is induced (Fuchs and Green, 1981). Under these conditions, mRNAs encoding the large keratins are synthesized.

2. *The Structure of the 8-nm Keratin Filaments of Epidermis*

Amino acid sequences of the epidermal keratins have recently become available from cDNA sequence analyses (Hanukoglu and Fuchs, 1982, 1983; Steinert *et al.*, 1983, 1984; Jorcano *et al.*, 1984a, 1984b). There are two distinct types of keratin sequences: type I keratins are smaller (M_r 40–59 K) and relatively acidic, whereas type II keratins are larger (M_r 52–67 K) and more

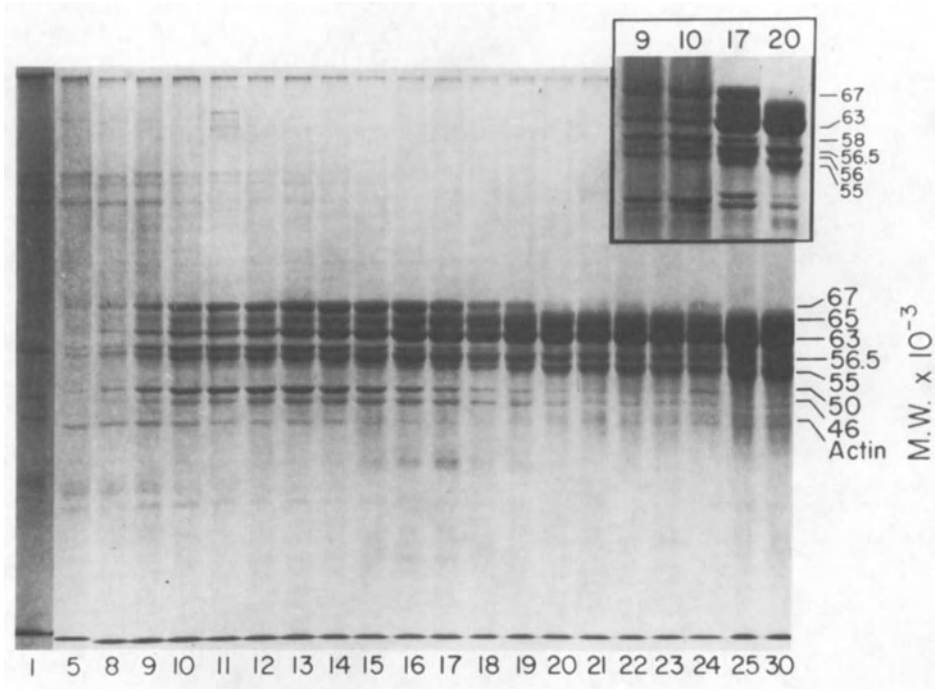


Figure 1. Changes in the keratin subunits at different stages of terminal differentiation in human epidermis. A 3-mm square of human plantar skin was sliced into 15-μm sections parallel to the epidermal surface. The keratins were extracted from each section and resolved electrophoretically. The numbers across the bottom of the gel denote consecutive sections beginning with the dermis and proceeding outward. Some sections were omitted, as no significant changes took place in them. Tracks 1–7 were dermal, tracks 8–16 were basal and spinous layers, tracks 17–19 were granular layers, and tracks 20–30 were stratum corneum. To see more clearly the replacement of the 56-K and 58-K keratins by 55-K and 56.5-K keratins, tracks 9, 10, 17, and 20 are magnified in the inset. (Reprinted from Fuchs and Green, 1980.)

basic (Fuchs *et al.*, 1981). In skin of all vertebrate organisms, both types of keratins are coordinately expressed at all times, and they are frequently expressed as pairs (Fig. 3; Moll *et al.*, 1982; Fuchs and Marchuk, 1983; Franke *et al.*, 1983; Kim *et al.*, 1983; Nelson and Sun, 1983; Eichner *et al.*, 1984). At all stages of epidermal differentiation, the balance of the ratio of type I and type II keratins is not disrupted (Kim *et al.*, 1984a). Thus, it seems that both types of sequences play an important role in forming the coiled-coil backbone of the 8-nm filaments. This conclusion has also been reached from *in vitro* filament assembly studies, which suggested that no one keratin subunit by itself was competent for filament formation (Steinert *et al.*, 1976; Lee and Baden, 1976; Milstone, 1981; Franke *et al.*, 1983).

The cDNA sequences of a 50-K type I (#14, i.e., K14 according to the nomenclature of Moll *et al.*, 1982) and a 56-K type II (#6, i.e., K6a) keratin of cultured human epidermal cells first revealed that despite the low degree of homology (24%) in amino acid sequence, the predicted secondary structures

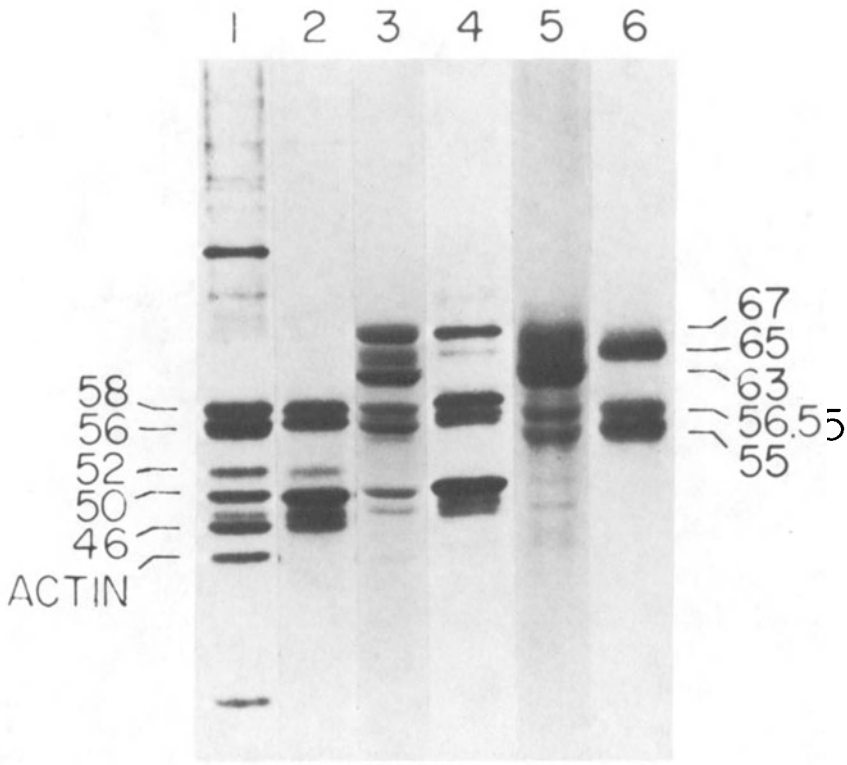


Figure 2. Synthesis of human epidermal keratins from poly(A)$^+$ RNA of cultured human basal epidermal cells and differentiating human epidermis. Poly(A)$^+$ RNAs from cultured basal epidermal cells and from adolescent human foreskin epidermis were isolated and translated *in vitro* and the synthesized products were immunoprecipitated (Fuchs and Green, 1980). The keratins were separated electrophoretically, and the gel was fluorographed. (Lane 1) [^{35}S]-methionine-labeled keratin extract from cultured cells; (lane 2) immunoprecipitated translation products from cultured cell RNA; (lane 3) [^{35}S]-methionine-labeled keratin extract from foot epidermis; (lane 4) immunoprecipitated translation products from foreskin epidermis RNA; (lane 5) Coomassie Blue-stained pattern of keratins extracted from the outermost stratum corneum layers of foot epidermis; (lane 6) Coomassie Blue-stained pattern of keratins extracted from the outermost stratum corneum layers of foreskin epidermis. MW values are in kD. Note that a keratin of 63 K is produced by plantar epidermis, but not by epidermis from foreskin or elsewhere.

of the polypeptide chains are highly similar (Hanukoglu and Fuchs, 1982; 1983; Tyner *et al.*, 1985; see Fig. 4). Additional sequence information has led to the generalization that all keratins have similar structures (Crewther *et al.*, 1980, 1983; Fuchs and Hanukoglu, 1983; Dowling *et al.*, 1983; Steinert *et al.*, 1983, 1984). The central 300-residue portion of the keratins is largely α-helical (marked by the bars in Fig. 4). However, the helical segment is demarcated by three regions in which helix-breaking residues are found. The significance of these breaks is not yet understood.

Throughout the helical domains of both the type I and the type II ker-

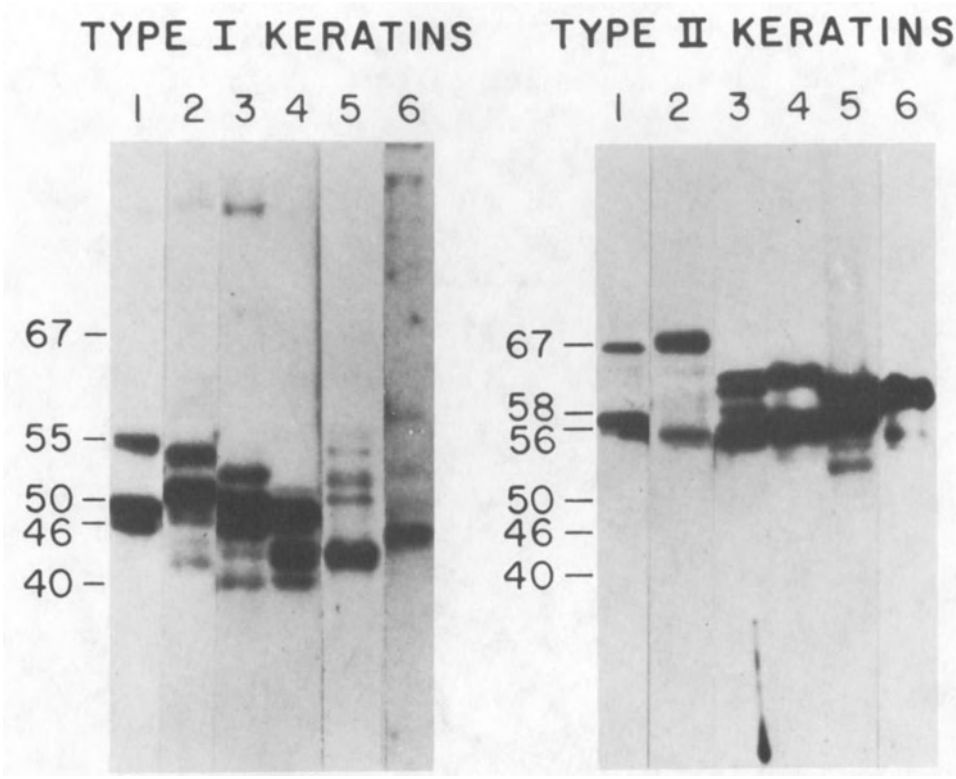


Figure 3. Keratins of type I (left) and type II (right) are both present in all vertebrate epidermis. Antibodies were raised against electrophoretically purified K14 (50 K, type I) and K6a (56 K, type II) human keratins. These antisera were used in immunoblot analysis to detect the presence of immunoreactive forms of both classes of keratins in other vertebrate epidermal keratinocytes. (Left) Immunoblot with anti-type I keratin antisera. (Right) Immunoblot with anti-type II keratin antisera. Tracks with different samples of vertebrate keratins: 1, whole human epidermis; 2, mouse; 3, rabbit; 4, bovine; 5, chicken; and 6, frog. MW values are in kD. (Reprinted from Fuchs and Marchuk, 1983).

atins is found a repeat of hydrophobic residues, where the first and the fourth residue of every seven is hydrophobic in nature (marked by dots in Fig. 4). This heptad repeat of hydrophobic residues was first described for tropomyosin (McLachlan and Stewart, 1975) and later for the wool keratins (McLachlan, 1978). It signifies a coiled-coil interaction between the α-helical polypeptide chains, a feature that was first predicted from the X-ray diffraction studies of the wool keratin filaments (Crick, 1953; Pauling and Corey, 1953). It has since been found in the helical segments of all intermediate-filament subunits.

Given the general design of the type I and type II keratins, it is easy to see that they have the capacity to form a coiled-coil structure. Although early physicochemical studies (Skerrow *et al.*, 1973; Steinert, 1978) suggested that

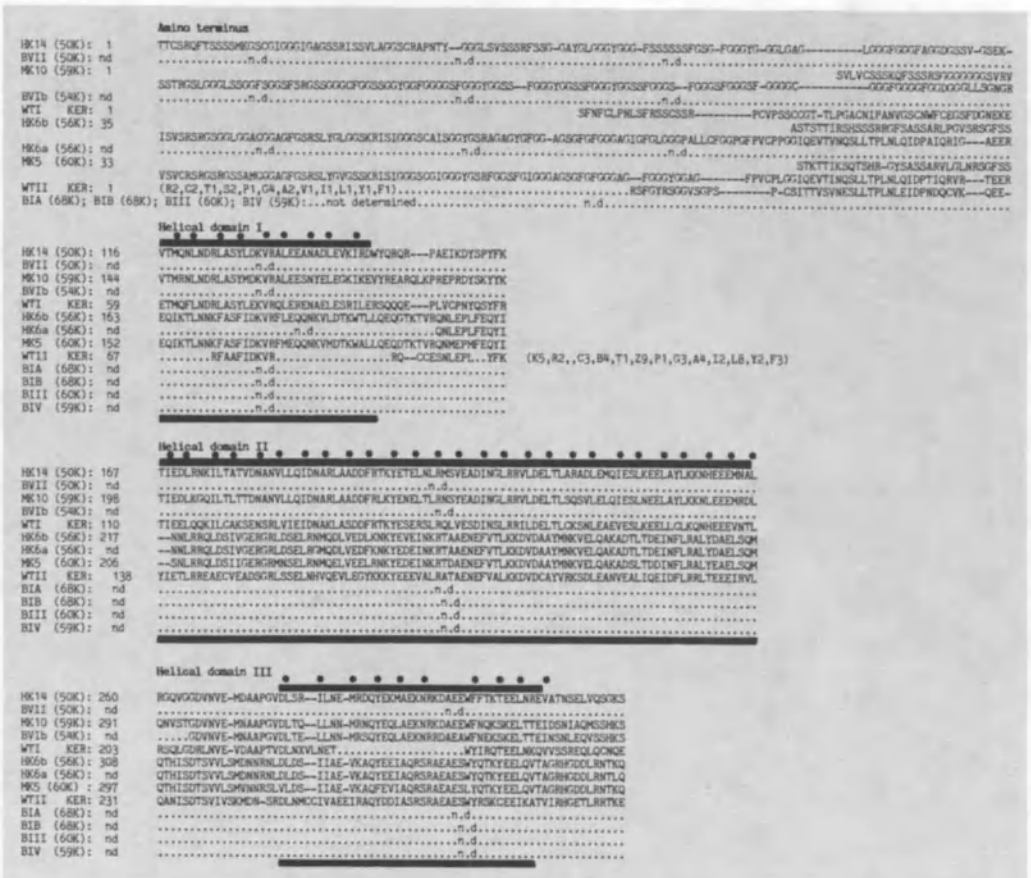

Amino terminus
Helical domain I
Helical domain II
Helical domain III

Figure 4. The amino acid sequences of type I and type II cytoskeletal and microfibrillar keratins and the common location of their predicted helical domains. The sequences are from the following sources: 1, HK14 (50 K), basal epidermal type I keratin from cultured human epidermal cells (Marchuk *et al.*, 1984); 2, BVII (50 K), type I keratin from bovine epidermis (Jorcano *et al.*, 1984b); 3, MK10 (59 K), type I keratin of terminally differentiating mouse epidermis (Steinert *et al.*, 1983); 4, BVIb (54 K), type I keratin from bovine epidermis Dowling *et al.*, 1983); 6, HK6b (56 K), human epidermal type II keratin (Tyner *et al.*, 1985); 7, HK6a (56 K), human epidermal type II keratin from cultured human epidermal cells (Hanukoglu and Fuchs, 1983); 8, MK5 (60 K) basal epidermal type II keratin from mouse (Steinert *et al.*, 1984); 9, WTII Ker, type II microfibrillar keratin from sheep wool (Crewther *et al.*, 1983 Dowling *et al.*, 1983). The small dots indicate missing sequence information (not determined, nd). In cases where the sequence of the amino terminus is known, the numbering on the left side of the sequences starts with the first amino terminal residue. Otherwise the numbering is not given. With the exception of WT-II, the sequences extend to the last carboxyterminal residue of the respective protein. The dashed lines in the sequences indicate gaps introduced in order to align the sequences for optimal homology. The positions of the helical domains (marked by overhead bars) are based on computerized secondary structure prediction analyses using the Chou and Fasman and Garnier, Osguthorpe, and Robson methods as previously described (Hanukoglu and Fuchs, 1982, 1983). The heptad repeats of hydrophobic residues are marked by the large overhead dots.

the basic subunit structure of the keratin filament was a coiled-coil trimer, more recent chemical cross-linking data (Woods and Gruen, 1981; Ahmadi and Speakman, 1978), model-building studies (McLachlan, 1978), and Fourier transform analyses (Crewther *et al.*, 1983) of complete keratin sequences have indicated that the keratin polypeptides most likely form dimers, which then pack into 8-nm filaments. Since the type I keratins are relatively acidic and the type II keratins are more basic, charge interactions may serve to further stabilize the cylindrical subunits.

A model of the way in which these putative subunit dimers pack into the overall 8-nm filamentous structure is illustrated in Fig. 5. End-to-end linkages of the cylindrical subunits seem to give rise to protofilaments of 2-nm diameter, which further intertwine to form 4.5-nm protofibrils (Pauling and Corey, 1953; Crick, 1953; Steinert, 1978; McLachlan, 1978; Aebi *et al.*, 1983; Steinert *et al.*, 1983; Crewther *et al.*, 1983; Fuchs and Hanukoglu, 1983). The protofibrils then appear to twist to give rise to a 21-nm axial periodicity, easily visible in the electron microscope (Milam and Erikson, 1982; Aebi *et al.*, 1983).

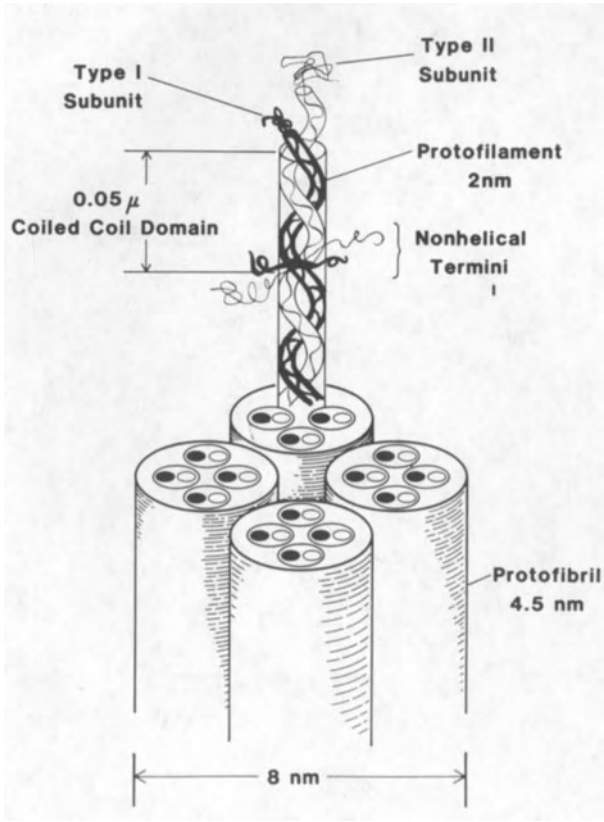


Figure 5. A structural model for the 8-mm keratin filament. The model is based collectively on X-ray diffraction data (Crick, 1953; Pauling and Corey, 1953), physicochemical studies (Skerrow *et al.*, Steinert, 1978; Steinert *et al.*, 1983), chemical cross-linking data (Ahmadi and Speakman, 1978; Woods and Gruen, 1981), model building using keratin sequences (McLachlan, 1978), electron microscopy studies (Aebi *et al.*, 1983; Steinert *et al.*, 1983), amino acid sequence data (Crewther *et al.*, 1980, 1983; Hanukoglu and Fuchs, 1982, 1983; Steinert *et al.*, 1983, 1984; Dowling *et al.*, 1983), and gene expression studies (Fuchs *et al.*, 1981; Moll *et al.*, 1982; Kim *et al.*, 1983; Roop *et al.*, 1983; Fuchs and Marchuk, 1983; Eichner *et al.*, 1984; Jorcano *et al.*, 1984c). The precise number of protofilaments per protofibril has not yet been unequivocally determined. Although the protofibrillar structures are shown as straight cylinders, they are most likely coiled in a left-handed helix with a periodicity of 21 nm (Milam and Erickson, 1982). Whether the coiled-coils are homo- or hetero-dimers has not yet been resolved, although both types of subunits seem to play an essential role in forming the filament.

Although the precise details of keratin filament assembly are only poorly understood, much of the information that dictates the assembly process seems to reside in the primary sequence of the polypeptide chains. *In vitro* filament formation does not appear to require any auxiliary proteins or factors (Steinert *et al.*, 1976; Franke *et al.*, 1983).

The nonhelical ends of the human epidermal keratins are variable in length, and they are rich in glycine and serine residues. This is readily apparent for the terminal sequences of K14 and K6b (Fig. 4). Recent sequence data for the epidermal keratins of other species confirm the universal presence in epidermal keratins of unusual inexact repeats of Gly–Gly–Gly–X (where X is frequently either Phe, Tyr, Leu, or Ala) interspersed with stretches of serine residues (Steinert *et al.*, 1983, 1984; Jorcano *et al.*, 1984a, 1984b). The larger keratins characteristic of terminally differentiating epidermis seem to be similar to the basal keratins, with the exception of longer stretches of these glycine–serine-rich repeats at the amino terminal end (Steinert *et al.*, 1983). A comparison of the amino terminus of the human K14 keratin of basal cells with that of the mouse MK10 keratin of terminally differentiating cells illustrates this point (Fig. 4). In contrast, the terminal ends of the wool keratins are rich in cysteine residues rather than glycine and serine (Crewther *et al.*, 1980, 1983; Dowling *et al.*, 1983). The differences between the end terminal sequences of the wool keratins (WT I and WT II, Fig. 4) and those of the epidermal keratins (K14 and K6b) are remarkable considering the striking similarities in the helical domains of the two type I keratins and the two type II keratins.

To summarize the protein structure work, it seems that the sequences as well as the general structural features of the coiled-coil helical domains of all keratin subunit pairs are highly similar, whereas the nonhelical end domains are related for each coordinately expressed pair of keratins, but are different for differentially expressed pairs of keratins. A clue to the significance of having different pairs of keratins with variable end domains is revealed when the functional role of the end domains is considered. When the keratins are subjected to mild chymotryptic digestion, the coiled-coil cylindrical dimer can form, but it cannot assemble into 8-nm filaments (Steinert, 1978). Examination of the keratins indicated that the treatment resulted in the digestion of the glycine–serine-rich end sequences, and that these sequences must therefore play a role in lateral and end-to-end interactions. By changing the strengths of these interactions in using different terminal sequences, the properties of the resulting filaments may change, without disrupting the overall structure *per se*. Thus, the existence of variable end domains for different pairs of keratins may enable a cell to tailor the properties of a keratin filament to suit its particular structural needs.

Several examples demonstrate that different keratin filaments do indeed have different properties. It is known that in wool and hair, the keratins bundle to form rigid macrofibrils, which become highly cross-linked through disulfide bonding (Fig. 6; for review, see Fraser *et al.*, 1972). These rigid macrofibrillar structures are ideal for the highly ordered cells of the hair

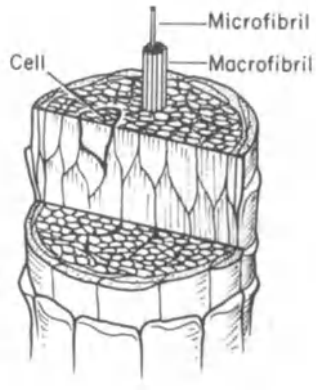


Figure 6. Model of the organization of keratin filaments in hair and wool. The keratin filaments in the terminally differentiated cells of hair and wool are highly ordered (for review, see Fraser *et al.*, 1972). Numerous disulfide bond cross-linkages create macrofibrillar bundles of rigid 8-nm filaments. (Taken from Dickerson and Geis, 1969).

shaft, but they would most likely be disastrous for dividing cells with normal metabolic functions. In terminally differentiating epidermal cells, the keratins are more soluble than those in hair, but less soluble than those in basal epidermal cells (Fuchs and Green, 1980). Their insolubility may be attributed to less organized bundles that form with the differentiation-specific keratin filaments. The long termini of these large keratins seem to protrude along the surface of the filament, thereby coating the filament with glycine–serine-rich sequences (Steinert *et al.*, 1983). The filaments then associate with another glycine–serine-rich protein, fillagrin, which causes bundling to occur (Fig. 7; Dale *et al.*, 1978). These cables seem to be more flexible in nature than the macrofibrils of wool, but nonetheless, they may be quite indestructible and would probably interfere with cell division. Indeed, the keratins of dividing epithelial cells do not seem to form macrofibrillar bundles *in vivo*.

3. The Genes Encoding the Human Epidermal Keratins

There are about 12 keratins that are differentially expressed in human epidermal cells at different stages of differentiation, and a total of about 20 different keratins if one considers all the human epithelial tissues (for review, see Moll *et al.*, 1982). When the two epidermal cDNAs encoding keratins K14 (50 K, type I) and K6a (56 K, type II) were used as probes at low stringency (750 mM NaCl, 50% formamide, 50°C), each hybridized with a different subset of about 10 genes in the human genome (Fig. 8; Fuchs *et al.*, 1981). Since it is not yet known whether all of the keratin genes are identified under these conditions, 20 must be considered a lower limit to the number of human keratin genes.

To begin to explore the complexity of the two keratin gene subfamilies, we screened a human genomic library in λ Charon 4A (Ed Fritsch, Genetics Institute) using the plaque hybridization method of Benton and Davis (1977) with radiolabeled cDNA to K14 and K6a as probe. We isolated a number of different clones containing putative keratin genes. These clones hybridized

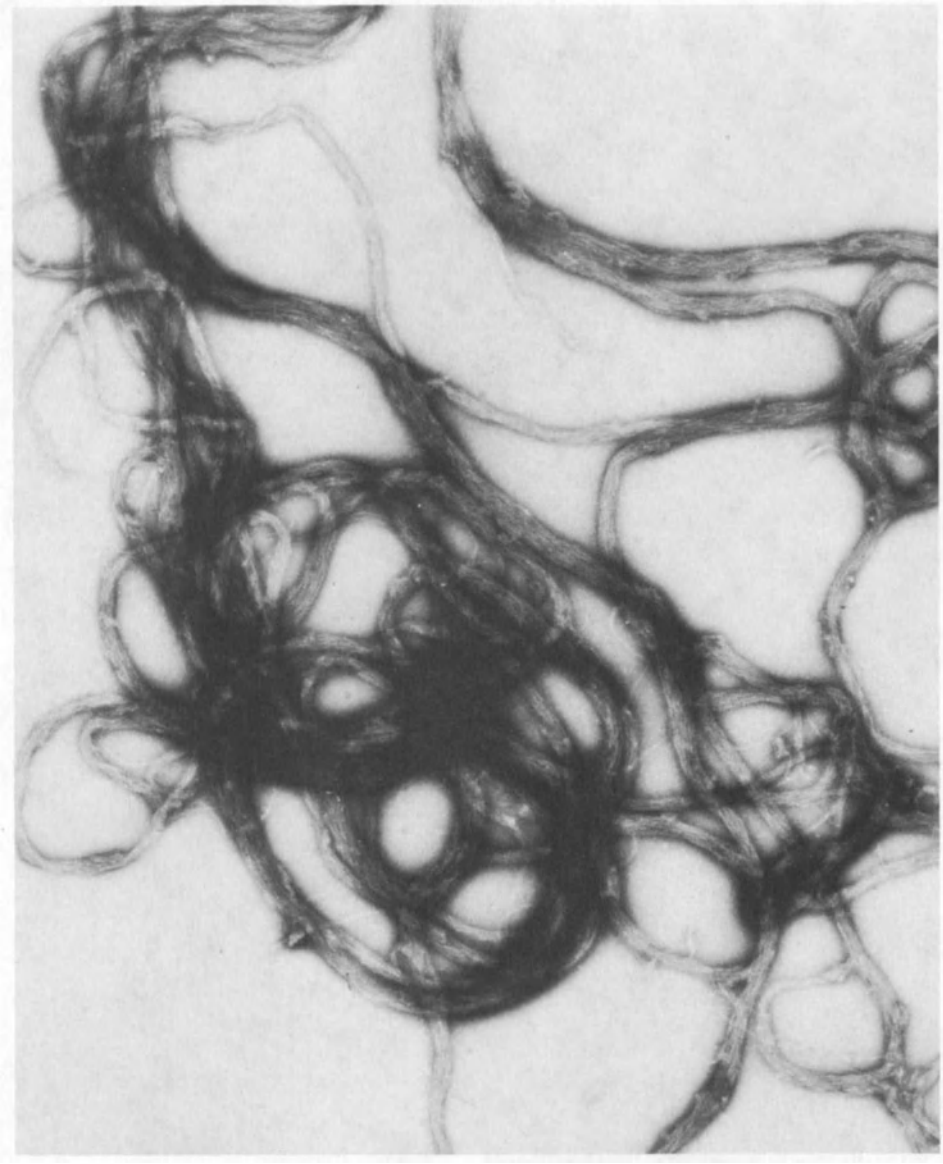


Figure 7. Electron micrograph of fillagrin-induced macrofibrillar bundles of keratin filaments. When epidermal cells are triggered to undergo terminal differentiation, new keratin filaments are produced that are comprised of keratin polypeptides larger than those of the basal epidermal cells (Fuchs and Green, 1980). These filaments ultimately form macrofibrillar bundles, which serve to protect the keratin filaments from the proteolytic enzymes released in the granular epidermal layer. The process of macrofibrillar bundling can be mimicked *in vitro*, as shown here, by the action of a differentiation-specific protein called fillagrin (Dale *et al.*, 1978), mixed at a ratio of 2:1 with epidermal keratin filaments. Macrofibrils were negatively stained with uranyl acetate. X46,000. (Figure kindly donated by Drs. Beverly Dale and Karen Holbrook, University of Washington, Seattle.)

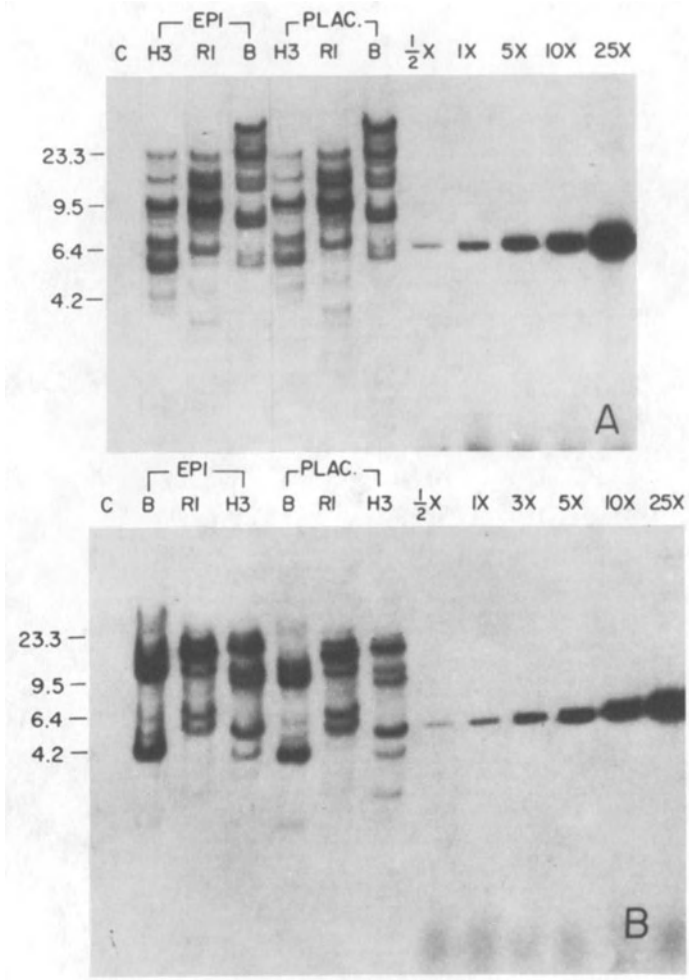


Figure 8. Identification of human genomic DNA restriction fragments bearing keratin genes. DNA (5 μg/lane) from cultured human epidermal cells was digested with a fivefold excess of each of three restriction endonucleases. Fragments were fractionated by electrophoresis through a 0.8% agarose gel and transferred to nitrocellulose paper (Fuchs *et al.*, 1981). The blots were hybridized against ^{32}P-labeled probes copied from cDNA encoding K6a, the 56-K keratin of human epidermal cells (A), and cDNA encoding K14, the 50-K keratin of human epidermal cells (B). Lanes contain either human epidermal DNA (EPI) or human placental DNA (PLAC) digested with Hind III (H3), Eco RI (RI), or Bam HI (B). Additonal lanes represent dilutions into 5 μg of sheared *Escherichia coli* DNA of the appropriate linearized hybrid plasmid, either pKA-1 encoding K6a (A), or pKB-2 encoding K14 (B), in amounts corresponding to 0.50–50 copies of keratin cDNA per haploid genome. Lane C shows a control in which the plasmid pBR322 was added in amount corresponding to 10 copies per haploid genome. Molecular weights in kilobases are shown at the left.

with varying degrees of homology to one or the other of the cDNA probes. Six sets of overlapping clones were identified from restriction endonuclease mapping of the genomic fragments within some of these clones (Marchuk, Tyner, and Fuchs, unpublished observations). From this group, we selected one clone, G-K1, containing the complete gene encoding keratin K14 (Marchuk *et al.*, 1984, 1985), and another clone, G-K2, containing the complete gene encoding a K6 keratin (Tyner *et al.*, 1985).

The complete nucleotide sequence of the gene contained in GK-1 (Marchuk *et al.*, 1984, 1985) confirmed that it encoded a K14 keratin mRNA identical to the KB-2 cDNA that had previously been isolated and sequenced (Hanukoglu and Fuchs, 1982). The complete sequence of the gene contained in GK-2 (Tyner *et al.*, 1985) indicated that it encoded a K6 keratin mRNA, but this mRNA was not identical to the K6a keratin mRNA of the KA-1 cDNA that had previously been isolated and sequenced (Hanukoglu and Fuchs, 1983). Three amino acid residue differences distinguish the partial (63%) sequence of the K6a mRNA and the equivalent sequence in GK-2 (K6b). In addition, whereas the first 125 base pairs (bp) of the 3′ noncoding sequence of GK-2 share a high degree of homology with that of KA-1, the remaining 375 bp of sequence is highly divergent (Tyner *et al.*, 1985). This divergent portion of the 3′ noncoding end of GK-2 was shown to hybridize strongly with a 2.1-kb mRNA expressed in abundance in human epidermal cells (Tyner *et al.*, 1985). The specific noncoding segment also hybrid-selected an mRNA that translated into a 56-K keratin *in vitro* (Tyner *et al.*, 1985). Since this segment shows no cross-hybridization with the corresponding segment of the KA-1 cDNA and yet each clone hybridizes strongly with an mRNA encoding a 56K keratin, it is likely that there are at least two distinct genes encoding 56K keratins that are coordinately expressed in human epidermal cells.

4. *Structure of the Two Types of Keratin Genes and Their Relation to Other Intermediate-Filament Genes*

When the GK-2 gene encoding the K6b type II keratin was compared with the GK-1 gene encoding the K14 type I keratin, it was discovered that even though the two coding sequences share only 24% homology with each other, the structures of their genes as well as the structures of their proteins are very nearly identical (Fig. 9). Six of the introns (marked by triangles) show identical or nearly identical positioning for the two proteins. These introns are located in the central portion of the coding region. Despite the strict conservation of intron position, neither the size nor the sequence of the introns has been conserved (Marchuk *et al.*, 1984, 1985; Tyner *et al.*, 1985). Even the sequences bordering the intron–exon junctions of the two polypeptides are not identical.

No correlation was found between the evolutionarily conserved amino acid residues of the two types of keratin proteins (black dots, Fig. 9) and the highly conserved positions of the introns. In some of these cases, the homolo-

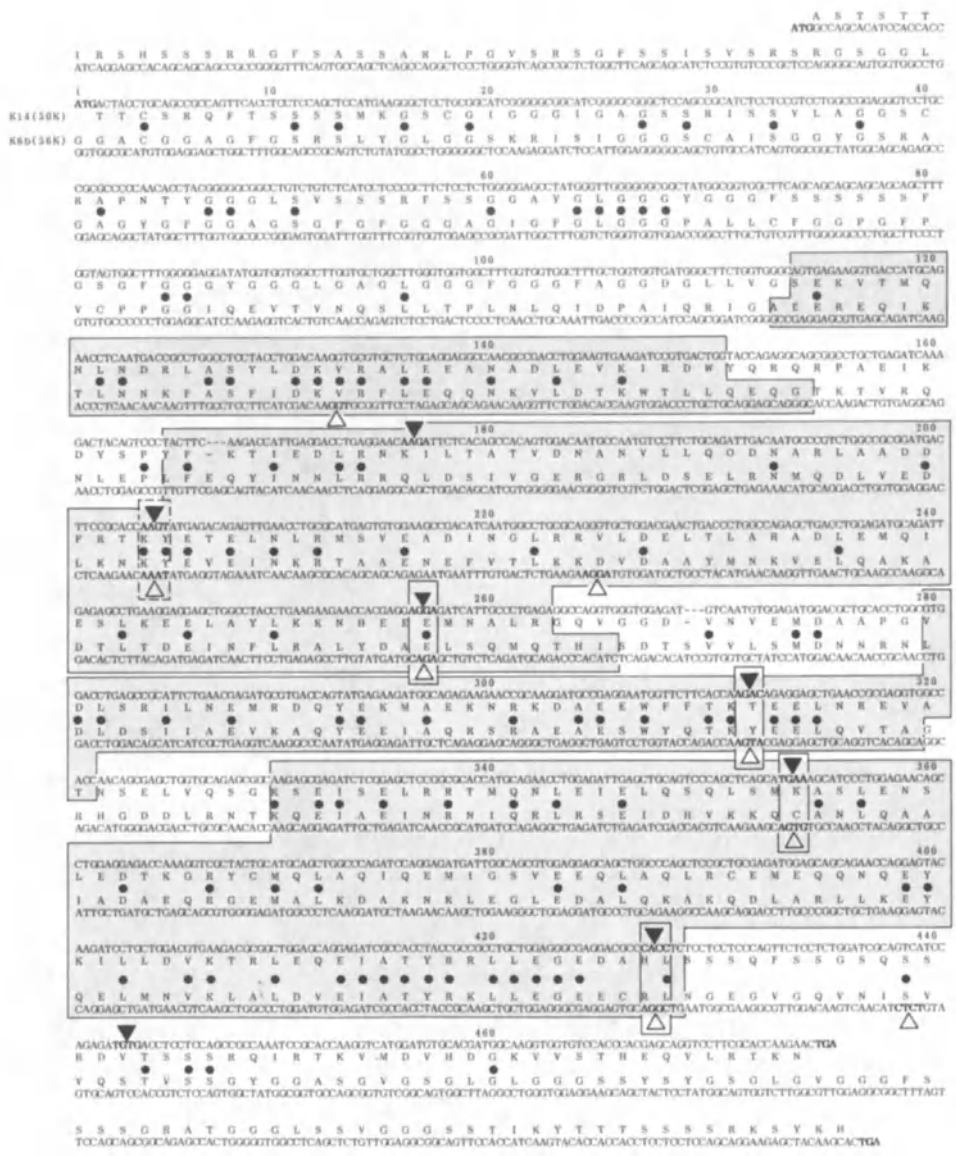


Figure 9. The relation between the protein/nucleic acid sequence homologies and the conservation of intron position in the keratin type I (K14) and the keratin type II (K6b) genes. The amino acid sequences of human K14 keratin (Marchuk *et al.*, 1984) and human K6b keratin (Tyner *et al.*, 1985) were aligned for optimal homology. The two sequences were shown to share only 24% homology at the amino acid sequence level (identical residues are indicated by black dots). Their secondary structures are nearly identical, however, and the four large helical domains are shown by grey boxes. The positions of the type I keratin introns are indicated by the solid triangles. The positions of the type II keratin introns are indicated by the open triangles. The exon–intron junctions that are positioned identically for both sequences are encompassed by boxes. Note that the positions of the introns do not seem to demarcate the boundaries of the helical domains of the keratin polypeptides.

gy of protein segments flanked by similarly positioned introns is fairly high (e.g., the exon corresponding to #4 of K14, 33%). In other cases, the homology is very low (e.g., the exon corresponding to exon #2 of K14, 11%). Sometimes, regions of high and low homologies, respectively, are contained within a single exon (e.g., the exon corresponding to #6 of K14). Not even the amino acid sequences immediately flanking the exon–intron junctions have been evolutionarily conserved. Thus, for the keratin genes, exons do not seem to have evolved as separate units, even though the evolutionary pressures on maintaining exon size have been considerable.

The grey boxes in Fig. 9 signify the four α-helical domains of the two types of keratins. The introns do not seem to demarcate the boundaries of these helical domains, nor do they separate the nonhelical glycine–serine-rich termini from the central helical region. Thus, its seems likely that the positioning of the introns is dependent on factors other than forming the boundaries of the structural domains of the keratins as they exist today.

The conservation of intron position in the two types of keratin genes is truly remarkable, considering the very distant relation between their proteins (24% homology). This conservation extends to the third class (type III) of intermediate filament genes, which encodes the glial fibrillary acidic (GFA) protein, and desmin and vimentin proteins that are differentially expressed in higher eukaryotic cells other than epithelia (Quax *et al.*, 1983). Thus, a common structure exists for most if not all IF genes, a feature that was also suggested from heteroduplex analyses (Lehnert *et al.*, 1984). Two very distant duplications must have led to the early formation of three genes: a type I and a type II keratin gene and a third, type III gene. The multiplicity of related sequences within these three classes seems to have arisen from more recent gene duplications. Whereas the three gene families share only 25–30% homology with each other, the individual members of a single class share 40–95% homology.

The significance of the extreme evolutionary pressure exerted on maintaining intron position in the IF gene family has not yet been elucidated. However, a clue to the possible importance is revealed by considering the consequences of shifting an intron, once it has been introduced at a particular position within a gene. Utilizing an alternative splice junction at a position 3n from the original ag or gt consensus sequence would introduce or delete one or more amino acid residues at the exon–intron border. Since these junctions are located within the helical domains of the IF polypeptide, such changes might disrupt the heptad repeat that is necessary for the proper formation of the coiled-coil. A perturbation in this structure may, in turn, alter the ability of the subunits to assemble into the 8-nm filaments.

We do not know whether the conservation of intron position in keratin and other IF genes might also be a clue to the boundaries of as yet unidentified functional domains that could be involved in interactions between the filaments and other cellular components. The proteins and cellular structures that associate with 8-nm filaments to form the overall cytoskeletal architecture remain largely unidentified. However, if such interactions did play a role in

defining important IF functional domains that were subsequently assembled piecemeal in the construction of the primordial IF gene, then these interactions must be common to the IF networks of many varied cell types.

5. Regulation of the Type I and Type II Keratin Genes

In the past 5 years, we have just begun to decipher some of the factors that are important in the regulation of keratin gene expression. Epithelial cell growth has been shown to be dependent on a number of different factors, e.g., epidermal growth factor (Savage and Cohen, 1972; Rheinwald and Green, 1977), cAMP-inducing agents (Green, 1978), hydrocortisone (Rheinwald and Green, 1975), calcium (Hennings *et al.*, 1980), and insulin and other serum factors (Maciag *et al.*, 1981). However, few, if any, of these factors seem to influence the choice of which pairs of keratins will be expressed in a cell and what the ultimate level of expression will be. To date, only a few cases have been documented where specific factors are known to regulate keratin gene expression. One of these factors, vitamin A, has long been recognized as a strong regulator of epithelial cell differentiation (Wolbach, 1954; Lotan, 1980; Wolf, 1980; Elias and Williams, 1981). This vitamin diminishes the expression of terminal differentiation in stratified squamous epithelia and accentuates the expression of differentiation in secretory epithelia. Deficiency of the vitamin can convert a secretory epithelium to a squamous epithelium (squamous metaplasia), and excess of the vitamin can convert a (cultured) stratified squamous epithelium to a secretory epithelium (mucous metaplasia). This vitamin is distinguished from all others by its ability to control epithelial differentiation.

When vitamin A is removed from cell culture medium by solvent extraction (delipidization), human epidermal cells begin to stratify and synthesize the large keratins characteristic of the terminal differentiative process (Fuchs and Green, 1981). When mucus-secreting epithelial cells are cultured in this medium, synthesis of the pairs of keratins characteristic of their type of differentiation is inhibited (Fuchs and Green, 1981; Kim *et al.*, 1984b). Reintroduction of the vitamin in the form of retinyl acetate (Fuchs and Green, 1981) or other retinoids (Gilfix and Green, 1984) restores the patterns of keratin synthesis to their normal state. These changes are clearly at the level of keratin mRNA synthesis and may be at the transcriptional level as well (Fuchs and Green, 1981; Kim *et al.*, 1984b; Eckert and Green, 1984).

It has been postulated that retinoids act in a fashion analagous to that of steroid hormones to regulate epithelial gene expression (for a review, see Chytil and Ong, 1979). A cytoplasmic 14-K protein, cellular retinoic acid binding protein (CRABP), has been purified, and it interacts strongly with a number of different retinoids (Ong and Chytil, 1975; Liau *et al.*, 1981; Ong *et al.*, 1982; Saari *et al.*, 1982). Embryonal cell mutants lacking CRABP have been shown to be unresponsive to retinoids, suggesting that the receptor indeed plays a role in mediating the action of vitamin A (Schindler *et al.*,

1981). We have found that in cultured human epidermal cells, the CRABP levels are high (Kim *et al.*, 1984b). Whether vitamin–receptor complexes interact directly with different keratin genes to regulate their expression, however, has yet to be investigated.

Although the expression of some pairs of keratins is clearly dependent on the levels of vitamin A in the culture medium, the vitamin does not seem to be involved in regulating the expression of the genes encoding the K14 type I and K6b type II keratins expressed in cultured epidermal cells (Fuchs and Green, 1981). These genes seem to be constitutively expressed at levels of up to 10% of the total protein (Sun and Green, 1978). The abundant levels of these keratins in the cultured cells suggests that the promotors for these genes might be particularly strong. However, the factors that allow the tissue-specific and abundant expression of these genes in epidermis and other epithelia have not yet been identified.

For some viral and cellular genes that are expressed in abundance in a tissue-specific manner, sequences containing a core octamer G-T-G-G-A-A-A-G have been found in either the introns or the 5′ upstream region of the genes. Through site-directed mutagenesis experiments, these nucleotides have been shown to specifically enhance the level of transcription of these genes (Moreau *et al.*, 1981; Laimins *et al.*, 1982; Gillies *et al.*, 1983). It has been proposed that different tissue-specific proteins might interact directly with enhancer elements to regulate the levels of expression of such a gene in a tissue-specific manner. Indeed, specific interactions between enhancer-containing sequences and cellular components have recently been identified (Scholer and Gruss, 1984).

When the 5′ upstream region for K14 gene is scanned, three regions are found that contain the sequences T-$(G)_{1\text{-}3}$-A-A-A-G (underlined in Fig. 10). We do not yet know whether the enhancerlike sequences that are upstream from the transcription initiation site of the K14 gene actually contribute in enhancing the transcription of the gene. If, indeed, these sequences do act as enhancer elements, then they do not appear to be a universal mechanism for abundant keratin gene expression, since the coordinately expressed K6b gene does not seem to contain these sequences in the corresponding 5′ upstream region of the gene (Tyner *et al.*, 1985).

When the 5′ upstream regions of the two epidermal genes were aligned for optimal homology, three 15 to 22-bp sequences in comparable positions were found to share about 75% homology (Fig. 10). The transcribed 5′ noncoding regions of the K6b and K14 genes also shared substantial homology. In contrast, much less homology was found between the 5′ upstream regions of the vimentin and keratin genes. Whether any of these features play a role in the coordinate regulation of the two epidermal keratin genes or the differential regulation of the vimentin gene remains to be investigated. Recent evidence that regulatory regions of a gene are not necessarily localized within the 5′ upstream sequences (Gillies *et al.*, 1983; Charnay *et al.*, 1984; Moore *et al.*, 1985) makes it very difficult to assess the significance of the homologous sequences identified in Fig. 10.

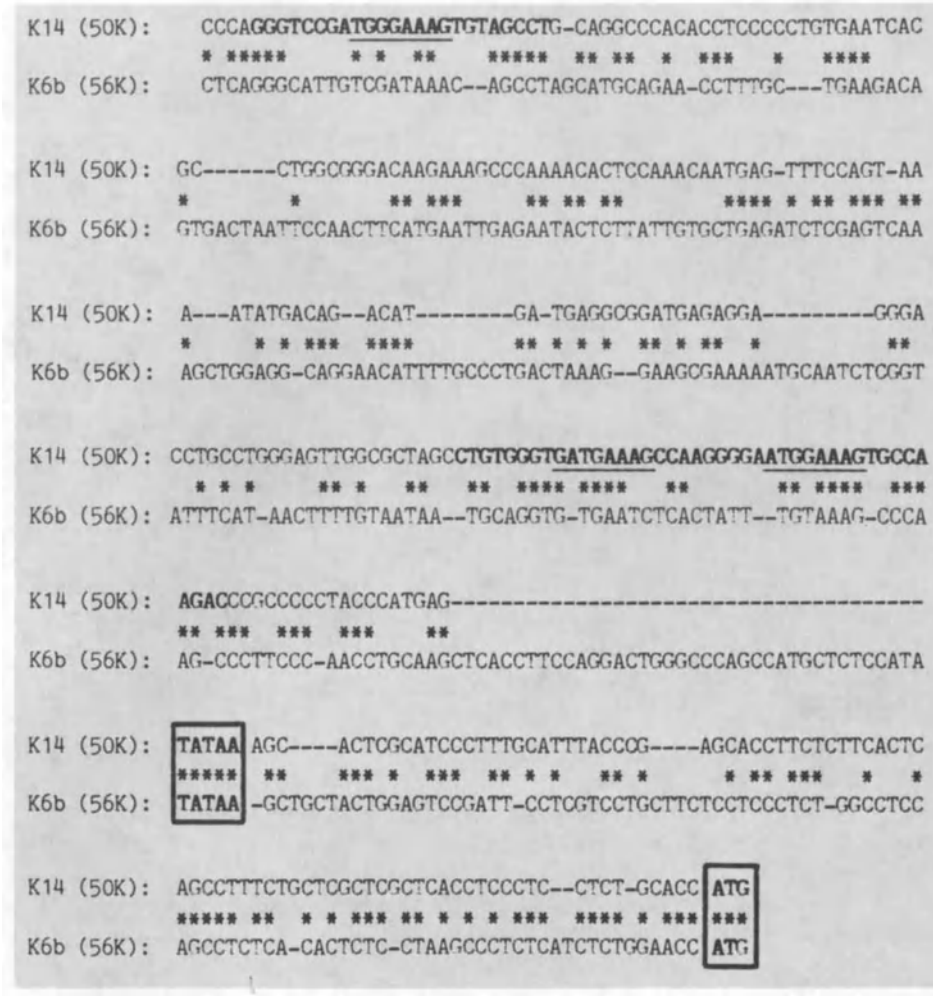


Figure 10. Alignment of the 5′ upstream regions of the type I (K14) and the type II (K6b) keratin genes. The 5′ upstream regions of the K14 (Marchuk *et al.*, 1985) and K6b (Tyner*et al.*, 1985) keratin genes were aligned for optimal homology. The TATA box, common to most eukaryotic promotors and the translation initiation site AUG are highlighted by boxes. The underlined sequences show regions of the K14 gene sharing a substantial degree of homology with the core enhancer sequence common to some viral and cellular genes (Marchuk *et al.*, 1985). Note that substantial homology exists in the noncoding transcribed upstream region and also in a few concentrated regions upstream from the TATA box. Whether any of these similarities are significant in the coordinate regulation of these genes remains to be investigated.

As we elucidate the sequences and structures of additional keratin genes, we hope to gain a better understanding of the regulation of the differential expression of specific pairs of keratins in different epithelia and at different stages of differentiation and development. Site-directed mutagenesis should help to define the regions of the genes that are responsible for transcriptional

regulation and vitamin A sensitivity. Site-directed mutagenesis of the coding portions of these genes should unravel additional clues as to the sequences within the polypeptide chains that are essential for filament assembly. At the moment, we have only begun to scratch the surface of understanding the molecular mechanisms underlying keratin gene expression in human epidermis.

ACKNOWLEDGMENTS. We would like to thank all the other members of the laboratory, past and present, who have been instrumental in contributing to our overall knowledge of the differential expression of keratin genes in human epidermis. Without their help, the data and theories put forth in this chapter could not have evolved.

This work was supported by grants from the National Institutes of Health. E. F. is a Searle Scholar, a Presidential Young Investigator, and the recipient of a Career Development Award from the N.I.H. A. T. and D. M. are predoctoral trainees funded by a grant from the N.I.H.

References

Aebi, U., Fowler, W. E., Rew, P., and Sun, T-T., 1983, The fibrillar substructure of keratin filaments unraveled, *J. Cell Biol.* **97:**1131–1143.

Ahmadi, B., and Speakman, P. T., 1978, Suberimidate crosslinking shows that a rod-shaped, low cystine, high helix protein prepared by limited proteolysis of reduced wool has four protein chains, *FEBS Lett.***94:**365–367.

Baden, H. P., and Lee, L. D., 1978, Fibrous protein of human epidermis, *J. Invest. Dermatol.*-**71:**148–151.

Benton, W. D., and Davis, R. W., 1977, Screening gt recombinant clones by hybridization to single plaques in situ, *Science* **196:**180–182.

Bladon, P. T., Bowden, P. E., Cunliffe, W. J., and Wood, E. J., 1982, Prekeratin biosynthesis in human scalp epidermis, *Biochem. J.* **208:**179–187.

Bowden, P. E., and Cunliffe, W. J., 1981, Modification of human prekeratin during epidermal differentiation, *Biochem. J.* **199:**145–154.

Bowden, P. E., Quinlan, R. A., Breitkreutz, D., and Fusenig, N. E., 1984, Proteolytic modification of acidic and basic keratins during terminal differentiation of mouse and human epidermis, *Eur. J. Biochem.***142:**29–36.

Charnay, P., Treisman, R., Mellone, P., Chao, M., Axel, R., and Maniatis, T., 1984, Differences in human α- and β-globin gene expression in mouse erythroleukemia cells: the role of intragenic sequences, *Cell* **38:**251–263.

Chytil, F., and Ong, D. E., 1979, Cellular retinol- and retinoic acid-binding proteins, *Adv. Nutr. Res.* **5:**13–29.

Crewther, W. G., Dowling, L. M., and Inglis, A. S., 1980, Amino acid sequence data from a microfibrillar protein of α-keratin, in: *Proceedings of the 6th Quinquennial International Wool Textile Conference*, Vol. 2, pp. 79–81, Pretoria, South Africa.

Crewther, W. G., Dowling, L. M., Steinert, P. M., and Parry, D. A. D., 1983, Structure of intermediate filaments, *Int. J. Biol. Macromol.***5:**267–274.

Crick, F. H. C., 1953, The packing of α-helices: simple coiled-coils, *Acta Cryst.***6:**689–697.

Dale, B. A., and Stern, I. B., 1975, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins of newborn rat skin. II. Keratohyalin and stratum corneum proteins, *J. Invest. Dermatol.***65:**223–227.

Dale, B. A., Stern, I. B., Rabin, M., and Huang, L-Y., 1976, The identification of fibrous proteins in fetal rat epidermis by electrophoretic and immunologic techniques, *J. Invest. Dermatol.* **66**:230–235.

Dale, B. A., Holbrook, K. A., and Steinert, P. M., 1978, Assembly of stratum corneum basic protein and keratin filaments in macrofibrils, *Nature* **276**:729–731.

Dickerson, R. E., and Geis, I., 1969, *The Structure and Action of Proteins*, p. 39, Harper and Row, New York.

Dowling, L. M., Parry, D. A. D., and Sparrow, L. G., 1983, Structural homology between α-keratin and the intermediate filament proteins desmin and vimentin, *Biosci. Rep.* **3**:73–78.

Eckert, R. L., and Green, H., 1984, Cloning of cDNAs specifying vitamin A-responsive human keratins, *Proc. Natl. Acad. Sci. USA* **81**:4321–4325.

Eichner, R., Bonitz, P., and Sun, T-T., 1984, Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression, *J. Cell Biol.* **98**:1388–1396.

Elias, P. M., and Williams, M. L., 1981, Retinoids, cancer and the skin, *Arch. Dermatol.* **117**:160–180.

Franke, W. W., Schiller, D. L., Hatzfeld, M., and Winter, S., 1983, Protein complexes of intermediate-sized filaments: Melting of cytokeratin complexes in urea reveals different polypeptide separation characteristics, *Proc. Natl. Acad. Sci. USA* **80**:7113–7117.

Fraser, R. D. B., MacRae, T. P., and Rogers, G. E., 1972, *Keratins:Their Composition, Structure and Biosynthesis*, pp. 56–121, Charles C. Thomas, Springfield, Ill.

Fuchs, E., and Green, H., 1979, Multiple keratins of cultured human epidermal cells are translated from different mRNA molecules, *Cell* **17**:573–582.

Fuchs, E., and Green, H., 1980, Changes in keratin gene expression during terminal differentiation of the keratinocyte, *Cell* **19**:1033–1042.

Fuchs, E., and Green, H., 1981, Regulation of terminal differentiation of cultured human keratinocytes by vitamin A, *Cell* **25**:617–625.

Fuchs, E., and Hanukoglu, I., 1983, Unraveling the structure of the intermediate filament, *Cell* **34**:332–334.

Fuchs, E., and Marchuk, D., 1983, Type I and type II keratins have evolved from lower eukaryotes to form the intermediate filaments in mammalian skin, *Proc. Natl. Acad. Sci. USA* **80**:5857–5861.

Fuchs, E., Coppock, S. M., Green, H., and Cleveland, D. W., 1981, Two distinct classes of keratin genes and their evolutionary significance, *Cell* **27**:75–84.

Fukuyama, K., and Epstein, W. L., 1968, Synthesis of RNA and protein during epidermal cell differentiation in man, *Arch. Dermatol.* **98**:75–79.

Fukuyama, K., Nakamura, T., and Berstein, I. A., 1965, Differentially localized incorporation of amino acids in relation to epidermal keratinization in the newborn rat, *Anat. Rec.* **152**:525–536.

Gilfix, B. M., and Green, H., 1984, Bioassay of retinoids using cultured human conjunctival keratinocytes, *J. Cell. Physiol.* **119**:172–174.

Gillies, S. D., Morrison, S. L., Oi, V. T., and Tonegawa, S., 1983, A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene, *Cell* **33**:717–728.

Green, H., 1978, Cyclic AMP in relation to proliferation of the epidermal cell: a new view, *Cell* **15**:801–811.

Hanukoglu, I., and Fuchs, E., 1982, The cDNA sequence of a human epidermal keratin: Divergence of sequence but conservation of structure among intermediate filament proteins, *Cell* **31**:243–252.

Hanukoglu, I., and Fuchs, E., 1983, The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratins, *Cell* **33**:915–924.

Hennings, H. D., Cheng, M. C., Steinert, P., Holbrook, K., and Yuspa, S. H., 1980, Calcium regulation of growth and differentiation of mouse epidermal cells in culture, *Cell* **19**:245–254.

Hoober, J. K., and Berstein, I. A., 1966, Protein synthesis related to epidermal differentiation, *Proc. Natl. Acad. Sci. USA* **56**:594–601.

Jorcano, J. L., Franz, J. K., and Franke, W. W., 1984a, Amino acid sequence diversity between bovine epidermal cytokeratin polypeptides of the basic (type II) subfamily as determined from cDNA clones, *Differentiation* **28**:155–163.

Jorcano, J. L., Rieger, M., Franz, J. K., Schiller, D. L., Moll, R., and Franke, W. W., 1984b, Identification of two types of keratin polypeptides within the acidic cytokeratin subfamily I, *J. Mol. Biol.* **179**:257–281.

Jorcano, J. L., Magin, T. M., and Franke, W. W., 1984c, Cell type-specific expression of bovine keratin genes as demonstrated by the use of complementary DNA clones, *J. Mol. Biol.* **176**:21–37.

Kim, K. H., Rheinwald, J. G., and Fuchs, E., 1983, Tissue specificity of epithelial keratins: differential expression of mRNAs from two multigene families, *Mol. Cell. Biol.* **3**:495–502.

Kim, K. H., Marchuk, D., and Fuchs, E., 1984a, Expression of unusually large keratins during terminal differentiation: balance of type I and type II keratins is not disrupted, *J. Cell Biol.* **99**:1872–1877.

Kim, K. H., Schwartz, F., and Fuchs, E., 1984b, Differences in keratin synthesis between normal epithelial cells and squamous cell carcinomas are mediated by vitamin A, *Proc. Natl. Acad. Sci. USA* **81**:4280–4284.

Laimins, L. A., Khoury, G., Gorman, C., Howard, B., and Gruss, P., 1982, Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus, *Proc. Natl. Acad. Sci. USA* **79**:6453–6457.

Lavker, R. M., and Matoltsy, A. G., 1970, Formation of horny cells: The fate of cell organelles and differentiation products in ruminal epithelium, *J. Cell. Biol.* **44**:501–512.

Lee, L. D., and Baden, H. P., 1976, Organization of the polypeptide chains in mammalian keratin, *Nature* **264**:377–379.

Lehnert, M. E., Jorcano, J. L., Zentgraf, H., Blessing, M., Franz, J. K., and Franke, W. W., 1984, Characterization of bovine keratin genes: Similarities of exon patterns in genes coding for different keratins, *EMBO J.* **13**:3279–3287.

Liau, G., Ong, D. E., and Chytil, F., 1981, Interaction of the retinol/cellular retinol-binding protein complex with isolated nuclei and nuclear components, *J. Cell Biol.* **91**:63–68.

Lotan, R., 1980, Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells, *Biochim. Biophys. Acta* **605**:33–91.

Maciag, T., Nemore, R. E., Weinstein, R., and Gilchrest, B., 1981, An endocrine approach to the control of epidermal growth: Serum-free cultivation of human keratinocytes, *Science* **211**:1452–1454.

Marchuk, D., McCrohon, S., and Fuchs, E., 1984, Remarkable conservation of structure among intermediate filament genes, *Cell* **39**:491–498.

Marchuk, D., McCrohon, S., and Fuchs, E., 1985, Complete sequence of a gene encoding a human type I keratin: Sequences homologous to enhancer elements in the regulatory region of the gene, *Proc. Natl. Acad. Sci. USA* **82**:1609–1613.

McLachlan, A. D., 1978, Coiled-coil formation and sequence regularities in the helical regions of α-keratin, *J. Mol. Biol.* **124**:297–304.

McLachlan, A. D., and Stewart, M., 1975, Tropomyosin coiled-coil interactions: evidence for an unstaggered structure, *J. Mol. Biol.* **98**:293–304.

Milam, L., and Erikson, E. P., 1982, Visualization of a 21 nm axial periodicity in shadowed keratin filaments and neurofilaments, *J. Cell Biol.* **94**:592–596.

Milstone, L. M., 1981, Isolation and characterization of two polypeptides that form intermediate filaments in bovine esophageal epithelium, *J. Cell Biol.* **88**:317–322.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R., 1982, The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells, *Cell* **31**:11–24.

Moore, D. D., Marks, A. R., Buckley, D. I., Kapler, G., Payvar, F., and Goodman, H. M., 1985, The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor, *Proc. Natl. Acad. Sci. USA* **82**:699–702.

Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M. P., and Chambon, P., 1981, The SV40 72

base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants, *Nucl. Acid Res.* **9:**6047–6068.

Nelson, W. G., and Sun, T-T. 1983, The 50- and 58-Kdalton keratin classes as molecular markers for stratified squamous epithelia: Cell culture studies, *J. Cell Biol.* **97:**244–251.

Ong, D. E., and Chytil, F., 1975, Specificity of cellular retinol-binding protein for compounds with vitamin A activity, *Nature* **255:**74–75.

Ong, D. E., Crow, J. A., and Chytil, F., 1982, Radioimmunochemical determination of cellular retinol- and cellular retinoic acid-binding proteins in cytosols of rat tissues, *J. Biol. Chem.* **257:**13385–13389.

Pauling, L., and Corey, R. B., 1953, Compound helical configurations of polypeptide chains: structure of proteins of the α-keratin type, *Nature* **171:**59–61.

Quax, W., Egberts, W. V., Hendriks, W., Quax-Jeuken, Y., and Bloemendal, H., 1983, The structure of the vimentin gene, *Cell* **35:**215–223.

Rheinwald, J. G., and Green, H., 1975, Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells, *Cell* **6:**331–343.

Rheinwald, J. G., and Green, H., 1977, Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes, *Nature* **265:**421–424.

Rice, R. H., and Green, H., 1977, The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein, *Cell* **11:**417–422.

Roop, D. R., Hawley-Nelson, P., Cheng, C. K., and Yuspa, S. H., 1983, Keratin gene expression in mouse epidermis and cultured epidermal cells, *Proc. Natl. Acad. Sci. USA* **80:**716–721.

Rowden, G., 1975, Ultrastructural studies of keratinized epithelia of the mouse, *J. Invest. Dermatol.* **64:**1–3.

Saari, J. C., Bredberg, L., and Garwin, G., 1982, Identification of the endogenous retinoids associated with three cellular retinoid binding proteins from bovine retina and retinal pigment epithelium, *J. Biol. Chem.* **257:**13329–13333.

Savage, C. R., Jr., and Cohen, S., 1972, Epidermal growth factor and a new derivative. Rapid isolation procedures and biochemical and chemical characterization, *J. Biol. Chem.* **247:**7609–7611.

Schindler, J., Matthaei, K. I., and Sherman, M. I., 1981, Isolation and characterization of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid, *Proc. Natl. Acad. Sci. USA* **78:**1077–1080.

Scholer, H. R., and Gruss, P., 1984, Specific interaction between enhancer-containing molecules and cellular components, *Cell* **36:**403–411.

Simon, M., and Green, H., 1984, Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte, *Cell* **36:**827–834.

Skerrow, D., and Hunter, I., 1978, Protein modifications during the keratinization of normal and psoriatic human epidermis, *Biochim. Biophys. Acta.* **537:**474–484.

Skerrow, D., and Skerrow, C. J., 1983, Tonofilament differentiation in human epidermis: isolation and polypeptide chain composition of keratinocyte subpopulation, *Exp. Cell Res.* **143:**27–35.

Skerrow, D., Matoltsy, G., and Matoltsy, M., 1973, Isolation and characterization of the helical regions of epidermal prekeratin, *J. Biol. Chem.* **248:**4820–4826.

Steinert, P. M., 1978, Structure of the three-chain unit of the bovine epidermal keratin filament, *J. Mol. Biol.* **123:**49–70.

Steinert, P. M., Idler, W. W., and Zimmerman, S. P., 1976, Self-assembly of bovine epidermal keratin filaments *in vitro*, *J. Mol. Biol.* **108:**547–567.

Steinert, P. M., Rice, R. H., Roop, D. R., Trus, B. L., and Steven, A. C., 1983, Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments, *Nature* **302:**794–800.

Steinert, P. M., Parry, D. A. D., Racoosin, E. L., Idler, W. W., Steven, A. C., Trus, B. L., and Roop, D. R., 1984, The complete cDNA and deduced amino acid sequence of a type II mouse epidermal keratin of 60,000 Da: Analysis of sequence differences between type I and type II keratins, *Proc. Natl. Acad. Sci. USA* **81:**5709–5713.

Sun, T-T., and Green, H., 1978, Keratin filaments of cultured human epidermal cells, *J. Biol. Chem.* **253**:2053–2060.

Tyner, A. L., Eichman, M. J., and Fuchs, E., 1985, The complete sequence of a type II keratin gene expressed in human epidermis: conservation of structure among all intermediate filament genes, *Proc. Natl. Acad. Sci. USA* **82**:4683–4687.

Viac, J., Staquet, M. J., Thivolet, J., and Goujon, C., 1980, Experimental production of antibodies against stratum corneum keratin polypeptides, *Arch. Dermatol. Res.* **267**:179–188.

Warhol, M. J., Pinkus, G. S., and Banks-Schlegel, S. P., 1983, Localization of keratin proteins in the human epidermis by a postembedding immunoperoxidase technique, *J. Hist. Cytochem.* **31**:716–723.

Watt, F. M. and Green, H., 1981, Involucrin synthesis is correlated with cell size in human epidermal cultures, *J. Cell Biol.* **90**:738–742.

Wolbach, S. B., 1954, Effects of vitamin A deficiency and hypervitaminosis A in animals, in: *The Vitamins* (W. H. Sebrell, Jr., and R. S. Harris, eds), Vol. 1, pp. 106–137, Academic Press, New York.

Wolf, G., 1980, Vitamin A, in: *Nutrition and the Adult: Micronutrients* (R. B. Alfin-Slater and D. Kritchevsky, eds.), pp. 97–103, Plenum Press, New York.

Woods, E. F., and Gruen, L. C., 1981, Structural studies on the microfibrillar proteins of wool: Characterization of the α-helix-rich particle produced by chymotryptic digestion, *Austr. J. Biol. Sci.* **34**:515–526.

Yardley, H. J., and Goldstein, D. J., 1976, Changes in dry weight and projected area of human epidermal cells undergoing keratinization as determined by scanning interference microscopy, *Br. J. Dermatol.* **95**:621–626.

5

Organization and Expression of the Vimentin and Desmin Genes

Wim Quax and Hans Bloemendal

1. The Discovery of Intermediate Filaments

The detailed view of the electron microscope has made it possible to identify a number of filamentous structures in the eukaryotic cell. Differences in general morphology and diameter, in addition to the susceptibility to certain drugs, have been used to classify the observed cytoskeletal filaments. Actin filaments (6 nm in diameter), microtubules (25 nm) in nonmuscle cells, and myosin filaments (15 nm) in muscle cells were readily characterized. However, the filaments with a diameter of 9–10 nm (intermediate between the diameter of actin-containing microfilaments and those of myosin containing thick filaments and microtubules) were initially not regarded as a different class of fibers.

A first step toward appreciation of these filaments as a different type was the observation that intermediate filaments (or 10-nm filaments) do not stain with heavy meromyosin (in contrast to actin filaments) and that they are resistant to treatment with colchicine (Ishikawa *et al.*, 1968). This drug, which dissociates microtubules into its subunits, caused the intermediate filaments to collapse into a perinuclear organization, but did not disintegrate the individual filaments. From these observations it was anticipated that intermediate filaments (IF) form a distinct cytoskeletal structure, which interacts with microtubules (Ishikawa *et al.*, 1968). Despite the clear distinction from other cytoskeletal fibers, progress in the biochemical characterization of IF was

Wim Quax and Hans Bloemendal • Department of Biochemistry, University of Nijmegen, 6525 EZ Nijmegen, The Netherlands. *Present address of Dr. Quax:* Department of Microbiology, Gistbrocades, 2600MA Delft, The Netherlands.

slow, mainly because of the insolubility of the filaments and the resulting difficulties in isolation of their subunits (Shelanski *et al.*, 1971; Eng *et al.*, 1971; Skerrow *et al.*, 1973; Hoffman and Lasek, 1975). Moreover, it was puzzling that number and sizes of subunits isolated from diverse sources such as nerve cells, smooth muscle cells, epithelia, and astrocytes were very different, although the 10-nm filaments from different tissues showed a similar morphology in electron microscopy studies (Hoffman and Lasek, 1975; Steinert and Idler, 1975; Small and Sobieszek, 1977). The full understanding of the diversity and wide distribution of 10-nm filaments was only achieved when suitable antibodies directed against the different subunits were used in immunofluorescence studies (Bignami *et al.*, 1972; Lazarides and Hubbard, 1976; Liem *et al.*, 1978; Hynes and Destree, 1978; Franke *et al.*, 1978a,b; Sun and Green, 1978). In the immunofluorescence microscope bright filamentous structures were observed after treatment of permeabilized cells with antibodies directed against IF proteins. The localization of these fluorescent fibers and the collapse into a perinuclear cap upon treatment with colchicine were the main arguments linking the brightly fluorescent network with the electron microscopic observations of 10-nm filaments (Osborn *et al.*, 1977; Hynes and Destree, 1978). With the aid of different antibodies it was now rapidly understood that IF show tissue specificity: for instance, antibodies raised against IF from epithelial cells were able to decorate filaments in all types of epithelial cells, but not in muscle cells or nerve cells (Franke *et al.*, 1978a; Lazarides and Hubbard, 1976; Bennet *et al.*, 1978).

During the last 5 years enormous progress has been made in classification and characterization of different IF subunits owing to the application of new techniques such as high-resolution two-dimensional gel electrophoresis, production of monoclonal antibodies, immunoblotting, and recently DNA cloning.

2. *The Diversity of IF Subunits*

Isolation procedures for IF subunits depended on a common chemical property of 10-nm filaments, namely their insolubility in buffers containing Triton X-100 (1%) and high or low salt concentrations (for references see Franke *et al.*, 1981). Solubilization of IF proteins is achieved only under denaturing conditions, e.g., 6 M urea. Surprisingly, it was found that after removal of urea, purified proteins reassembled to form authentic 10-nm filaments *in vitro* (Steinert *et al.*, 1976). This shows that the polymerization of IF subunits to filaments is a reversible process that takes place spontaneously without the need of energy or accessory proteins. By carrying out subsequent disassembly/reassociation steps, some workers used this phenomenon to develop a procedure to purify IF proteins (for references see Steinert *et al.*, 1981). On the other hand, SDS-polyacrylamide gel electrophoresis of the Triton X-100 insoluble fraction was sufficient in most cases to obtain a pure antigen for the production of antibodies. Antisera obtained in this way have

been used to classify the different IF proteins. In the past few years this classification has been confirmed and refined by data on the primary structure of the different proteins. Part of these data were obtained by direct amino acid sequencing of the purified proteins (Geisler and Weber, 1981, 1982, 1983; Geisler *et al.*, 1983, 1984). Most of the data, however, were obtained by analyzing the DNA information encoding the IF subunits (Hanukoglu and Fuchs, 1982, 1983; Steinert *et al.*, 1983, 1984; Quax-Jeuken *et al.*, 1983; Quax *et al.*, 1983, 1984a,b; Hofmann and Franz, 1984; Lewis *et al.*, 1984; Jorcano *et al.*, 1984b). Based on sequence data and immunological observations we have summarized the classification of IF in Table 1.

The expression pattern of the different IF shows close parallels to established patterns of embryological differentiation (Table 1). In muscle cells we find desmin, in neurons neurofilaments, in cells of glial origin (astrocytes) glial filaments, in cells of mesenchymal origin vimentin, and in epithelial tissue keratins. This tissue specificity is one of the main causes for the growing interest in these proteins, because this renders them, to very specific differentiation markers, useful in developmental biology and tumor diagnosis (Osborn and Weber, 1982; Ramaekers *et al.*, 1981). In contrast to the nonepithelial IF subunits, the keratins form a large group of related proteins encoded by a family of related genes. Subdivision of keratins has been performed on the basis of isoelectric point and mutual homology. This has resulted in the definition of a subclass of acidic (type I) and basic (type II) proteins. Within one group the keratins show about 60–70% homology (identical amino acids at corresponding positions). Keratins of different groups are only about 30% homologous. The homology in primary structure between the keratins, on the one hand, and the nonepithelial IF subunits, on the other hand, is low, but significant (±30% homology). The mutual homology of nonepithelial IF proteins is higher (>50%), with vimentin, desmin, and glial fibrillary acidic protein being the most closely related (>65%).

Although the divergence in primary structure of IF proteins suggests rather dramatic differences between the subunits, the secondary structure (as it can be predicted from the primary structure) and the physical properties of the proteins show striking similarities. It appears that all IF molecules have a central polypeptide domain of about 310 residues that is mainly in α-helical conformation (as was first observed from circular dichroism measurements). Within this α-helical region there is a regular heptade distribution of hydrophobic amino acids, which is typical for α-helices that can interact to form coiled-coils. An identical heptade distribution has thus far been found in all IF molecules, suggesting the same lengths and interruptions for the coiled-coils. These findings have resulted in the prediction of a general model for IF proteins. In Fig. 1 the model is outlined on the basis of the vimentin sequence (Quax-Jeuken *et al.*, 1983). The central α-helical domain can be considered as the constant domain of IF, since all subunits show a nearly identical secondary structure in this region. Consequently, this is also the region of highest homology in primary structure among the various IF sequences. The nonhelical NH_2 and COOH parts account for the diversity of IF. Homologous sequences

Table 1. Overview of the Various Intermediate Filament Subunits as Identified by Immunological and Biochemical Techniques[a]

Cell type	Subunits	#	Molecular weight	Sequence	Reference
Muscle	Desmin	1	52 kd	Chicken (100%)	Geisler and Weber, 1982
				Chicken (COOH 20%)	Capetanaki *et al.*, 1984
			54 kd	Hamster (COOH 70%)	Quax *et al.*, 1984a
				Hamster (100%)	Quax *et al.*, 1985a
Mesenchymal	Vimentin	1	53.5 kd	Hamster (COOH 95%)	Quax-Jeuken *et al.*, 1983
				Hamster (100%)	Quax *et al.*, 1983
				Pig (COOH 30%)	Geisler and Weber, 1981
Glial	GFAP	1	50 kd	Pig (NH_2 + COOH 30%)	Geisler and Weber, 1983
				Mouse (COOH 90%)	Lewis *et al.*, 1984
Neuronal	Neurofilaments	3	68 kd[b]	Pig (50%)	Geisler *et al.*, 1983
				Mouse (COOH 60%)	Lewis and Cowan, 1985
			160 kd[b]	Pig (NH2 30%)	Geisler *et al.*, 1984
			200 kd[b]	Pig (30%)	Geisler *et al.*, 1983, 1985
Epithelial	Keratins	Many[c]			
	Man				
	Basic	8	52.5–68 kd	Human 56 kd (COOH 60%)	Hanukoglu and Fuchs, 1983
	Acidic	11	40–64 kd	Human 50 kd (COOH 85%)	Hanukoglu and Fuchs, 1982
				Human 50 kd (100%)	Marchuk *et al.*, 1984
	Cow				
	Basic	±15		Cow 1A (COOH 30%)	Jorcano *et al.*, 1984c
				Cow 1B (COOH 30%)	Jorcano *et al.*, 1984c
	Acidic	±11	43–57 kd	Cow VIB (COOH 40%)	Jorcano *et al.*, 1984b
				Cow VII (COOH 20%)	Jorcano *et al.*, 1984b
	Mouse				
	Basic	±14	54–70 kd	Mouse 59 kd (100%)	Steinert *et al.*, 1983
	Acidic	±12	45–58 kd	Mouse 69 kd (100%)	Steinert *et al.*, 1984
	Xenopus				
	Basic	>3	63–65 kd		
	Acidic	>3	49–53 kd	*Xenopus* 51 kd (COOH 25%)	Hoffmann and Franz, 1984

[a]The first column (cell type) refers to the tissue in which the corresponding subunit (column 2) is expressed. Column 3 (#) represents the number of primary gene products and column 4 the molecular weight determined for that subunit type. Column 5 (Sequence) summarizes the elucidation of primary structures of various IF proteins as performed by direct amino acid sequencing or by prediction from the corresponding DNA sequence.

[b]There is an enormous discrepancy between the molecular weight estimations performed on SDS–PAGE gels and those determined by other methods (Kaufmann *et al.*, 1984).

[c]For the number and isoelectric point of the human cytokeratins, see the human keratin catalogue (Moll *et al.*, 1982); for other species, see Franke *et al.* (1981).

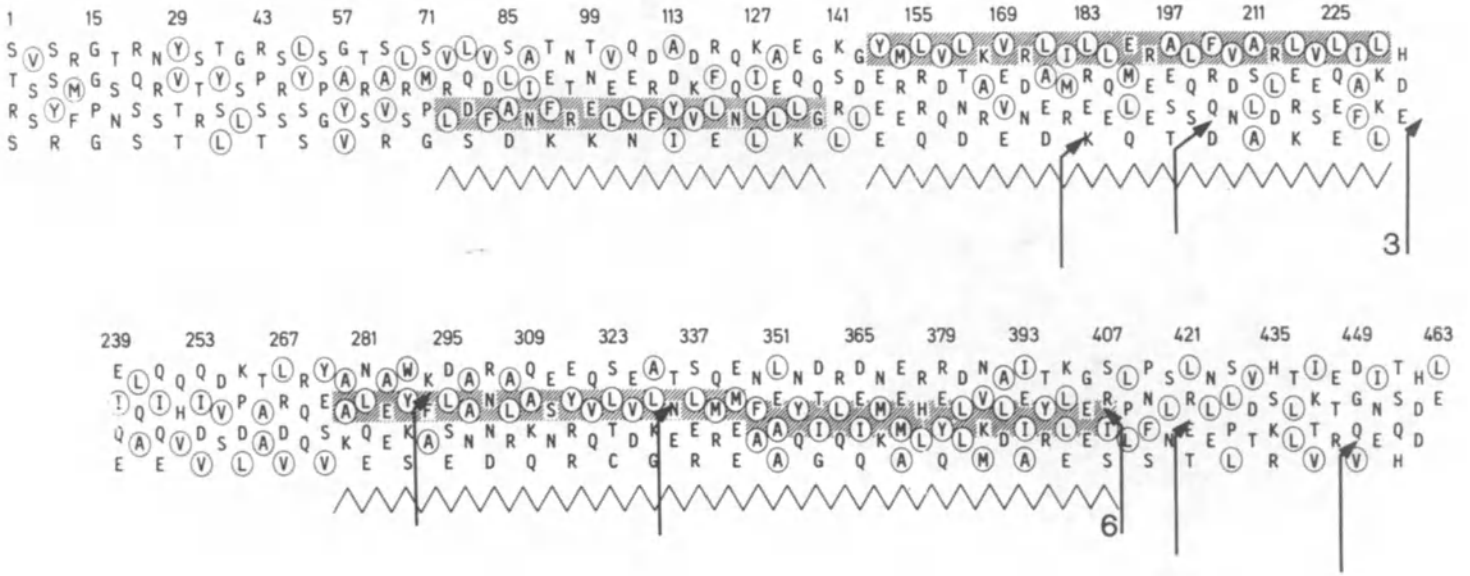


Figure 1. The general intermediate-filament model and intron positions. The complete primary structure of vimentin, as deduced from the nucleotide sequence of the gene (Quax *et al.*, 1983), has been arranged in a periodic distribution with a regularity of seven (heptade convention). Nonpolar amino acids [alanine (A), valine (V), methionine (M), isoleucine (I), leucine (L), tyrosine (Y), phenylalanine (F), and tryptophan (W)] are circled. Shaded regions show a heptade distribution of hydrophobic residues and are therefore thought to be in α-helical conformation (Quax-Jeuken *et al.*, 1983). It can be seen that the arrows, representing the intron positions in the vimentin gene, do not match with protein domain borders apart from intron 3 and 6. The latter two intervening sequences are the only ones that comply with the terms of the Gilbert hypothesis (Gilbert, 1978).

in these domains are found only between very closely related members; e.g., vimentin is about 45% identical to desmin and GFAP in the COOH domain, but homology with neurofilaments or keratins is completely absent in this region.

The length of the NH_2 terminal domain varies among different IF molecules from short in GFAP (45 residues) to long in the 57-kD mouse keratin (125 residues). The COOH tailpiece is extremely variable in length: from 45 in human keratin 50 kD to probably more than 700 amino acids in the highest-molecular-weight neurofilament subunit (Geisler *et al.*, 1984; Kaufmann *et al.*, 1984). The extremely long extensions of the two high-molecular-weight neurofilament subunits (NF-M and NF-H) are probably not involved in filament formation. Amino acid composition data indicate that these tailpieces are extremely rich in charged residues, particularly glutamic acid and lysine (Geisler *et al.*, 1983, 1984). These charged residues are located surface-exposed at the outside of neurofilaments. Whether these charged polypeptide chains are involved in nerve conduction is unknown.

As already mentioned, isolated IF subunits can polymerize *in vitro*. However, only the nonepithelial members are able to form homopolymer filaments consisting of a single subunit (Small and Sobieszek, 1977; Geisler and Weber, 1980). The keratins always form a heteropolymer, built up from at least one type I and one type II subunit (Franke *et al.*, 1983; Quinlan *et al.*, 1984). Purified monomers of one type do not form filaments *in vitro* (Steinert *et al.*, 1981). In the case of the nonepithelial IF, homopolymer filaments represent the common situation. However in cell types that contain more than one subunit, heteropolymers can also form (Quinlan and Franke, 1982, 1983). This concerns mostly cells in culture, since cultured cells as a rule start synthesizing vimentin in addition to the original IF subunit (for references see Franke *et al.*, 1981). Polymerization of keratins and vimentin into the same filaments has never been observed.

The abundance of data on the structure of IF subunits is in sharp contrast to the lack of understanding of the polymerization process by which individual subunits assemble into 10-nm filaments. It is now generally accepted that the first polymerization step is the formation of a dimer through a coiled-coil interaction and the subsequent alignment of two dimers to form a tetramer. The number and the arrangement of these tetramers within 10-nm filaments are, however, still the subject of speculation (Ip *et al.*, 1985).

3. The Genes Encoding IF

The introduction of recombinant DNA technology has also put its mark on IF research. cDNA clones have not only made it possible to derive the primary structure of many subunits from the nucleotide sequence, but they have also made it possible to study chromosomal information and gene expression of IF. Keratin cDNAs, which were the first to be constructed (Fuchs *et al.*, 1981), allowed the distinction of two different keratin subclasses on the

basis of hybridization experiments. On genomic DNA each cDNA hybridized with multiple restriction fragments indicating that there are many closely related genes encoding keratins: a multigene family. Vimentin cDNA, which was isolated next (Dodemont *et al.*, 1982; Quax *et al.*, 1982), hybridized only with a single chromosomal locus. Hence, vimentin represented the first cytoskeletal protein that was proven to be encoded by a single-copy gene. Recently it was shown that GFAP (Lewis *et al.*, 1984) and desmin (Quax *et al.*, 1984a) are also encoded by single-copy genes. More detailed studies of keratin cDNA (Jorcano *et al.*, 1984a; Lehnert *et al.*, 1984) showed that keratin genes behave like single-copy genes if one applies highly stringent hybridization conditions. This would mean that each IF protein that can be visualized in a specific position on a two-dimensional gel is encoded by a single gene detectable on genomic blots. These findings distinguish the IF proteins from actins and tubulins, the other cytoskeletal proteins. The latter proteins are encoded by multiple, very closely related genes (e.g., >20 actin genes in man; Soriano *et al.*, 1982; Quax *et al.*, 1982) that encode identical or nearly identical proteins with nearly equal migration behavior in two-dimensional gel electrophoresis.

Despite the diversity of IF proteins, the homology at the DNA and protein level favors the concept that IF emerged from a common ancestral gene. In the search for the evolutionary origin of IF genes, cDNA probes have been used to detect homologous sequences in the genomic DNA of vertebrate and invertebrate species. In all vertebrates tested, vimentin cDNA (Quax *et al.*, 1982, 1984a), keratin cDNA (Fuchs and Marchuk, 1983), and desmin cDNA (Quax *et al.*, 1984a) were able to detect hybridizing fragments under normal stringent conditions (50% formamide, 0.6 M NaCl, 42 °C). However, detection of positive hybridizing fragments in invertebrates was not possible under the same conditions. This implies that the coding information for IF is evolutionary lesser conserved than the genes for actin and tubulin, a finding that could be anticipated because of the diversity of IF proteins.

As far as the structure of IF genes is concerned, data have only recently become available (Quax *et al.*, 1983; Lehnert *et al.*, 1984; Johnson *et al.*, 1984). The hamster vimentin gene was the first IF gene whose structure was unraveled. Nine exons, which are spread over ± 9kb of DNA, carry the coding capacity for vimentin mRNA (1848 bases) (Fig. 2). Relating the intron positions to the general model of IF (Fig. 1), it becomes clear that some introns match with a protein domain border (intron 3 and 6), but most do not. This correlation of gene and protein structure, which is found in many genes, is thought to be a remainder of processes of exon shuffling and gene duplication during evolution (Gilbert, 1978; Blake, 1978). From the structure of the vimentin gene one could anticipate that those intron positions that do not map at domain borders of the IF model are unique for the vimentin gene and therefore not evolutionary conserved. Surprisingly, however, precisely the same borders are also found in the hamster desmin gene (Fig. 3), showing that the intron positions of these two genes have been conserved (Fig. 4) since their divergence. The conclusion concerning the evolutionary relationship could be drawn owing to elucidation of the complete nucleotide sequences of the vi-

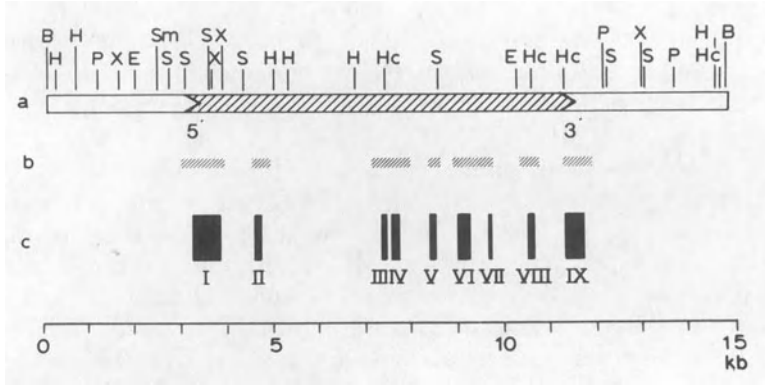


Figure 2. Physical map of the vimentin gene. (a) The sites for some often used restriction enzymes in the λ-Havim insert are shown. B, Bam H1; E, EcoR1; H, Hind111; Hc, Hinc11; P, Pvu11; S, Sst1; Sm, Sma1; X, Xhol. 5′ and 3′ correspond to the positions where the 5′ and 3′ specific probes derived from the cDNA hybridized, respectively. (b) Shaded bars = regions whose sequence have been reported elsewhere (Quax *et al.*, 1983). (c) The black bars represent the position and size of the vimentin exons. They are numbered with Roman numerals.

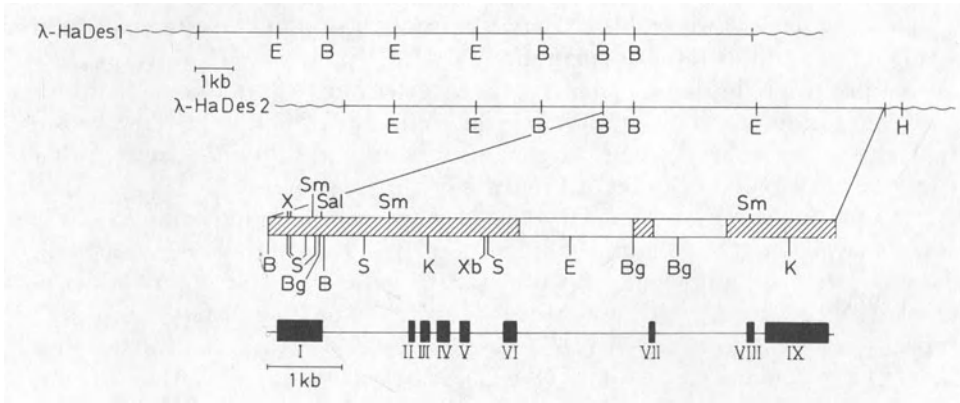


Figure 3. Physical map of the hamster desmin gene. The two overlapping Charon phages carrying desmin gene sequences (λ-HaDes1 and λ-HaDes2) are shown in the upper two lines. The wavy lines represent Charon 28 sequences. The large (7.4-kb) central EcoR1–EcoR1 fragment of λ-HaDes2 and the 3.8-kb fragment, ranging from the rightmost EcoR1 site of a λ-Hades2 to the Hpa1 site of Charon 28, were subcloned in pBR322. The thick bar is the region that was analyzed in detail with the striated parts representing the sequenced areas (Quax *et al.*, 1985a). The lowermost line is a schematic drawing of the desmin gene: the closed boxes represent the exons and the thin lines in between them the introns. B, BamH1; Bg, Bg11I; E, EcoR1; H, Hpa1; K, Kpn1; S, Sac1; Sal, Sal1; Sm, Sma1; X, Xho1; Xb, Xba1.

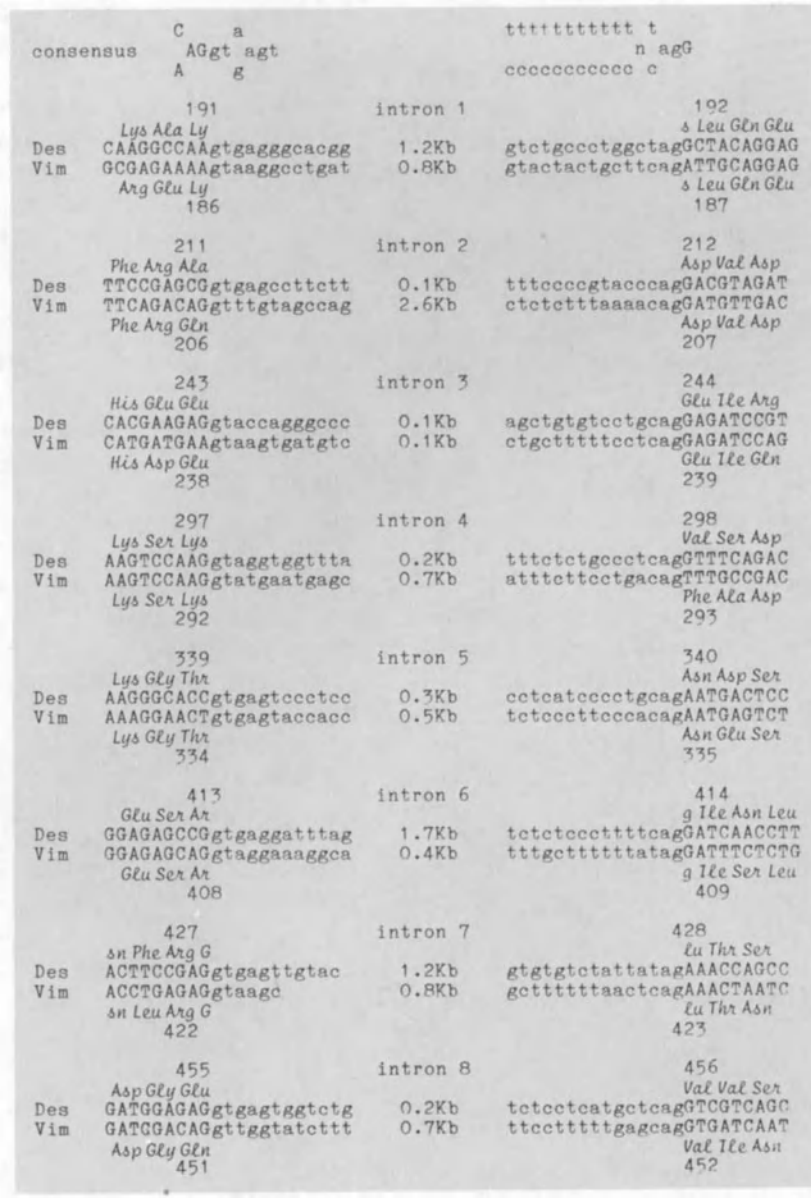


Figure 4. Exon–intron junctions of the desmin gene compared to the vimentin gene. The nucelotides surrounding the splice points of the desmin (Des) and the vimentin gene (Vim) are compared to consensus 5′ splice point (left) and a consensus 3′ splice sequence (right), which has been deduced by comparing a great number of intron borders (Mount, 1982). Exon sequences are in capital letters, the number on top of, or below the amino acids represents the relative number in the desmin polypeptide or the vimentin polypeptide, respectively. The sizes of the introns are shown in the middle column. Apart from the dinucleotides "gt" and "ag," the homology between intron border sequences of the two genes is low or absent.

mentin and desmin gene, which are shown in alignment (Fig. 5). Divergence of the vimentin and desmin genes is thought to have taken place long before the evolution of vertebrate species (Quax *et al.*, 1984a).

Recently some data on the exon/intron pattern of keratin genes have been reported. R-looping analysis of four bovine keratin genes suggests that the number of introns in the keratin genes is the same (8) or one less (7) as in the vimentin and desmin genes (Lehnert *et al.*, 1984). Although it seems that not all intron positions are equal to the vimentin gene, some of them are. One of the exon/intron borders was sequenced (λKB1a, Lehnert *et al.*, 1984), and this correlates exactly with vimentin and desmin intron 6, the intron that interrupts the gene precisely behind the information for the coiled-coil α-helical domain. Another preliminary report (Johnson *et al.*, 1984) states that the gene for mouse keratin 67 kD is also interrupted precisely behind the information encoding the coiled coil. The most extensive information on keratin gene structure has recently become available for the human gene encoding the 50-kD keratin (Marchuk *et al.*, 1984). A very remarkable conservation of intron positions among IF genes was revealed by this study. Five out of seven introns of the human keratin gene are located at positions that fully correspond to intron positions of the vimentin gene. This provides additional evidence that IF genes emerged from a common ancestral gene.

The expression of IF genes seems to be regulated at the transcriptional level. mRNA quantities have been found to correlate correctly with the presence of proteins. The mRNA expression of the vimentin gene was puzzling in chicken cells, because two mRNA-size bands could be detected in northern blots, although only one gene was found (Dodemont *et al.*, 1982; Zehner and Paterson, 1983; Capetanaki *et al.*, 1983). A comparison of the structure of the 3′ part of the chicken vimentin gene and the vimentin cDNA showed that multiple mRNAs originated from the alternative use of more polyadenylation sites at the 3′ end of the gene (Zehner and Paterson, 1983).

4. Expression of the Cloned Desmin Gene

4.1. In Lens Cells

One of the most revolutionary impacts of the availability of cloned IF genes is the possibility to transfer and express genes into heterologous cells. This makes it possible to study the behavior of newly synthesized IF proteins of a certain type in cells in which the introduced gene is normally inactive.

The cloned IF genes, in combination with suitable expression systems, also form the obvious way to study the molecular mechanism that is at the basis of the tissue-specific transcription of IF. Promoter regions, "activators" or "enhancers" that might be responsible for the onset or shutoff of gene expression, can be characterized by testing parts of the cloned gene inserted into suitable eukaryotic vectors in different cell lines.

First we asked two questions: Is the cloned DNA biologically active after

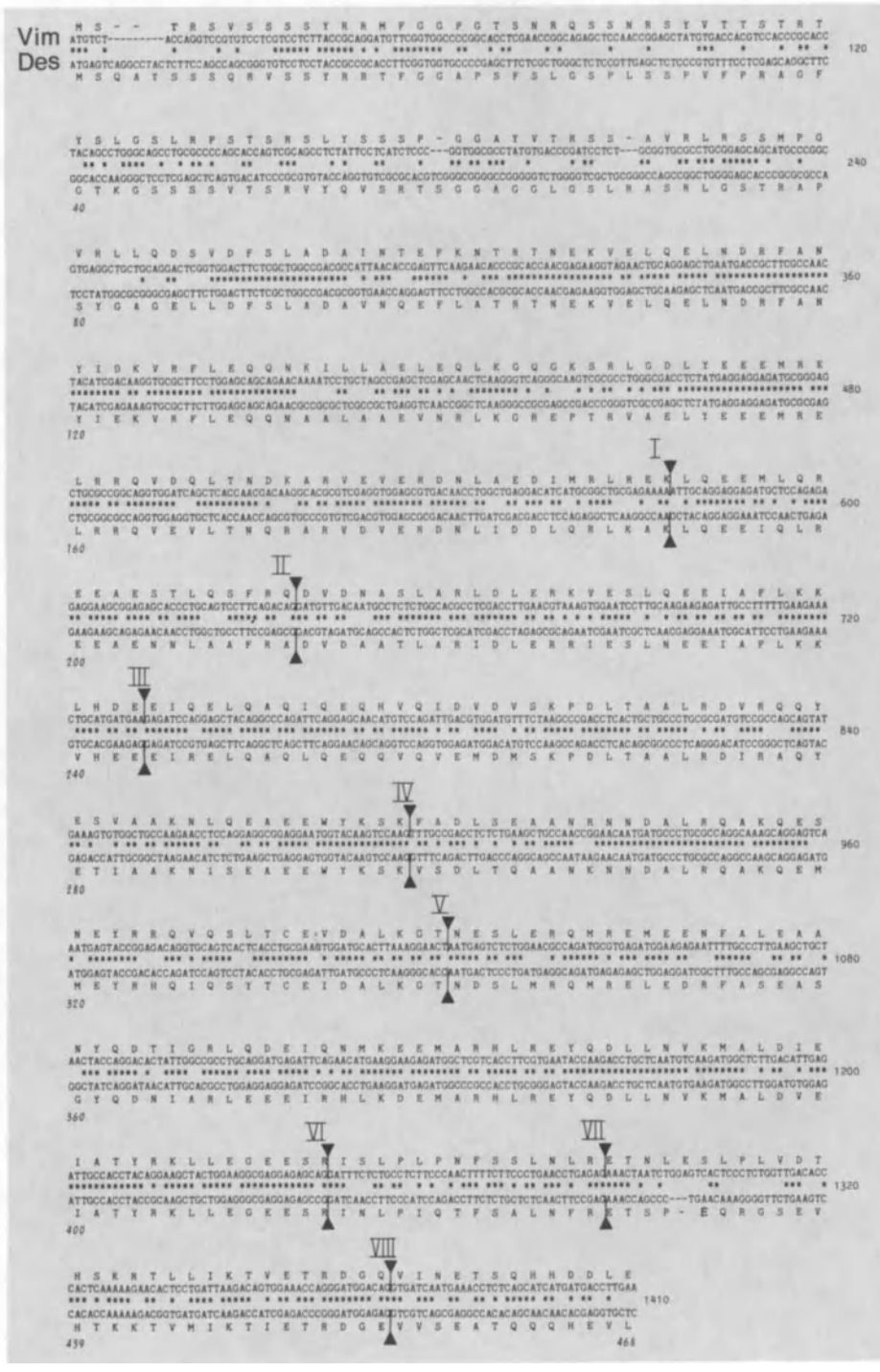


Figure 5. Alignment of the complete coding sequences of the hamster vimentin and desmin genes. Intron positions are indicated by arrows.

transfection into cultured cells, and if so, is it possible to generate a desmin IF cytoskeleton in cells that normally do not contain desmin filaments. We therefore constructed a plasmid, which contains the 7.4 EcoR1–EcoR1 and the 3.8 EcoR1–Hpa1 fragment of λ-HaDes2 together with the EcoR1–PvuII fragment carrying the origin and the ampicillin resistance gene of pBR322 (Fig. 6). The complete desmin transcription unit plus 3.5 kb of 5′ upstream sequences is present in this construct. A calcium phosphate precipitate containing this plasmid was transfected onto a monolayer of hamster lens cells, a culture previously shown to contain vimentin as the sole IF subunit (Bloemendal *et al.*, 1980). Two days after transfection the cells were fixed on the coverslips, which were used as substratum, and incubated with antibodies directed against desmin. After being stained with an appropriate fluorescein-conjugated second antibody, the monolayers were monitored for desmin-positive cells. About 0.5–2% of the cells showed the filament-staining pattern typical for IF (Fig. 7). This percentage of positive cells is within the normal range for the calcium phosphate technique (Sompayrac and Danna, 1981; Graham and van der Eb, 1973). In control experiments without DNA or with plasmids without desmin sequences, desmin-positive cells were never observed. From Fig. 7b,c it is obvious that the newly synthesized desmin is able to form bona fide filaments. Using double immunofluorescence staining with

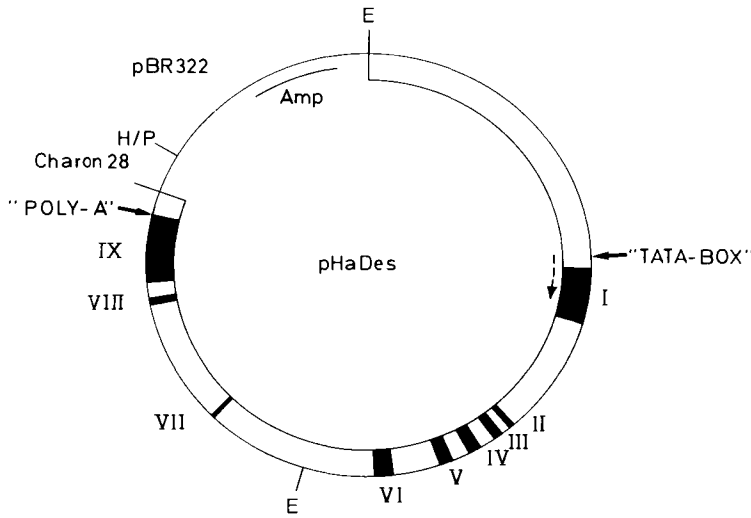


Figure 6. Structure of pHaDes: the construction used for gene transfer. The 7.4 EcoR1–EcoR1 and the 3.8 EcoR1–Hpa1 fragment of λ-HaDes2 were subcloned into pBR322, which was cut with the enzymes EcoR1 and PvuII. The hamster genomic fragment, represented by the thick bar, carries the complete desmin gene. "TATA-BOX" and "POLY-A" are the transcription start and stop signals of the desmin gene. The closed parts of the bar represent the exons (numbered I to IX). The pBR322 part of the construct carries the origin of replication and the ampicillin gene allowing the use of *Escherichia coli* for propagation of the construct. Charon 28 indicates the fragment of the rightmost arm of the gene cloning vector Charon 28, which was introduced by subcloning the 3.8 EcoR1–Hpa1 fragment of λ-HaDes2. The total size of pHaDes is 13.5 kb.

polyclonal or monoclonal vimentin antibodies in combination with a monoclonal or polyclonal desmin antibody, respectively (Fig. 7d,e), we observed that the newly formed fibrillar desmin staining pattern colocalizes with the vimentin filaments, which are always present in lens cells. This demonstrates that the newly synthesized desmin is able to form a heterogeneous cytoskeleton with vimentin IF. This cytoskeleton probably also consists of heteropolymer filaments as described for BHK-21 cells (Quinlan and Franke, 1982).

Next to the authentically looking desmin filaments in most cells, in a small subset of cells we observed lumplike aggregates that were deeply stained with the desmin antibody (Fig. 7f,g). The origin of these aggregates is unclear, but it is possible that the synthesis of desmin in those cells occurs at such high levels that the IF proteins precipitate. An indication for this hypothesis comes from the observation that the vimentin fluorescence of these cells showed exactly the same aggregated distribution (Fig. 7h,i). Furthermore, in some of the cells the aggregate was not concentrated in one lump, but rather distributed in a number of dotlike structures (Fig. 7g). Whether the dots in those cells originate from an excess synthesis or from an uncontrolled or ineffective filament assembly is obscure at this moment. The fact that desmin-positive cells are in many instances found in clusters (Fig. 7d), in combination with the observation that occasionally cells seem to go through some stages of mitosis, gives a strong indication that these hamster cells are still able to divide and multiply, independent of the synthesis and assembly of desmin polypeptides.

4.2. Expression of the Hamster Desmin Gene in Human Epithelial (HeLa) Cells

To test whether there is any species specificity in the transcription of the desmin gene or in the subsequent formation of desmin filaments, we decided to transfect the plasmid pHaDes also into HeLa cells. These epithelial cells express, in addition to vimentin, cytokeratins 7, 8, 17, and 18 (Moll *et al.*, 1982), but no desmin. Transfection of pHaDes caused about 0.5–2% of the cells to react positively with desmin antibodies after 2 days (Fig. 8a,b), indicating that there is no species barrier for the transcription signals of the hamster gene. The desmin-staining reaction shows a similar fibrillar pattern as in the hamster lens cells (Fig. 8b). Double immunofluorescence of the same cell with a monoclonal desmin and a polyclonal vimentin antibody (Fig. 8c, d) shows that at least the major part of the newly synthesized desmin subunits becomes completely integrated in the preexisting vimentin cytoskeleton of HeLa cells. When aggregation or precipitation of desmin IF was seen, the vimentin IF could also be shown to exist (partly) in an aggregated form (Fig. 8e,f). This is in favor of the assumption that the aggregates on these cells result from precipitation of an abundance of synthesized desmin protein forming a coprecipitate with the preexisting vimentin molecules.

Since no cell type has been described to express both desmin and cytokeratins so far, we wondered how the two types of filaments would interact in the manipulated HeLa cells. Therefore, we applied the double-label immu-

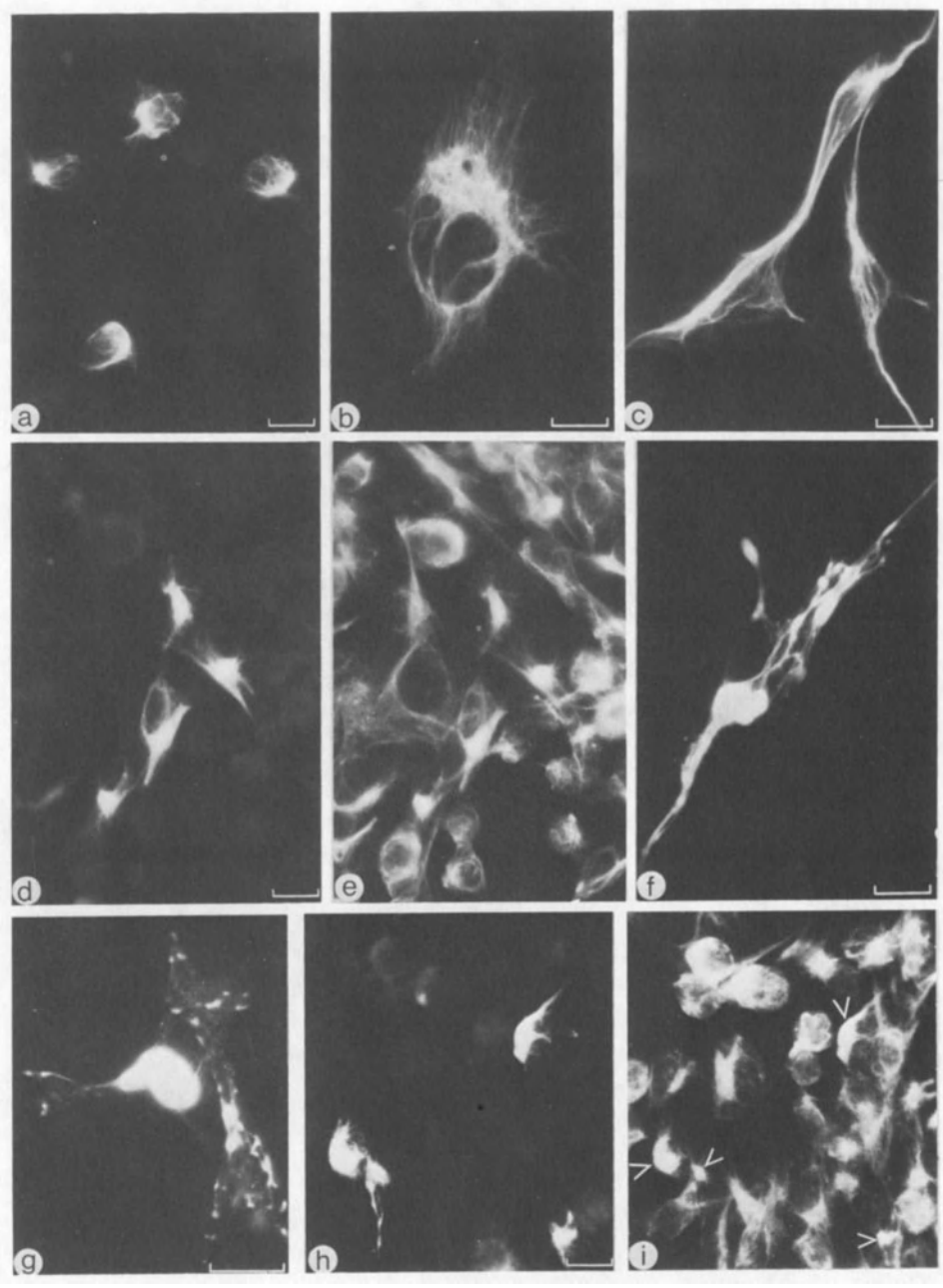


Figure 7. Expression and assembly into intermediate filaments of desmin in cultured hamster lens cells, 45 hr after transfection with the cloned desmin gene. (a–c) Immunofluorescence microscopy showing synthesis of desmin and assembly into authentic intermediate filaments, as monitored with the polyclonal desmin antiserum. (d, e) Double-label immunofluorescence microscopy with the monoclonal antiserum (RD301) (d) and the polyclonal antivimentin (e) showing complete colocalization of both IF protein types. (f, g) Occasionally, desmin-positive immunofluorescence is observed in large, lumplike aggregates or in smaller dotlike (g) structures (polyclonal antidesmin). (h, i) Double-label immunofluorescence microscopy showing perfect matching of desmin (as stained with RD301) and vimentin (stained with polyVim) immunofluorescence in intermediate filament aggregates (indicated by arrowheads in i). Bars: 20μm.

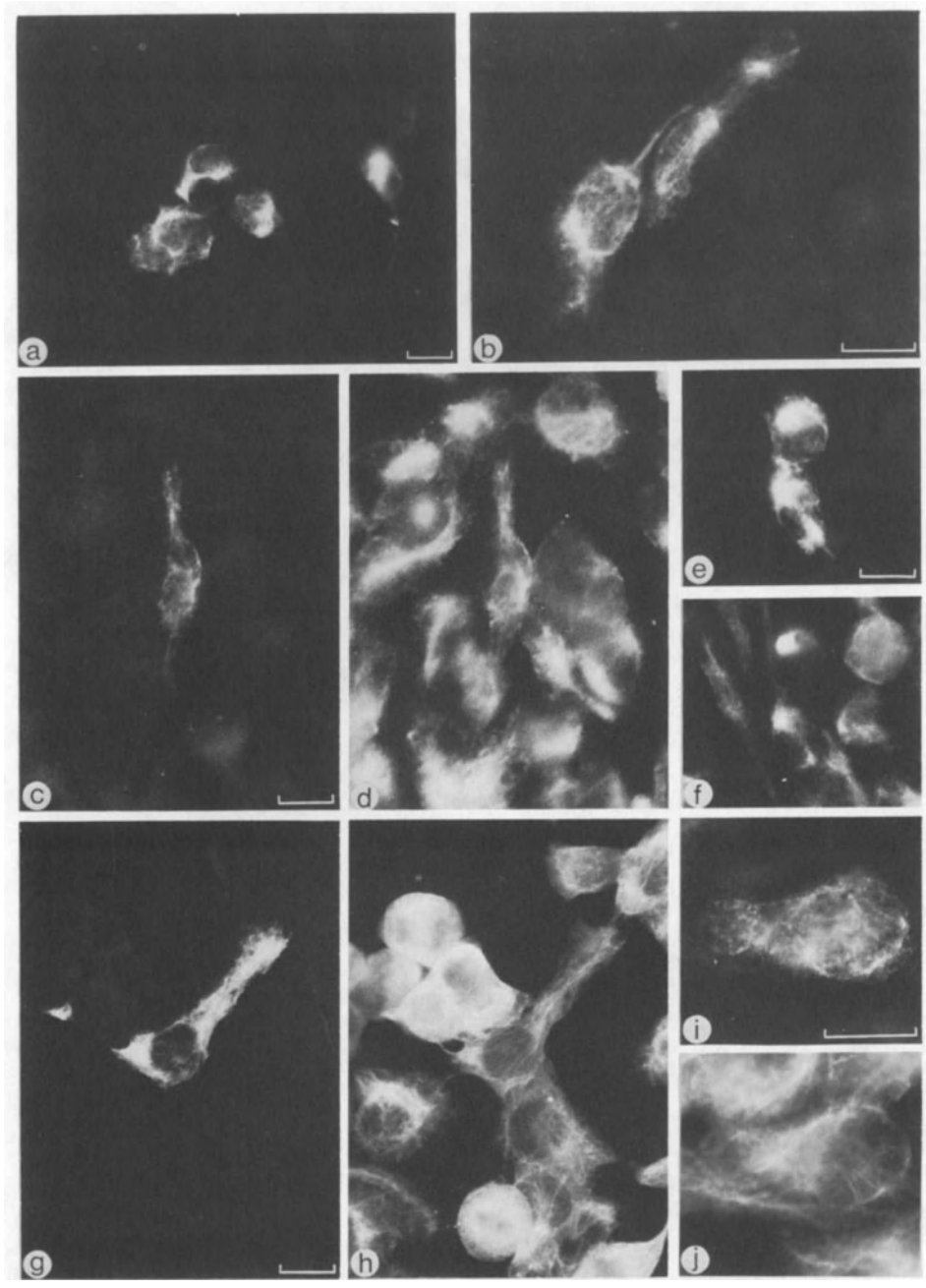


Figure 8. Expression and assembly of desmin into filaments in HeLa 45 hr after transfection with pHaDes. (a, b) Single-label immunofluorescence microscopy of desmin producing HeLa cells, as detected by the polyclonal desmin antiserum (polyDes). Note fibrillar organization in b. (c–f) Double-label immunofluorescence microscopy showing desmin intermediate filaments (c, e) and colocalization with vimentin (d, f) both in network structures (c, d) and in aggregates (e, f). Antibodies used were monoDes, RD301 (c, e) and polyVim (d, f). (g–j) Micrographs demonstrating that no real spatial relationship exists between the newly made desmin (g, i; stained with polyDes) and the HeLa cytokeratin filaments (h, j; stained with the monoclonal antibody to cytokeratin 18, RGE 53). Bars: 20 μm.

nofluorescence technique with polyclonal desmin antiserum and the monoclonal antibody to cytokeratin 18. As can be seen in Fig. 8g–j, the desmin fluorescence does not show the same overall distribution pattern as the cytokeratin fluorescence, although in some regions both types of IF seem to occupy the same positions. Because of the small size of the cells, however, it is difficult to determine whether this involves true colocalization in these regions. In additional experiments, where we used monoclonal desmin antibody in combination with a polyclonal keratin antiserum, the two types of filaments did not seem to be precisely colocalized either. Finally, desmin-producing HeLa cells also seem to be able to go through mitosis, as can be concluded on the basis of the same arguments as put forward for lens cells.

We believe that the experiments described here are an important addition to the methods of experimentally altering the IF cytoskeleton. It can be concluded from the ability of the formed protein to react with both monoclonal and polyclonal desmin antibodies, in combination with the observed correct formation of desmin filaments, that the product formed by the transferred gene is authentic desmin. This conclusion is strongly supported by our observations in double-label experiments with vimentin and cytokeratin antibodies. In accord with findings by other authors (for example, Quinlan and Franke, 1982) we have found that the desmin formed in both cell types used in this study colocalizes almost completely with the vimentin filament network. In the HeLa cells, however, in general no or less colocalization of cytokeratin and desmin in filaments is observed. Focal alignments or intermingling of these two types of IF systems is occasionally seen, but such an apparent spatial relationship may be explained by crowding of filaments of different types within a thin rim of cytoplasm, as is often the case in the cells used in this study.

One advantage of the use of a cloned gene is the ability to study the expression and filament formation of a single subunit at a time without introducing unknown and possibly interacting proteins, which is what happens when an impure mRNA mixture is used. Therefore, we conclude that in our experiments the assembly of the de novo synthesized desmin molecules into filaments is not dependent on muscle-specific accessory proteins. Second, the expression of the cloned desmin gene makes clear that there is no blockade on the transcriptional level as well as on the translational level in heterologous cells as well as in a heterologous species. This apparent nonobeyance of a gene to the normally observed tissue specificity has also been found for other genes in a transient expression assay (Gunning *et al.*, 1984). Probably the regulation of gene expression is not functioning perfectly when a great number of episomal DNA molecules carrying the gene are present in the nucleus.

Third, an exciting advantage of using a cloned gene is the possibility of engineering the DNA *in vitro* prior to transfection. This will enable the construction of genes that express altered IF proteins and make it possible to study the effects of these alterations on filament formation. Ultimately, the sequence requirements for the structural domains of IF, which have been deduced merely from comparison of different sequences, can be experimentally tested.

5. *Chromosome Localization of the Human Vimentin and Desmin Gene*

For the assignment of IF genes we used a panel of five mouse × human and a panel of nine chinese hamster × human somatic cell hybrids. The cryopreserved cell lines, which were screened for the human chromosomes and chromosome-specific markers during previous studies (Herbschleb-Voogt *et al.*, 1981a,b; Meera Khan *et al.*, 1983, 1984), were brought back to culture, and the cell pellets of each hybrid line were divided into two portions. One part was used to verify the human chromosome content by retesting for chromosome-specific enzyme markers. The updated information is summarized in Table 2. The other part was used to isolate total DNA. This DNA was digested with an appropriate restriction enzyme, electrophoresed in an agarose gel, and transferred to a nitrocellulose filter. Filters were hybridized to the nick-translated insert of pVim-1 (Quax-Jeuken *et al.*, 1983) or to one of the previously described desmin-specific M13 probes (Quax *et al.*, 1985b), respectively. For pVim-1 it was found that the restriction enzyme EcoR1 gave a good resolution of the mouse and human bands. In the case of the hamster × human cell hybrids BamH1 gave the best results. The 12.5-kb human EcoR1 fragment was detected in cell lines B and E. The 12.0-kb human BamH1 fragment was found in lines H, K, and N (not shown). Comparison of these results with the chromosome information of the hybrids (Table 2) and calculation of a discordance score indicates that the vimentin gene (VIM) is probably in chromosome 10. The only discordant is line E, which is negative for chromosome 10, but positive for vimentin. About 10% of discordancy is frequently observed among syntenic pairs of loci, especially when they happen to be situated wide apart on the chromosome, owing to an occasional occurrence of chromosomal breakage and rearrangement in the interspecific somatic cell hybrids (Ruddle, 1970). However, the use of another panel of nine cell hybrid lines also favored the assignment of the vimentin gene to chromosome 10.

The specific desmin probe, desB, gave the best resolution between the human bands and those of mouse or hamster with the restriction enzyme Hind111. The human 3.7-kb Hind111 fragment formed a relatively more intense band in cell lines K and M than in lines L and P (not shown). The data in Table 2 exclude every other chromosome but chromosome 2 for localization of the desmin gene. In the mouse × man panel no cell line was positive for human desmin, which is consistent with its assignment to chromosome 2. The isocitrate dehydrogenase-1 (IDH-1) and malate dehydrogenase-1 (MDH-1) were used as chromosome 2 specific enzyme markers (Herbschleb-Voogt *et al.*, 1981a) in the present study. Both these markers were found to be absent in the mouse × man panel, while their pattern of segregation and relative intensities of expression in the individual hybrids belonging to the hamster × man panel was paralleled by that of the human 3.7-kb Hind 111 band mentioned previously. Such a consistent dosage relationship between the gene products expressed in a set of hybrids exhibiting the segregation of human chromosomes has been suggested to provide additional evidence that the concerned loci are situated on the same human chromosome (Westerveld

Table 2. Distribution of Human Chromosomes in Cell Hybrids Segregating Vimentin and Desmin Genes[a]

Clone	Human chromosome																							VIM	DES
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X		
A PG Me 1-1	+		+	+	+	+	+	+	+		+	+		+		+	+		+	+	+	+	+		
B PG Me 4-1			+	+	+	+	+	+	+	+	+	+		+	+		+			+	+		+	+	
C PG Me 5-1							+				+	+	+				+		+				+		
D PG Me 6-1								+																	
E PG Me 13-1			+				+	+	+			+	+					+	+		+	+	+	+	
H a3Y 14-2	+		+	+	+			+		+	+	+	+	+	+	+	+		+	+	+	+		+	
I a3Y 13-1	+		+				+	+			+	+	+	+	+	+	+		+	+	+		+		
J a3G 5-2	+				+	+		+					+		+	+	+	+		+		+			
K E36 33.7-2	+	+	+	+		+		+		+				+		+	+	+					+	+	+
L E36 33.11-2	+	+					+					+	+	+	+	+	+			+	+		+		+
M E36 10 CB 1-2	+	+	+	+			+	+			+	+	+			+	+				+		+		+
N E36 10 CB 22B2				+				+	+	+	+					+					+		+	+	
P E36 78-3-13-2	+	+			+						+				+	+		+	+		+		+		+
Discordant # Vimentin	9	7	4	3	6	5	8	5	3	1	7	7	8	5	7	8	8	5	7	7	6	5	7		
Discordant # Desmin	4	0	7	6	7	6	7	10	8	6	8	8	7	6	6	5	7	4	8	8	7	8	6		

[a]Cell lines A–E are mouse × man hybrids and lines H–P are Chinese hamster × man somatic hybrids. Plus (+) indicates the presence of vimentin sequences, desmin sequences, human chromosomes, and chromosome-specific isozyme markers. The lowest discordance for vimentin is with chromosome 10 and for desmin with chromosome 2. The discordancy of line E—vimentin sequences detectable, but no enzyme marker present—may result from breakage and loss of a part of chromosome 10 in this line.

and Meera Khan, 1972). In order to further confirm the localization of the desmin gene on chromosome 2, we hybridized the hamster × human panel to another DNA probe, which is known to be located on chromosome 2, namely lens-γ-crystallin. The crystallin probe hybridized with the same lanes and in the same intensities as our desmin probe, strengthening the assignment of the human desmin gene to chromosome 2.

References

Bennet, G. S., Fellini, S. A., Croop, J. M., Otto, J. J., Bryan, J., and Holzer, H., 1978, Differences among 100 A filament subunits from different cell types, *Proc. Natl. Acad. Sci. USA* **75**:4364.

Bignami, A., Eng, L. F., Dahl, D., and Uyeda, C. T., 1972, Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence, *Brain Res.* **43**:429.

Blake, C. C. F., 1978, Do genes-in-piece imply proteins-in-piece? *Nature* **273**:267.

Bloemendal, H., Lenstra, J. A., Dodemont, H., Ramaekers, F. C. S., Groeneveld, A. A., and Benedetti, E. L., 1980, SV-40 transformed hamster lens epithelial cells: A novel system for the isolation of cytoskeletal messenger RNAs and their translation products, *Exp. Eye Res.* **31**:513.

Capetanaki, Y. G., Ngai, J., Flytzanis, C. N., and Lazarides, E., 1983, Tissue-specific expression of two mRNA species transcribed from a single vimentin gene, *Cell* **35**:411.

Capetanaki, Y. G., Ngai, J., and Lazarides, E., 1984, Characterization and regulation in expression of a gene coding for the intermediate filament protein desmin, *Proc. Natl. Acad. Sci. USA* **81**:6909.

Dodemont, H. J., Soriano, P., Quax. W. J., Ramaekers, F., Lenstra, J. A., Groenen, M. A., Bernardi, G., and Bloemendal, H., 1982, The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertebrates, *EMBO J.* **1**:167.

Eng, L. F., Vanderhaegen, J. J., Bignami, A., and Gerstl, B., 1971, An acidic protein isolated from fibrous astrocytes, *Brain Res.* **28**:351.

Franke, W. W., Weber, K., Osborn, M., Schmid, E., and Freudenstein, C., 1978a, Antibody to prekeratin: Decoration of tonofilament-like arrays in various cells of epithelial character, *Exp. Cell Res.* **116**:429.

Franke, W. W., Schmid, E., Osborn, M., and Weber, K., 1978b, Different intermediate-sized filaments distinguished by immunofluorescence microscopy, *Proc. Natl. Acad. Sci. USA* **75**:5034.

Franke, W. W., Schmid, E., Schiller, D. L., Winter, S., Jarasch, E. D., Moll, R., Denk, H., Jackson, B. W., and Illmensee, K., 1981, Differentiation-related patterns of expression of proteins of intermediate-sized filaments in tissues and cultured cells, *Cold Spring Harbor Symp. Quant. Biol.* **46**:431.

Franke, W. W., Schiller, D. L., Hatzfeld, M., and Winter, S., 1983, Protein complexes of intermediate-sized filaments: melting of cytokeratin complexes in urea reveals different polypeptide separation characteristics, *Proc. Natl. Acad. Sci. USA* **80**:7113.

Fuchs, E., and Marchuk, D., 1983, Type I and type II keratins have evolved from lower eukaryotes to form the epidermal intermediate filaments in mammalian skin, *Proc. Natl. Acad. Sci. USA* **80**:5857.

Fuchs, E. V., Coppock, S. M., Green, H., and Cleveland, D. W., 1981, Two distinct classes of keratin genes and their evolutionary significance, *Cell* **27**:75.

Geisler, N., and Weber, K., 1980, Purification of smooth-muscle desmin and a protein-chemical comparison of desmins from chicken gizzard and hog stomach, *Eur. J. Biochem.* **111**:425.

Geisler, N., and Weber, K., 1981, Comparison of proteins of two immunologically distinct intermediate-sized filaments by amino acid sequence analysis: Desmin and vimentin, *Proc. Natl. Acad. Sci. USA* **78**:4120.

Geisler, N., and Weber, K., 1982, The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins, *EMBO J.* **1**:1649.

Geisler, N., and Weber, K., 1983, Amino acid sequence data on glial fibrillary acidic protein (GFA); Implications for subdivision of intermediate filaments into epithelial and non-epithelial members, *EMBO J.* **2**:2059.

Geisler, N., Kaufmann, E., Fischer, S., Plessmann, U., and Weber, K., 1983, Neurofilament architecture combines structural principles of intermediate filaments with carboxy-terminal extensions increasing in size between triplet proteins, *EMBO J.* **2**:1295.

Geisler, N., Fischer, S., Vandekerckhove, J., Plessmann, U., and Weber, K., 1984, Hybrid character of a large neurofilament protein (NF-M): Intermediate filament type sequence followed by long and carboxy-terminal extension, *EMBO J.* **3**:2701.

Geisler, N., Fischer, S., Vandekerckhove, J., Van Damme, J., Plessmann, U., and Weber, K., 1985, Protein chemical characterization of NF-H, the largest mammalian neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension, *EMBO J.* **4**:57.

Gilbert, W., 1978, Why genes in pieces? *Nature* **271**:501.

Graham, F. L., and van der Eb, A. J., 1973, A new technique for the assay of infectivity of human adenovirus 5 DNA, *Virology* **52**:456.

Gunning, P., Ponte, P., Kedes, L., Hickey, R. J., and Skoultchi, A. I., 1984, Expression of human cardiac actin in mouse L cells: A sarcomeric actin associates with a nonmuscle cytoskeleton, *Cell* **36**;709.

Hanukoglu, I., and Fuchs, E., 1982, The cDNA sequence of a human epidermal keratin: Divergence of sequence but conservation of structure among intermediate filament proteins, *Cell* **31**:243.

Hanukoglu, I., and Fuchs, E., 1983, The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structural domains among keratins, *Cell* **33**:915.

Herbschleb-Voogt, E., Grzeschik, K-H., Pearson, P. L., and Meera Khan, P., 1981a, Assignment of ADCP gene(s) to human chromosome 2 in rodent-human somatic cell hybrids, *Hum. Genet.* **59**:317.

Herbschleb-Voogt, E., Pearson, P. L., Vossen, J. M., and Meera Khan, P., 1981b, Basic defect in the expression of ADA in ADA-SCID disease investigated through the cells of an obligate heterozygote, *Hum. Genet.* **56**:379.

Hoffman, P. N., and Lasek, R. T., 1975, The slow component of axonal transport, *J. Cell Biol.* **66**:351.

Hoffman, W., and Franz, J. K., 1984, Amino acid sequence of the carboxy-terminal part of an acidic type I cytokeratin of molecular weight 51,000 from xenopus laevis epidermis as predicted from the cDNA sequence, *EMBO J.* **3**:1301.

Hynes, R. O., and Destree, A. T., 1978, 10 nm filaments in normal and transformed cells, *Cell* **13**:151.

Ip, W., Harzer, M. K., Pang, S., and Robson, R. M., 1985, Assembly of vimentin *in vitro* and its implication concerning the structure of intermediate filaments, *J. Mol. Biol.* **183**:365.

Ishikawa, A., Bischoff, R., and Holtzer, H., 1968, Mitosis and intermediate-sized filaments in developing skeletal muscle, *J. Cell Biol.* **38**:538.

Johnson, L., Idler, W., Zhou, X-M., and Steinert, P., 1984, Structure of the gene for the human epidermal keratin of 67 kD, *J. Cell. Biol.* **99**:317a.

Jorcano, J. L., Magin, T. M., and Franke, W. W., 1984a, Cell type-specific expression of bovine keratin genes as demonstrated by the use of complementary cDNA clones, *J. Mol. Biol.* **176**:21.

Jorcano, J. L., Rieger, M., Franz, J. K., Schiller, D. L., Moll, R., and Franke, W. W., 1984b, Identification of two types of keratin polypeptides within the acidic cytokeratin subfamily I, *J. Mol. Biol.* **179**:257.

Jorcano, J. L., Franz, J. K., and Franke, W. W., 1984c, Amino acid sequence diversity between bovine epidermal cytokeratine polypeptides of the basic (type II) subfamily as determined from cDNA clones, *Differentiation* **28**:155.

Kaufmann, E., Geisler, N., and Weber, K., 1984, SDS-page strongly overestimates the molecular masses of the neurofilament proteins, *FEBS Lett.* **170**:81.

Lazarides, E., and Hubbard, B. D., 1976, Immunological characterization of the subunit of the 100 A filaments from muscle cells, *Proc. Natl. Acad. Sci. USA* **73**:4344.

Lehnert, M. E., Jorcano, J. L., Zentgraf, H., Blessing, M., Franz, J. K., and Franke, W. W., 1984, Characterization of bovine keratin genes: Similarities of exon patterns in genes coding for different keratins, *EMBO J.* **3**:3279.

Lewis, S. A., and Cowan, N. J., 1985, Genetics, evolution, and expression of the 68,000-mol-wt neurofilament protein: Isolation of a cloned cDNA probe, *J. Cell. Biol.* **100**:843.

Lewis, S. A., Balcarek, J. M., Krek, V., Shelanski, M., and Cowan, N. J., 1984, Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: Structural conservation of intermediate filaments, *Proc. Natl. Acad. Sci. USA* **81**:2743.

Liem, R. K. H., Yen, S. H., Salomon, G. D., and Shelanski, M. L., 1978, Intermediate filaments in nervous tissues, *J. Cell. Biol.* **79**:637.

Marchuk, D., McCrohon, S., and Fuchs, E., 1984, Remarkable conservation of structure among intermediate filament genes, *Cell* **39**:491.

Meera Khan, P., Wijnen, L. M. M., Wijnen, J. Th., and Grzeschik, K-H., 1983, Electrophoretic characterization and genetics of human biliverdin reductase (BLVR; EC 1.3.1.24); assignment of BLVR to the p14 cen region of human chromosome 7 in mouse-human somatic cell hybrids, *Biochem. Genet.* **21**:123.

Meera Khan, P., Wijnen, L. M. M., Hagemeijer, A., and Pearson, P. L., 1984, Human formaldehyde dehydrogenase (FDH) and its assignment to chromosome 4, *Cytogenet. Cell Genet.* **38**:112.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R., 1982, The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells, *Cell* **31**:11.

Mount, S. M., 1982, A catalog of splice junction sequences, *Nucleic Acids Res.* **10**:459.

Osborn, M., and Weber, K., 1982, Intermediate filaments: Cell-type-specific markers in differentiation and pathology, *Cell* **31**:303.

Osborn, M., Franke, W. W., and Weber, K., 1977, Visualization of a system of filaments 7–10 nm thick in cultured cells of an epithelial line (Pt K2) by immunofluorescence microscopy, *Proc. Natl. Acad. Sci. USA* **74**:2490.

Quax, W. J., Dodemont, H. J., Lenstra, J. A., Ramaekers, F., Soriano, P., van Workum, M. E. S., Bernardi, G., and Bloemendal, H., 1982, Genes coding for vimentin and actin in mammals and birds, in: *Stability and Switching in Cellular Differentiation* (R. M. Clayton and D. E. S. Truman, eds.), p. 349, Plenum Press, New York.

Quax, W., Vree Egberts, W., Hendriks, W., Quax-Jeuken, Y., and Bloemendal, H., 1983, The structure of the vimentin gene, *Cell* **35**:215.

Quax, W., van den Heuvel, R., Vree Egberts, W., Quax-Jeuken, Y., and Bloemendal, H., 1984a, Intermediate filament cDNAs from BHK-21 cells: Demonstration of distinct genes for desmin and vimentin in all vertebrate classes, *Proc. Natl. Acad. Sci. USA* **81**:5970.

Quax-Jeuken, Y., Quax, W., and Bloemendal, H., 1983, Primary and secondary structure of hamster vimentin predicted from the nucleotide sequence, *Proc. Natl. Acad. Sci. USA* **80**:3548.

Quax, W., van den Brock, L., Vree Egberts, W., Ramaekers, F., and Bloemendal, H., 1985a, Characterization of the hamster desmin gene: Expression and formation of desmin filaments in nonmuscle cells after gene transfer, *Cell* **43**:327.

Quax, W., Meera Khan, P., Quax-Jeuken, Y., and Bloemendal, H., 1985b, The human desmin and vimentin genes are located on different chromosomes, *Gene* **38**:189.

Quax, W., Quax-Jeuken, Y., van den Heuvel, R., Vree Egberts, W., and Bloemendal, H., 1984b, Organization and sequence of the genes for desmin and vimentin, *Cold Spring Harbor Symp. Molecular Biology of the Cytoskeleton*, p. 445.

Quinlan, R. A., and Franke, W. W., 1982, Heteropolymer filaments of vimentin and desmin in vascular smooth muscle tissue and cultured baby hamster kidney cells demonstrated by chemical crosslinking, *Proc. Natl. Acad. Sci. USA* **79**:3452.

Quinlan, R. A., and Franke, W. W., 1983, Molecular interactions in intermediate-sized filaments revealed by chemical crosslinking, *Eur. J. Biochem.* **132**:477.

Quinlan, R. A., Cohlberg, J. A., Schiller, D. L., Hatzfeld, M., and Franke, W. W., 1984, Heterotypic tetramer (A_2D_2) complexes of non-epidermal keratins isolated from cytoskeletons of rat hepatocytes and hepatoma cells, *J. Mol. Biol.* **178**:365.

Ramaekers, F. C. S., Puts, J. J. G., Moesker, O., Jap, P. H. K., and Vooijs, G. P., 1981, Use of antibodies to intermediate filaments in the characterization of human tumors, *Cold Spring Harbor Symp. Quant. Biol.* **46:**331.

Ruddle, F. H., 1970, Utilization of somatic cells for genetic analysis: Possibilities and problems, in: *Control Mechanisms in Expression of Cellular Phenotypes* (M. A. Padykula, ed.), p. 233, Academic Press, New York.

Shelanski, M. L., Albert, S., DeVries, G. H., and Norton, W. T., 1971, Isolation of filaments from brain, *Science* **174:**1242.

Skerrow, D., Matoltsy, A. G., and Matoltsy, M. N., 1973, Isolation and characterization of the alpha-helical regions of epidermal prekeratin, *J. Biol. Chem.* **248:**4820.

Small, J. V., and Sobieszek, A., 1977, Studies on the function and composition of the 10 nm filaments of vertebrate smooth muscle, *J. Cell Sci.* **23:**243.

Sompayrac, L. M., and Danna, K. J., 1981, Efficient infection of monkey cells with DNA of simian virus 40, *Proc. Natl. Acad. Sci. USA* **78:**7575.

Soriano, P., Szabo, P., and Bernardi, G., 1982, The scattered distribution of actin genes in the mouse and human genomes, *EMBO J.* **1:**579.

Steinert, P. M., and Idler, W. W., 1975, The polypeptide composition of bovine epidermal alpha-keratin, *Biochem. J.* **151:**603.

Steinert, P. M., Idler, W. W., and Zimmerman, S. B., 1976, Self-assembly of bovine epidermal keratin filaments *in vitro*, *J. Mol. Biol.* **108:**547.

Steinert, P., Idler, W., Aynardi-Whitman, M., Zackroff, R., and Goldman, R. D., 1981, Heterogeneity of intermediate filaments assembled *in vitro*, *Cold Spring Harbor Symp. Quant. Biol.* **46:**465.

Steinert, P. M., Rice, R. H., Roop, D. B., Trus, B. L., and Steven, A. C., 1983, Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments, *Nature* **302:**794.

Steinert, P. M., Parry, P. A. D., Racoosin, E. L., Idler, W. W., Steven, A. C., Trus, B. L., and Roop, D. R., 1984, The complete cDNA and deduced amino acid sequence of a type II mouse epidermal keratin of 60.000 Da: analysis of sequence differences between type I and type II keratins, *Proc. Natl. Acad. Sci. USA* **81:**5709.

Sun, T-T., and Green, H., 1978, Immunofluorescent staining of keratin fibers in cultured cells, *Cell* **14:**469.

Westerveld, A., and Meera Khan, P., 1972, Evidence for linkage between human loci for 6-phosphogluconate dehydrogenase and phosphoglucomutase$_1$ in man-Chinese hamster somatic cell hybrids, *Nature* **236:**30.

Zehner, Z. E., and Paterson, B. M., 1983, Characterization of the chicken vimentin gene: single copy gene producing multiple mRNAs, *Proc. Natl. Acad. Sci. USA* **80:**911.

6

Actin

A REGULATOR OF CELL GROWTH AND DIFFERENTIATION

Stephen R. Farmer

1. Introduction

Traditionally, nonmuscle actin has been considered a housekeeping gene, constitutively expressed to form a microfilament network that is necessary to maintain cell shape and allow movement. This chapter provides evidence that actin is not constitutively expressed, but is tightly regulated. It also presents a speculative discussion, based on current research, which argues that actin is a regulator of cell growth and differentiation. This regulation may be a direct action on the nucleus or may be indirect acting through the microfilaments. A comparison is also made between actin and other recently characterized regulatory proteins: the nuclear localized protooncogene products.

2. Cell Morphology and Growth and Differentiation

2.1 Cell Configuration

The requirement of some cells for a solid substrate upon which to grow has been known for many years and is termed "anchorage dependence" (MacPherson and Montagnier, 1964; Stoker *et al.*, 1968). Loss of this phenotype is usually associated with transformation to tumorigenicity. Placing normal anchorage-dependent cells on a semisolid substratum (in suspension culture) causes them to assume a rounded morphology and prevents contact with a

Stephen R. Farmer • Department of Biochemistry, Boston University Medical School, Boston, Massachusetts 02118.

solid support. The result is inhibition of proliferation and arrest in the G1 phase of the cell cycle (Otsuka and Moskowitz, 1975). In an attempt to understand why normal cells are dependent on proper anchorage for growth, several researchers have studied separately the roles of cell shape and contact. The pioneering studies of Folkman and co-workers (Folkman and Greenspan, 1975; Folkman and Moscona, 1978) first demonstrated that cell shape is a fundamental regulator of proliferation. These investigators varied cell conformation from flat to nearly round by culturing endothelial cells on poly(2-hydroxyethyl methacrylate) poly(HEMA)-coated surfaces. By changing the thickness of the poly(HEMA) coating, they were able to control the degree of adhesiveness and thus the extent of cell spreading. A direct correlation between degree of spreading and growth rate, as measured by DNA synthesis, was observed. Hence, the surface adhesiveness can direct cell shape, which in turn influences a cell's ability to proliferate. Other aspects of growth control may also be explained by such a phenomenon. For instance, density-dependent or cell-to-cell contact inhibition of growth may result from changes in shape due to overcrowding.

It is unlikely that cell shape is the sole determinant in growth control. In the poly(HEMA) studies, the extent of cell contact with the substratum was also a variable. Investigations by Penman and co-workers (Ben-Ze'ev *et al.*, 1980) have therefore extended Folkman's studies to show that surface contact as well as cell shape has important effects on proliferation. Furthermore, these investigators revealed that most of the macromolecular metabolism in the cell is responsive to cell configuration (Benecke *et al.*, 1978, 1980; Farmer *et al.*, 1978). When anchorage-dependent fibroblasts were placed in suspension culture, DNA, RNA, and protein synthesis were all markedly inhibited. After reattachment of these cells to a solid culture dish surface, protein synthesis recovered rapidly. This recovery required only cell contact and was shape insensitive. In contrast, the recovery of nuclear events (DNA, rRNA, and mRNA synthesis) was much slower and depended on extensive cell spreading.

We must emphasize that in all these experiments there was a continued requirement for growth factors supplied as serum. It is likely that the mechanisms that control growth involve synergy between mitogenic factors and cell configuration. Certain cells proliferate in response to different growth factors when their configuration is changed by culturing on different extracellular matrices. Corneal epithelial cells grown on plastic respond to fibroblast growth factor (FGF), but not to epidermal growth factor (EGF), whereas when cultured on collagen the same cells respond to EGF (Gospodarowitz *et al.*, 1978). In this system plastic induces a rounder configuration than collagen.

Differentiation of a variety of cells, both *in vivo* and in culture, requires intimate association with an extracellular matrix, not only to initiate phenotypic change, but also to maintain the differentiated state (for review see Hay, 1981; Kleinman *et al.*, 1981; Bissell *et al.*, 1982). During early embryogenesis the interaction of primitive streak mesenchymal cells with the basal laminae facilitates migration of these cells, which is necessary for formation of

the primary tissues. Coincident with these migratory events, the cells undergo major shape changes, which are believed necessary for expression of differentiated functions (Hay, 1981).

The early embryo is not easily accessible for biochemical analysis. Therefore, most of our conclusions concerning the importance of cell configuration in differentiation have come from a limited number of cell culture model systems. These systems have provided some interesting insights into how cells respond to signals derived from changes in cell configuration. Benya and Shaffer (1982, 1983) found that dedifferentiation of adult rabbit articular chondrocytes, brought about by serial subcultivation in monolayer culture, could be reversed if the cells were maintained in suspension. Other investigators have manipulated shape by culturing cells on surfaces coated with specific extracellular matrices. The studies of Emerman, Pitelka, and collaborators (Emerman and Pitelka, 1977; Emerman *et al.*, 1977) first demonstrated that isolated mammary epithelial cells maintained their differentiated characteristics only when plated onto the appropriate collagen matrix. More recently, Bissell and co-workers (Lee *et al.*, 1985) showed that plating these cells on collagen gels has a profound effect on expression of the milk protein casein. In the case of differentiation of 3T3 preadipocytes to adipocytes, the presence of fibronectin dramatically alters cell shape and subsequently inhibits lipogenic gene expression (Spiegelman and Ginty, 1983). In these examples and others cited recently by Bissell *et al.* (1982), a striking feature of these cell–matrix interactions is an alteration in cell configuration with an attendant change in gene expression.

2.2. Cytoskeletal Organization

A recurring theme in many of the investigations cited in Section 2.1 is that changes in cell configuration, induced by different degrees of attachment to various surfaces, may directly affect organization of the cytoskeleton, and that it is through the cytoskeleton that changes in gene expression are effected.

Anchorage-dependent cells having well-controlled growth adopt a flattened morphology in culture and contain well-ordered arrays of actin-containing microfilaments. However, transformed cells usually lack or have reduced levels of such "stress fibers" (Pollack *et al.*, 1975; Goldman *et al.*, 1977). Addition of fibronectin to transformed cells leads to increased spreading and to reorganization of the microfilament bundles (Yamada *et al.*, 1976; Ali *et al.*, 1977). Other experiments suggest a close relationship between fibronectin- and actin-containing filaments. When cells were treated with actin-depolymerizing agents, i.e., cytochalasin B or D, there was a rapid release of fibronectin from the cell surface (Mautner and Hynes, 1977; Ali and Hynes, 1977; Kurkinen *et al.*, 1978). Double-label immunofluorescence demonstrated a close correspondence between fibronectin on the exterior of the cell and actin bundles on the interior (Hynes and Destree, 1978). The present view is that the cell attaches to a surface or its extracellular matrix at specific contact

points called adhesion plaques (Geiger *et al.*, 1984). In these regions the bundles of actin filaments are connected to fibronectin across the cell membrane by some as yet unidentified transmembrane component(s). With this model in mind, it is reasonable to propose that the extracellular matrix elicits its functional response during growth and differentiation by directly reorganizing the cytoskeleton.

As mentioned in Section 2.1, the role of the many growth factors and hormones must be incorporated into any proposal for growth regulation. Most of these factors, such as EGF or insulin, elicit their response by binding to surface receptors (Shecter *et al.*, 1978, 1979). Edelman (1976) was the first to propose that the cytoskeleton may modulate the mobility of mitogen receptors and thus affect the mitogen's ability to promote DNA synthesis. Since then several studies have tested this idea. The involvement of the microtubule system in this model is supported by the demonstration that colchicine and colcemid, drugs capable of depolymerizing microtubules, enhance the initiation of DNA synthesis in mitogenically stimulated cells (Teng *et al.*, 1977; Freidkin *et al.*, 1979; Otto *et al.*, 1979). Other studies suggested that microtubule depolymerization alone was sufficient to initiate DNA synthesis (McClain and Edelman, 1980). These studies however, were performed in medium containing serum making it difficult to determine the contribution of serum factors. Crossin and Carney (1981a) addressed this by performing similar experiments in serum-free medium and showed that microtubule depolymerization early in the cell cycle was sufficient to initiate DNA synthesis. Furthermore, they (Crossin and Carney, 1981b) found that microtubule stabilization by taxol inhibits the initiation of DNA synthesis normally elicited by thrombin and EGF. In a similar manner, studies have implicated the microfilament system in the transmission of growth factor signals. Maness and Walsh (1982) demonstrated that cytochalasin D disrupted the actin structure of 3T3 fibroblasts and prevented quiescent cells from entering S phase even in the presence of serum growth factors. Microtubules and microfilaments may act independently or may interact to coordinate growth control. The possible involvement of the intermediate filaments has not been investigated mainly because drugs that specifically disrupt such structures are not available.

Cytoskeletal organization is probably also important in mediating the effects of extracellular matrix on cytodifferentiation. In particular, the microfilament system has been implicated in transmiting various cell configuration signals. Benya and Shaffer (1983) demonstrated that dedifferentiated chondrocytes reexpress differentiation specific collagen when grown as monolayer cultures in the presence of cytochalasin B. During the differentiation of 3T3 preadipocytes, cells undergo a major morphological change, which is accompanied by a decrease in the actin cytoskeleton as visualized by immunofluorescence (Spiegelman and Green, 1980) or electron microscopy (Novikoff *et al.*, 1980). Spiegelman and Ginty (1983) showed that preadipocytes cultured on fibronectin fail to disassemble actin filaments and fail to differentiate. They further showed that addition of cytochalasin D reversed the inhibition of

differentiation by fibronectin. Thus, extracellular matrix-induced changes in gene expression can be strongly modulated by drugs that alter cytoskeletal structure.

3. Regulation of Actin Gene Expression

Involvement of the cytoskeleton in control of growth and differentiation suggests the possibility that such mechanisms may depend directly on the expression of the cytoskeletal genes. This notion was particularly inspired by the observation that depolymerization of microtubules specifically inhibits expression of tubulin mRNA (Ben-Ze'ev *et al.*, 1979b; Cleveland *et al.*, 1981).

Growth stimulation of cultured cells is accompanied by an increase in cell rounding and motility. Studies were initiated to investigate whether actin gene expression was also changed in response to the configuration changes accompanying growth. Pardee and co-workers (Riddle *et al.*, 1979; Riddle and Pardee, 1980) had found that the induction of actin synthesis occurred within a few hours after activation of serum arrested 3T3 cells. They suggested that this may be a specific marker for passage from G0 to G1 of the cell cycle. To relate such regulation directly to cell configuration changes, we analyzed actin gene expression in cells that were growth-arrested following suspension in methocel (see Section 2.1). We could show that actin gene expression undergoes unique regulatory responses with respect to suspension and to cell reattachment (Farmer *et al.*, 1983). Suspension of 3T3 cells caused a specific reduction in actin synthesis, which greatly exceeded the overall inhibition of protein synthesis (Fig. 1). Upon reattachment, protein synthesis recovered rapidly to 80–90% of control values, whereas in the same interval actin synthesis was induced 200% above control values. The enhanced synthesis of actin was maximal 4–8 hr after reattachment and returned to control values by 17–24 hr, prior to initiation of DNA synthesis (Fig. 1). This response is very similar to that observed for the stimulation of serum-arrested cells (Riddle *et al.*, 1979; Riddle and Pardee, 1980) and suggests that suspended 3T3 cells may also be arrested in the G0 phase of the cell cycle. Our studies revealed that the induction of actin synthesis is due to an increased level of actin mRNA in the cytoplasm, as judged by *in vitro* translation (Fig. 2) and by hybridization to a specific actin cDNA (Fig. 3).

Since these initial observations on growth regulation of actin synthesis, several other reports have appeared in the literature confirming that increased expression of actin is associated with the G0–G1 transition (Campisi *et al.*, 1984; Elder *et al.*, 1984; Greenberg and Ziff, 1984; McCairns *et al.*, 1984). More important, some reports have indicated that such regulation is at a transcriptional level and may be mediated by specific growth factors. Activation of quiescent AKR-2B mouse embryo cells with EGF resulted in a rapid and specific induction of both β- and γ-actin mRNA production, reaching maximal levels 2–4 hr after stimulation. The response was transient, since the levels of actin mRNA declined as cells entered S phase (Elder *et al.*, 1984).

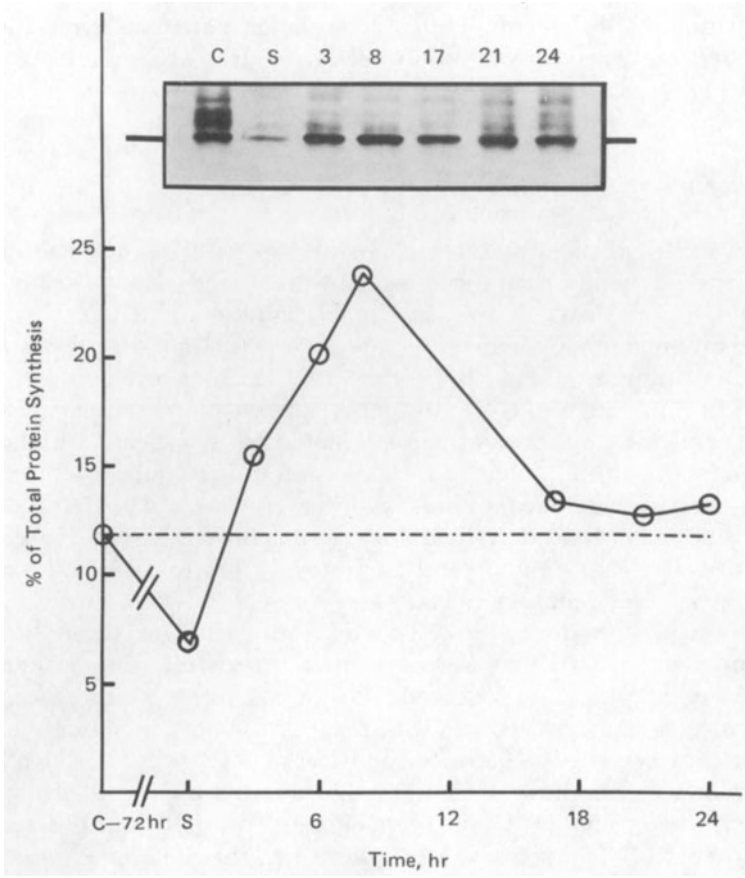


Figure 1. Synthesis of actin at different times after the reattachment of suspended 3T3 cells. 3T3 fibroblasts suspended in Methocel for 3 days were allowed to reattach to a culture dish surface for the times shown and were pulsed with [^{35}S]methionine for 45 min. Cytoplasmic proteins (equivalent to equal amounts of radioactivity) were analyzed on a 10% polyacrylamide SDS gel. The relative rate of actin synthesis was quantitated by scanning the gels and is expressed as a percentage of total protein synthesis. The insert is the actin region of the gel used to derive these data. C, control cells; S, suspended cells. Data are from Farmer *et al.*, 1983.

Nuclear runoff transcriptional assays revealed that mRNA accumulation is preceded by an increase in actin gene transcription.

Greenberg and Ziff (1984) also noticed dramatic changes in actin gene transcription in response to other growth factor signals. Within minutes of administering growth factors [i.e., 15% calf serum; platelet derived growth factor (PDGF); FGF; or 12-0-tetradecanoylphorbol-13-acetate] to quiescent 3T3 cells, there was a large increase in transcription of actin and also of c-fos, a protooncogene; c-fos transcription transiently increased more than 15-fold by 15 min after stimulation, returning to its initial levels by 30 min. Actin gene expression was more complex. An initial increase and subsequent decrease paralleled that of c-fos. However, after 60 min actin transcription again in-

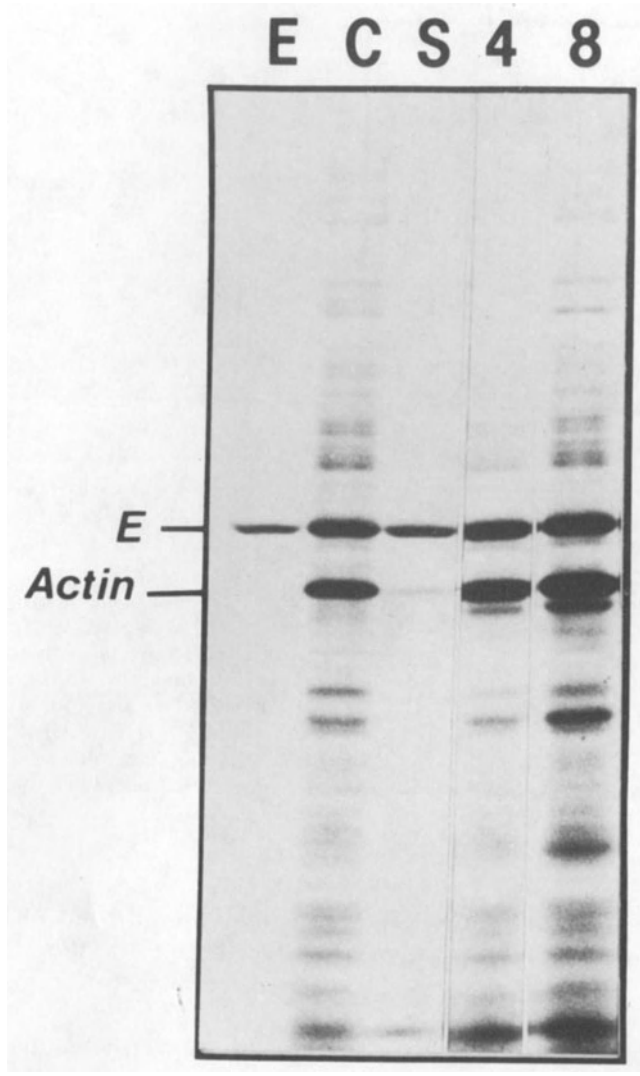


Figure 2. Analysis of actin mRNA levels in 3T3 cells by *in vitro* translation. Equal amounts of polyA+ RNA isolated from 3T3 cells maintained under different culture conditions were translated in a reticulocyte lysate *in vitro* system, and synthesized polypeptides were analyzed on a 10% polyacrylamide SDS gel. E, endogenous background; C, control; S, suspension; 8, 8 hr after reattachment. Data from Farmer *et al.*, 1983.

creased, reaching a second peak at 2–4 hr before returning to the quiescent level prior to initiation of DNA synthesis. This second peak of activity probably corresponds to the actin mRNA increases observed previously by others (Farmer *et al.*, 1983; Campisi *et al.*, 1984; Elder *et al.*, 1984; McCairns *et al.*, 1984). The very early response paralleling c-fos transcription is interesting and certainly raises many important questions, which will be discussed later.

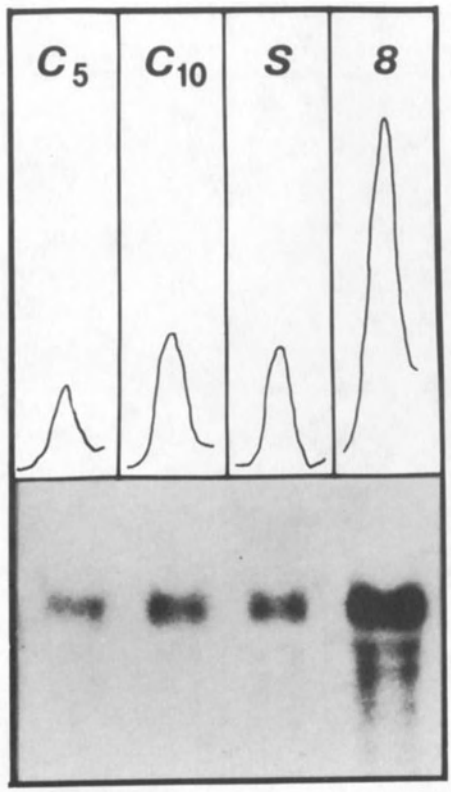


Figure 3. Hybridization analysis of actin mRNA levels in 3T3 fibroblasts. Total cellular RNA isolated from 3T3 cells maintained under different culture conditions was analyzed by Northern blot hybridization using an actin cDNA clone. C_5 and C_{10}, 5 and 10 μg, respectively, of control RNA; S, 10 μg suspended-cell RNA; 8, 10 μg of RNA from cells reattached for 8 hr.

Such a rapid response has recently been observed by Getz and co-workers (M. Getz, personal communication) in their studies on the effect of EGF on actin gene expression. It appears, therefore, that the specific stimulation of actin transcription during the G0–G1 transition, initiated by various growth factors, is biphasic.

We have recently embarked on a study of growth regulation *in vivo* using liver regeneration as a model system and have also observed a biphasic change in actin mRNA production (Fig. 4). Removal of two-thirds of the rodent liver triggers a controlled regrowth, which involves stimulation of all the remaining quiescent hepatocytes to leave G0 and enter the cell cycle. DNA synthesis occurs approx 24–36 hr after surgery. Analysis of actin mRNA levels at various times after the hepatectomy revealed an initial peak of actin production at 1–3 hr and the second peak at 12 hr (Fig. 4). This later change in actin mRNA accumulation has also been observed by Friedman *et al.*, (1984). In their studies, they stated that the increase was due to both transcriptional and posttranscriptional regulatory mechanisms.

It is interesting that in most of the studies discussed here tubulin mRNA levels did not change as a result of the G0–G1 transition. It is possible that if tubulin mRNA levels do change, they do so at a much later stage of the cell

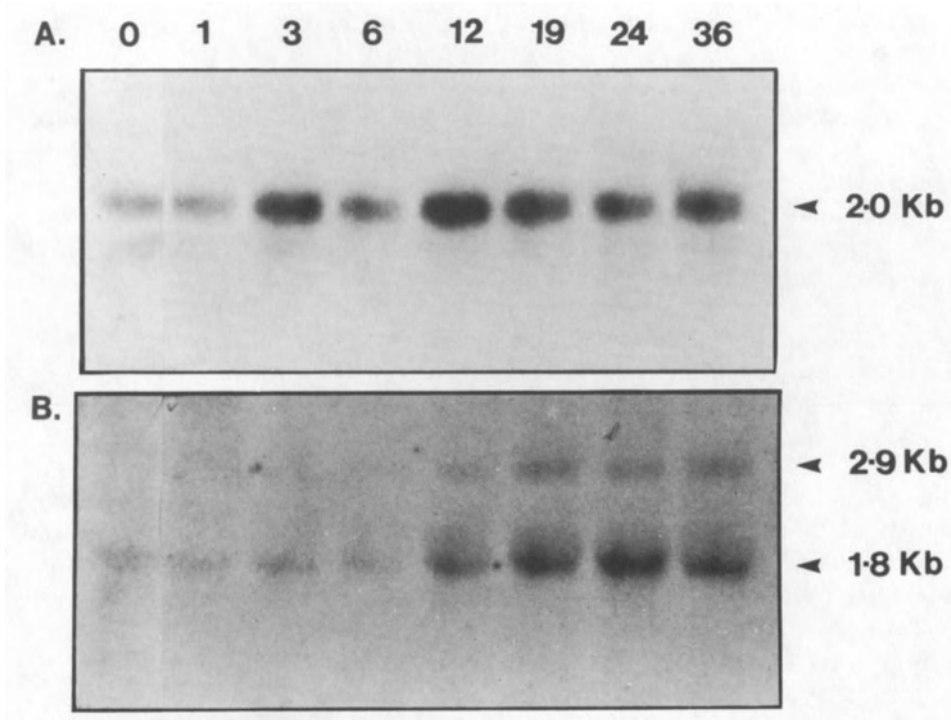


Figure 4. Quantitation of actin and tubulin mRNAs during rat liver regeneration. Total RNA was isolated from regenerating liver at different times (hr) after hepatectomy and equal amounts were analyzed using the Northern blot hybridization procedure. The filter was hybridized first to an actin cDNA (A) and then to a tubulin cDNA (B).

cycle, probably coincident with passage into S phase. This pattern of expression is particularly apparent during liver regeneration. Tubulin mRNAs accumulate only 19–36 hr after hepatectomy (Fig. 4). Since tubulin is not expressed early, we suggest that the induction of actin gene expression is specific and not simply a requirement for new overall cytoskeleton synthesis. It is possible that there is a specific need early in the cell cycle for only the microfilaments or just actin alone, but this is yet to be determined.

How growth factor signals are transmitted to the nucleus to initiate actin and specific protooncogene transcription is presently of particular interest to many investigators. The studies described here, which involve changes in cell configuration, support the concept that the cytoskeleton plays a crucial role in the mechanisms. A recent study comparing the response of 3T3 and 3T6 mouse fibroblasts to suspension culture provides even more support for this hypothesis. 3T3 cells are more tightly growth regulated than 3T6 cells. 3T3 cells are more susceptible to contact inhibition of growth, grow to lower densities at confluence, and have a more normal spread fibroblast morphology than 3T6 cells, which are considered minimally transformed. Mouse fibroblasts responded to suspension culture quite differently depending on their

degree of growth regulation and expression of the transformed phenotype (Wittelsberger *et al.*, 1981). The inhibition of growth and macromolecular metabolism was much less pronounced in the transformed cells. It is interesting that we also observe a difference between 3T3 and 3T6 cells in the response of actin synthesis to changes in cell configuration (Fig. 5). For example, suspension culture caused a dramatic inhibition of actin synthesis in 3T3 cells, and subsequent reattachment initiated a rapid induction of actin synthesis. The same culture manipulations with 3T6 cells had a significantly diminished effect on actin synthesis.

Is it possible that the change in configuration of the microfilaments themselves is responsible for the regulation of actin gene expression? As mentioned previously, there is a precedent for the direct control of the cytoskeletal genes in response to the polymerization state of the corresponding structural elements (Ben-Ze'ev *et al.*, 1979b; Cleveland *et al.*, 1981). Whether a similar feedback mechanism exists for the microfilaments has yet to be thoroughly investigated. One study, by Tannenbaum and Godman (1983), demonstrated that treatment of human hepatoma cells (HEp2) with cytochalasin D induced an increase in actin production. These investigators have not pursued this observation to determine whether such treatment alters actin gene expression directly.

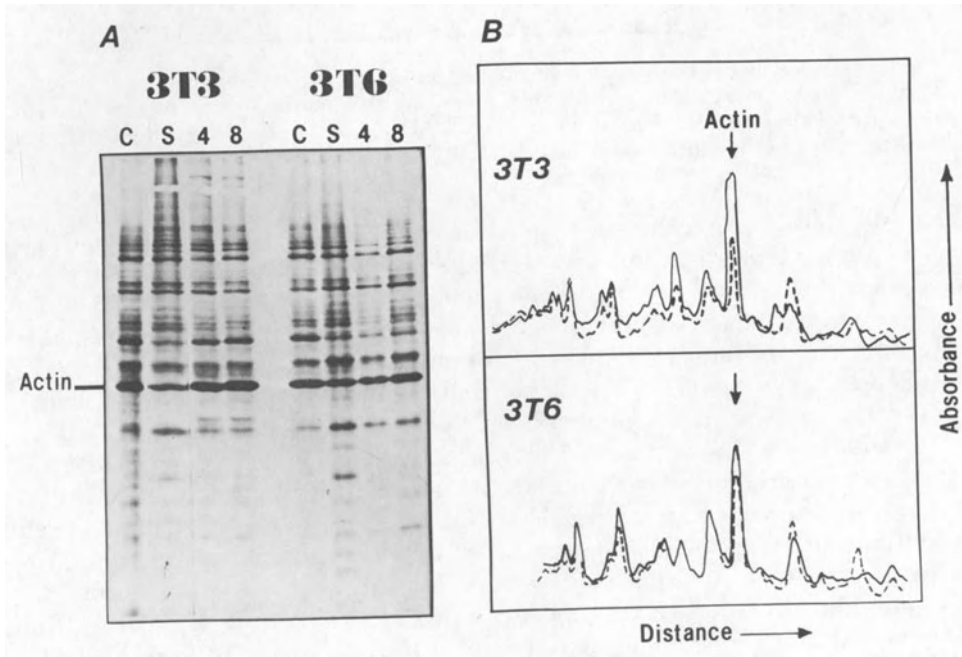


Figure 5. Relative rate of actin synthesis during suspension and reattachment of 3T3 and 3T6 fibroblasts. Cells suspended for 3 days were allowed to reattach to a surface for 4 or 8 hr and were pulsed with [^{35}S]methionine for 30 min. (A) Equivalent amounts of radioactively labeled polypeptides were electrophoresed on a 10% polyacrylamide SDS gel. (B) Densitometric scan of gel tracks C (dashed line) and 8 (solid line) corresponding to 3T3 and 3T6 cells.

In the case of preadipocyte differentiation, as discussed earlier, it appears that a change in cytoskeletal configuration is responsible for expression of differentiated functions and is also controlled at the level of cytoskeletal protein gene expression. To investigate the temporal relation between adipocyte morphological change and cytoskeleton synthesis, Spiegelman and Farmer (1982) analyzed both parameters during differentiation. We observed that the morphological change began 2–3 days after the culture reached confluence and that 50% of the cells were affected after 4–5 days. However, decreased actin and tubulin synthesis preceded this by at least 2 days. These decreases were due to a specific reduction in the levels of actin and tubulin mRNAs in the cytoplasm. The early occurrence of decreased cytoskeletal–protein synthesis suggested that the subsequent biosynthetic events specific to adipocyte differentiation may be influenced by alterations in the cytoskeleton. In fact, Spiegelman and Ginty (1983) were able to show that there is an intimate role for actin-containing structures in the differentiation process. In these studies fibronectin prevented the breakdown of the microfilaments and inhibited lipogenic gene expression, although actin gene expression was unaffected This implies that the initial event in the adipocyte differentiation pathway is a shutoff of actin and other cytoskeletal protein synthesis, which results in the decay of the cytoskeleton, and this, in turn, facilitates lipogenic gene expression.

A very interesting recent study was performed by Gerstenfeld *et al.*, (1985) in which evidence was presented for a direct relationship between β-actin and fibronectin gene expression and determination of the phenotypic characteristics of chicken fibroblasts and chondrocytes. These investigators showed that treatment of these two cell types with Phorbol-12-myristate-13-acetate (PMA) had opposite effects on cell morphology and β-actin gene expression. When fibroblasts are treated with PMA, they round up, form foci of densely packed cells, and show decreased levels of mRNA coding for their major actin isotype, β-actin. Similar treatment of floating sternal chondrocytes induces them to attach to the culture dish surface, to spread out, and to switch the major expressed actin isotype from γ-actin to β-actin. The chondrocytes also decrease expression of the cartilage-specific extracellular matrix proteins, i.e., collagen type II, and start synthesizing collagen type I and fibronectin. These studies suggest that β-actin gene expression is a phenotypic characteristic of cells with a dedifferentiated fibroblastic morphology. Since such cells have stress fibers, it seems likely that β-actin is the major component of these particular microfilament bundles.

4. Is Actin a Protooncogene?

The data described in Section 3 reveals that there is a correlation between actin gene expression and the growth and differentiation state of cells. Increased actin mRNA production certainly is associated with the G0–G1 transition of cell growth, occurring both in culture and *in vivo*. Furthermore, down-

regulation of this gene coincides with terminal differentiation of a variety of cells in culture (see Section 3) and *in vivo*. This is particularly apparent during the development of the liver (Friedman *et al.*, 1984) and the brain (Bond and Farmer, 1983).

This pattern of gene expression is also seen among a group of protooncogenes. Increased levels of the c-myc (Kelly *et al.*, 1983; Campisi *et al.*, 1984) and c-fos (Greenberg and Ziff, 1984) transcripts are detected very soon after activation of quiescent cells in culture. Interestingly, a recent study has also shown that increased c-myc mRNA production accompanies the early stages (within 1–3 hr) of rat liver regeneration (Makino *et al.*, 1984). On the other hand, c-myc expression was downregulated when F9 teratocarcinoma stem cells (Campisi *et al.*, 1984) were induced to terminally differentiate. Furthermore, both c-myc and c-myb mRNA expression was diminished during the differentiation of certain hematopoietic stem cells (Westin *et al.*, 1982a, 1982b; Craig and Bloch, 1984; Gonda and Metcalf, 1984). Thus, actin, c-myc, c-myb, and c-fos gene expression is closely associated with the proliferative state of cells.

Is it possible that expression of these genes is coordinately regulated and involved in a common cellular function? In this regard, actin and c-myc share another common feature, the involvement of a negative regulator of gene expression. The induction of actin (Elder *et al.*, 1984) and c-myc (Kelly *et al.*, 1983) expression by growth factors is potentiated significantly by cycloheximide. Such a superinduction can also occur independent of growth. In the case of actin, Ringold *et al.*, (1984) found that treatment of CHO cells with cycloheximide alone induced a 2.5-fold increase in actin transcription. While injection of cycloheximide into rats not only potentiated c-myc expression during liver regeneration, it also increased c-myc expression in untreated rats (Makino *et al.*, 1984). These studies suggest that both actin and c-myc gene expression is regulated by a very short-lived protein.

The close correlation between actin and protooncogene expression in the early phases of cell growth raises the question of whether actin itself is a protooncogene. It is interesting that the Gardner–Rasheed feline virus, designated v-fgr, encodes a fusion protein, a portion of which is homologous to a 128-amino-acid segment of actin (Naharro *et al.*, 1984). This actin sequence is fused to a protein-bearing extensive homology to the tyrosine-specific protein kinase portion of v-yes. Whether the actin region of this oncogene is contributing to the transformation of the feline cells is not known. It is possible that the aberrant actin segment directly causes disruption of the microfilaments. Alternatively, the actin sequences may direct the tyrosine-specific kinase to particular cytoskeletal target sites, or the actin region may not play any role in the transformation.

However, a role for actin in maintaining normal cell growth has been implicated from studies of clonal neoplastic cell lines derived by treatment of human diploid fibroblasts with the chemical carcinogen 4-nitroquinolin-1-oxide. The original transformed cell line, HUT-14, contained a mutant β-actin that was initially identified by its abnormal electrophoretic behavior.

The aberration was due to a single amino acid exchange of glycine to aspartic acid at position 244 of the polypeptide (Leavitt and Kakunaga, 1980; Vanderckhove *et al.*, 1980). This biochemical change was accompanied by a subtle reduction in incorporation of the variant β-actin into the detergent-insoluble cytoskeleton of the HUT-14 cells. Actin was not the only protein change observed in these cells; in fact, nearly 20% of the polypeptide species were modulated during the neoplastic state (Leavitt *et al.*, 1982b). Thus, it is possible that the mutation in β-actin may be a coincidence and may not contribute to the transformed phenotype. However, later studies supported the evidence that there is a role for the mutated β-actin in transformation. This conclusion was derived from establishing subclones of the original HUT-14 cell line that expressed a greater degree of anchorage-independent growth and tumorigenicity. These subclones were then injected into nude mice and formed fibrosarcomas. From one fibrosarcoma a highly tumorigenic cell line, HUT-14T, was derived. These cells expressed a more variant β-actin, which differed from the original mutant polypeptide by an additional net negative charge; it had a short half-life in the cell and a greatly diminished ability to incorporate into the cytoskeleton (Leavitt *et al.* 1982a). Examination of subclonal cells from intermediate stages of the HUT-14T derivation pathway established a progression in expression of mutant β-actin: the more variant the β-actin, the greater the degree of malignancy (Leavitt *et al.*, 1982a). The genes corresponding to these mutant and wild-type β-actins have been isolated (Leavitt *et al.*, 1984). It should now be possible, through DNA transfection, to test whether a mutant β-actin alone is capable of transforming cells.

Recently, Leavitt and collaborators (Leavitt *et al.*, 1985) have also demonstrated that an α-actin isoform is a transformation-sensitive marker for mouse NIH 3T3 and Rat 2 cells. When these cells were transformed to tumorogenicity, either by exposure to carcinogenic chemicals or oncogenic DNA, the accumulation of α-actin mRNA and α-actin synthesis was greatly inhibited. These authors suggest that this transformation sensitive actin is the smooth muscle α-isotype. However, they do state that they cannot exclude the possibility that it may be an as yet undefined α-actin isotype.

5. *Mechanisms by Which Actin May Regulate Cell Growth and Differentiation*

It is apparent from these studies that actin plays a crucial role in the control of cell growth and differentiation. Although the molecular basis of this regulation is not clear, recent studies provide information to propose some plausible working models.

Besides the apparent association of cell surface components with the microfilaments at focal adhesion contacts (Geiger *et al.*, 1984), it is likely that most of the integral membrane proteins are linked to the underlying network of cytoskeletal elements (Ben-Ze'ev *et al.*, 1979a). The binding of growth

factors to specific receptors may therefore trigger the growth response by first altering cytoskeletal organization. A recent study demonstrated that addition of PDGF to quiescent Balb/c-3T3 cells caused a rapid and reversible alteration in the distribution of vinculin and actin (Herman and Pledger, 1985). Within 2–3 min after addition of PDGF, vinculin disappeared from the adhesion plaques and accumulated in punctate spots in the perinuclear region. This response was followed by disruption of actin-containing stress fibers. Vinculin reappeared in adhesion plagues 60 min after treatment, whereas stress fibers reformed only after 2–3 hr. These investigators also showed that this cytoskeletal reorganization was a specific response to PDGF and other competence-inducing growth factors. Factors required for progression through the cell cycle (i.e., EGF and somatomedin C) did not elicit the response. Interestingly, EGF did cause a disappearance of vinculin from adhesion plagues, only if the cells were pretreated with a low dose of PDGF that by itself had no observable effect. This observation suggests that PDGF can sensitize cells to the action of progression factors by imperceptibly destabilizing the cytomatrix within adhesion plaques.

Fibroblasts transformed by Rous sarcoma virus have an altered distribution of vinculin. These cells express a transforming oncogene product, pp60src, which is localized in the adhesion plaques (Rohrschneider, 1980). pp60src is a tyrosine-specific kinase that phosphorylates vinculin (Sefton *et al.*, 1981). PDGF and EGF receptors also possess tyrosine-specific kinase activities; pp60src, the growth factor receptors, and certain other protooncogene products all phosphorylate a partially overlapping set of unidentified cellular proteins at tyrosine residues (Heldin and Westermark, 1984). Cytoskeletal proteins other than vinculin may be among the substrates for such kinases. Thus, binding of a growth factor to its receptor may initiate phosphorylation of specific actin-binding proteins. This single event, which would lead to disruption of the microfilaments, may be the first step in the transition from quiescence (G0) into G1. Differentiation may involve a similar set of mechanisms, triggered by interaction of extracellular matrix proteins with corresponding receptors. It will be interesting to determine whether, for instance, the fibronectin receptor is also a tyrosine-specific kinase.

How the disorganization of cytoskeletal elements conveys information to the nucleus is not clear. If the cytoplasmic filaments are attached to the nucleus, then disruption of microfilaments may directly alter the configuration of the nucleus. Alternatively, if the nucleus and cytoplasm compete for the same pool of microfilament proteins, then changes in the polmerization state of the cytoplasmic actin may dramatically affect nuclear actin function.

The existence of nuclear actin is now well established; however, the exact conformational state of this actin is not known. A significant amount of nuclear actin can be extracted from oocytes in a nonpolymerized form (Clark and Rosenbaum, 1979; Gounon and Karsenti, 1981). This feature may be unique to such specialized cells. A series of ultrastructure studies combined with biochemistry have recently revealed the presence of a nuclear matrix,

which appears to be intimately involved in metabolic processes occurring in the nucleus, i.e., DNA replication, RNA transcription and processing (for a review see Berezney, 1983). A major protein associated with this structure is actin (Capco *et al.*, 1982). Recent studies suggest that the nuclear and cytoplasmic matrices are physically associated as a result of the attachment of intermediate filaments to the nuclear lamina (Fey *et al.*, 1984). It is conceivable, therefore, that extracellular signals are directed to the nucleus along a converging system of cellular filaments. For instance, the microfilaments and microtubules, which are in contact with the surface receptors, may transfer their signals to the intermediate filaments, which in turn connect to the nuclear matrix.

An additional study implicates a direct involvement of nuclear actin in the transcription of lampbrush chromosomes (Scheer *et al.*, 1984). These investigators microinjected actin-binding proteins and antiactin antibodies into nuclei of viable oocytes of *Pleurodeles waltii* and demonstrated a selective inhibition of transcription mediated by RNA polymerase II. They also showed that extended meshworks of actin filaments appear in the vicinity of the lampbrush chromosomes upon inactivation of transcription. It will be important to repeat these experiments with somatic cells that have a defined nuclear matrix.

It is likely that oocytes as well as somatic cells do have a nuclear matrix. Such a structure is probably extremely dynamic, whereby actin and a variety of other associated proteins undergo continuous gel–sol transitions. If the matrix is in close association with chromatin (see Berezney, 1983, and Fey *et al.*, 1984) then such transitions may alter expression of transcriptionally active genes. Interestingly, a recent report demonstrates that correct initiation of transcription by RNA polymerase II *in vitro* requires a semipurified factor that contains actin (Egly *et al.*, 1984).

The very early induction of actin gene expression during the G0/G1 transition may be required to synthesize actin that is destined for the nucleus. It is relevant that other protooncogene proteins expressed at this time are nuclear associated. As discussed in Section 4, the early-induced proteins (actin, c-myc, c-myb, and c-fos) may serve a common function. In light of this present discussion, such a function may involve association of the nuclear matrix with active genes. A question that arises from such a notion is: what would direct the G0/nuclear actin exclusively into the nucleus, after its translation in the cytoplasmic environment of microfilaments? A nuclear specific actin-binding protein (fos, myc?) could be coordinately expressed with actin at the G0/G1 period.

If there are different actin-containing structures in the cell, the existence of only two nonmuscle actin proteins (β and γ) would require that the actin-binding proteins create the diversity. Further studies into the expression of not only the actin genes, but also the actin-binding protein genes, during growth and differentiation are necessary to determine whether this matrix model is correct.

ACKNOWLEDGMENTS. The author is indebted to Dr. Judith Campisi for many useful suggestions and for editing this manuscript. The original work reported in this chapter was supported by NIH grant GM29630.

References

Ali, I. U., and Hynes, R. O., 1977, Effects of cytochalasin B and colchicine on attachment of a major surface protein of fibroblasts, *Biochim. Biophys. Acta* **471**:16.

Ali, I. U., Mautner, V. M., Lanza, R. P., and Hynes, R. O., 1977, Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein, *Cell* **11**:115.

Benecke, B-J., Ben-Ze'ev, A., and Penman, S., 1978, The control of RNA production, translation and turnover in suspended and reattached anchorage dependant fibroblasts, *Cell* **14**:931.

Benecke, B-J., Ben-Ze'ev, A., and Penman, S., 1980, The regulation of RNA metabolism in suspended and reattached anchorage dependant fibroblasts, *J. Cell. Physiol.* **103**:247.

Ben-Ze'ev, A., Duerr, A., Solomon, F., and Penman, S., 1979a, The outer boundary of the cytoskeleton: A lamina derived from plasma membrane proteins, *Cell* **17**:859.

Ben-Ze'ev, A., Farmer, S. R., and Penman, S., 1979b, Mechanisms regulating tubulin synthesis in cultured mammalian cells, *Cell* **17**:319.

Ben-Ze'ev, A., Farmer, S. R., and Penman, S., 1980, Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage-dependant fibroblasts, *Cell* **21**:365.

Benya, P. D., and Shaffer, J. D., 1982, Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels, *Cell* **33**:215.

Benya, P. D., and Shaffer, J. D., 1983, Microfilament involvement in the reexpression of the differentiated chondrocyte collagen phenotype, *Ortho. Trans.* **7**:263.

Berezney, R., 1983, Organization and functions of the nuclear matrix, in: *Chromosomal Nonhistone Proteins* (L. S. Hnilica, ed.), Vol. IV, pp. 119–180, CRC Press, Boca Raton, FL.

Bissell, M. J., Hall, G., and Parry, G., 1982, How does the extracellular matrix direct gene expression, *J. Theor. Biol.* **99**:31.

Bond, J. F., and Farmer, S. R., 1983, Regulation of tubulin and actin mRNA production in rat brain: expression of a new β-tubulin mRNA with development, *Mol. Cell Biol.* **3**:1333.

Campisi, J., Gray, H. E., Pardee, A. B., Dean, M., and Sonenshein, G. E., 1984, Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation, *Cell* **36**:241.

Capco, D. G., Wan, K. M., and Penman, S., 1982, The nuclear matrix: Three dimensional architecture and protein composition, *Cell* **29**:847.

Clark, T. G., and Rosenbaum, J. L., 1979, An actin filament matrix in hand isolated nuclei of *X. laevis* oocytes, *Cell* **18**:1101.

Cleveland, D. W., Lopata, M. A., Sherline, P., and Kirschner, M. W., 1981, Unpolymerized tubulin modulates the level of tubulin mRNAs, *Cell* **25**:537.

Craig, R. W., and Bloch, A., 1984, Early decline in c-myb oncogene expression in the differentiation of human myeloblastic leukemia (ML-1) cells induced with 12-0-tetradecanoylphorbol-13-acetate, *Cancer Res.* **44**:442.

Crossin, K. L., and Carney, D. H., 1981a, Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis, *Cell* **23**:61.

Crossin, K. L., and Carney, D. H., 1981b, Microtubule stabilization by taxol inhibits initiation of DNA synthesis by thrombin and by epidermal growth factor, *Cell* **27**:341.

Edelman, G. M., 1976, Surface modulation in cell recognition and cell growth, *Science* **192**:218.

Egly, J. M., Miyamoto, N., Moncollin, V., and Chambon, P., 1984, Is actin a transcriptional factor for RNA polymerase B? *EMBO J.* **3**:2363.

Elder, P. K., Schmidt, L. J., Ono, T., and Getz, M. J., 1984, Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide, *Proc. Natl. Acad. Sci. USA* **81**:7476.

Emerman, J. T., and Pitelka, D. R., 1977, Maintenence and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes, *In Vitro* **13:**316.

Emerman, J. T., Enami, J., Pitelka, D. R., and Nandi, S., 1977, Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes, *Proc. Natl. Acad. Sci. USA* **74:**4466.

Farmer, S. R., Ben-Ze'ev, A., Benecke, B-J., and Penman, S., 1978, Altered translatability of messenger RNA from suspended anchorage-dependant fibroblasts: reversal upon cell attachment to a surface, *Cell* **15:**627.

Farmer, S. R., Wan, K. M., Ben-Ze'ev, A., and Penman, S., 1983, Regulation of actin mRNA levels and translation responds to changes in cell configuration, *Mol. Cell. Biol.* **3:**182.

Fey, E. G., Wan, K., and Penman, S., 1984, The epithilial cytoskeletal framework and nuclear matrix–intermediate filament scaffold: Three dimensional organization and protein composition, *J. Cell Biol.* **19:**1973.

Folkman, J., and Greenspan, H. P., 1975, Influence of geometry on control of cell growth, *Biochim. Biophys. Acta* **417:**217.

Folkman, J., and Moscona, A., 1978, Role of cell shape in growth control, *Nature (London)* **273:**345.

Friedkin, M., Legg, A., and Rozengurt, E., 1979, Antitubulin agents enhanced the stimulation of DNA synthesis by polypeptide growth factors in 3T3 mouse fibroblasts, *Proc. Natl. Acad. Sci. USA* **76:**3902.

Friedman, J. M., Chung, E. Y., and Darnell Jr, J. E., 1984, Gene expression during liver regeneration, *J. Mol. Biol.* **179:**37.

Geiger, B., Avnur, Z., Kreis, T. E., and Schlessinger, J., 1984, The dynamics of cytoskeletal organization in areas of cell-contact, in: *Cell and Muscle Motility* (J. W. Shay, ed.), Vol. 5, pp. 195–235, Plenum Press, New York.

Gerstenfeld, L. C., Finer, M. H., and Boedtker, H., 1985, Altered β-actin gene expression in phorbol myristate acetate treated chondrocytes and fibroblasts, *Mol. Cell. Biol.* **5:**1425.

Goldman, R. D., Yerna, M. J., and Schloss, J. A., 1977, Localization and organization of microfilaments and related proteins in normal and virus-transformed cells, in: *Cell Shape and Surface Architecture* (J-P. Revel, U. Henning, and C. F. Fox, eds.), pp. 107–135, Liss, New York.

Gonda, T. J., and Metcalf, D., 1984, Expression of myb, myc, and fos proto-oncogenes during the differentiation of a murine myeloid leukemia, *Nature* **310:**249.

Gospodarowitz, D., Greenburg, G., and Birdwell, C. R., 1978, Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth, *Cancer Res.* **38:**4155.

Gounon, P., and Karsenti, E., 1981, Involvement of contractile proteins in the changes in consistency of oocyte nucleoplasm of the newt *Pleurodeles waltlii*, *J. Cell Biol.* **88:**410.

Greenberg, M. E., and Ziff, E. B., 1984, Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene, *Nature (London)* **311:**433.

Hay, E. D., ed., 1981, *The Cell Biology of the Extracellular Matrix*, Plenum Press, New York.

Heldin, C-H., and Westermark, B., 1984, Growth factors: mechanism of action and relation to oncogenes, *Cell* **37:**9.

Herman, B., and Pledger, W. J., 1985, Platelet-derived growth factor-induced alterations in vinculin and actin distribution in balb/c-3T3 cells, *J. Cell Biol.* **100:**1031.

Hynes, R. O., and Destree, A. T., 1978, Relationships between fibronectin (LETS protein) and actin, *Cell* **15:**875.

Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P., 1983, Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor, *Cell* **35:**603.

Kleinman, H. K., Klebe, R. J., and Martin, G. R., 1981, Role of collagenous matrices in the adhesion and growth of cells (review), *J. Cell Biol.* **88:**473.

Kurkinen, M., Wartiovaara, J., and Vaheri, A., 1978, Cytochalasin B releases a major surface associated glycoprotein, fibronectin, from cultured fibroblasts, *Exp. Cell Res.* **111:**127.

Leavitt, J., and Kakunaga, T., 1980, Expression of a variant form of actin and additional polypep-

tide changes following chemical induced *in vitro* neoplastic transformation of human fibroblasts, *J. Biol. Chem.* **255:**1650.

Leavitt, J., Bushar, G., Kakunaga, H., Hamada, H., Hirakawa, T., Goldman, D., and Merril, C., 1982a, Variations in mutant β-actin expression accompanying incremental increases in human fibroblast tumorigenicity, *Cell* **28:**259.

Leavitt, J., Goldman, D., Merril, C., and Kakunaga, T., 1982b, Changes in gene expression accompanying chemically induced malignant transformation of human fibroblasts, *Carcinogenesis* **3:**61.

Leavitt, J., Gunning, P., Porreca, P., Ng, S-Y., Lin, C-S., and Kedes, L., 1984, Molecular cloning and characterization of mutant and wild-type human β-actin genes, *Mol Cell. Biol.***4:**1961.

Leavitt, J., Gunning, P., Kedes, L., and Jariwalla, R., 1985, Smooth muscle α-actin is a transformation-sensitive marker for mouse NIH 3T3 and Rat 2 cells, *Nature* **316:**840.

Lee, E. Y-H., Lee, W-H., Kaetzel, C. S., Parry, G., and Bissell, M. J., 1985, Interaction of mouse mammary epithelial cells with collagen substrata: Regulation of casein gene expression and secretion, *Proc. Natl. Acad. Sci. USA* **82:**1419.

MacPherson, I., and Montagnier, L., 1964, Agar suspension culture for the selective assay of cells transformed by polyoma virus, *Virology* **23:**291.

Makino, R., Hayashi, K., and Sugimura, T., 1984, c-myc transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment, *Nature* **310:**697.

Maness, P. F., and Walsh, R. C., 1982, Dihydrocytochalasin B disorganizes actin cytoarchitecture and inhibits initiation of DNA synthesis in 3T3 cells, *Cell* **30:**253.

Mautner, V., and Hynes, R. O., 1977, Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells, *J. Cell Biol.* **75:**743.

McCairns, E., Fahey, D., Muscat, G. E. O., Murray, M., and Rowe, P. B., 1984, Changes in levels of actin and tubulin mRNAs upon lectin activation of lymphocytes, *Mol. Cell. Biol.* **4:**1754.

McClain, D. A., and Edelman, G. M., 1980, Density-dependent stimulation and inhibition of cell growth by agents that disrupt microtubules, *Proc. Natl. Acad. Sci. USA* **77:**2748.

Naharro, G., Robbins, K. C., and Reddy, E. P., 1984, Gene product of v-fgr onc: Hybrid protein containing a portion of actin and a tyrosine-specific protein kinase, *Science* **223:**63.

Novikoff, A. B., Novikoff, P. M., Rosen, O. M., and Rubin, C. S., 1980, Organelle relationships in cultured 3T3-L1 preadipocytes, *J. Cell Biol.* **87:**180.

Otsuka, H., and Moskowitz, M., 1975, Arrest of 3T3 cells in G1 phase in suspension culture, *J. Cell Physiol.* **87:**213.

Otto, A., Zumbe, A., Gibson, L., Kubler, A. M., and DeAsua, L. J., 1979, Cytoskeleton-disrupting drugs enhance effect of growth factors and hormones on initiation of DNA synthesis, *Proc. Natl. Acad. Sci. USA* **76:**6435.

Pollack, R., Osborn, M., and Weber, K., 1975, Patterns of organization of actin and myosin in normal and transformed non-muscle cells, *Proc. Natl. Acad. Sci. USA* **72:**994.

Riddle, V. G. H., and Pardee, A. B., 1980, Quiescent cells but not cycling cells exhibit enhanced actin synthesis before they synthesize DNA, *J. Cell. Physiol.* **103:**11.

Riddle, V. G. H., Dubrow, R., and Pardee, A. B., 1979, Changes in the synthesis of actin and other cell proteins after stimulation of serum-arrested cells, *Proc. Natl. Acad. Sci. USA* **76:**1298.

Ringold, G. M., Dieckmann, B., Vannice, J. L., Trahey, M., and McCormick, F., 1984, Inhibition of protein synthesis stimulates the transcription of human β-interferon genes in Chinese hamster ovary cells, *Proc. Natl. Acad. Sci. USA* **81:**3964.

Rohrschneider, L. B., 1980, Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product, *Proc. Natl. Acad. Sci. USA* **77:**3514.

Scheer, U., Hinssen, H., Franke, W. W., and Jockusch, B. M., 1984, Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes, *Cell* **39:**111.

Sefton, B. M., Hunter, T., Ball, E. H., and Singer, S. J., 1981, Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus, *Cell* **24:**165.

Schecter, Y., Hernaez, L., and Cuatracasas, P., 1978, Epidermal growth factor: Biological activity

requires persistent occupation of high affinity cell surface receptors, *Proc. Natl. Acad. Sci. USA* **75**:5788.

Shecter, Y., Chang, K. J., Jacobs, S., and Cuatracasas, P., 1979, Modulation of binding and bioactivity of insulin by anti-insulin antibody: Relation of possible role of receptor self-aggregation in hormone action, *Proc. Natl. Acad. Sci. USA* **76**:2720.

Spiegelman, B., and Farmer, S. R., 1982, Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3-adipocytes, *Cell* **29**:53.

Spiegelman, B., and Ginty, C. A., 1983, Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes, *Cell* **35**:657.

Spiegelman, B., and Green, H., 1980, Control of specific protein biosynthesis during adipose conversion of 3T3 cells, *J. Biol. Chem.* **255**:8811.

Stoker, M., O'Neill, C., Berryman, S., and Waxman, V., 1968, Anchorage and growth regulation in normal and virus transformed cells, *Int. J. Cancer* **3**:683.

Tannenbaum, J., and Godman, G. C., 1983, Cytochalasin D induces increased actin synthesis in HEp-2 cells, *Mol. Cell Biol.* **3**:132.

Teng, M., Bartholmew, J. C., and Bissell, M. J., 1977, Synergism between antimicrotubule agents and growth stimulants in enhancement of cell cycle traverse, *Nature* **268**:739.

Vanderchkove, J., Leavitt, J., Kakunaga, T., and Weber, K., 1980, Coexpression of a mutant β-actin and two normal β- and cytoplasmic actins in a stably transformed human cell line, *Cell* **22**:893.

Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Baluda, M. A., Aaronson, S. A., and Wong-Staal, F., 1982a, Differential expression of the amv gene in human hematopoietic cells, *Proc. Natl. Acad. Sci. USA* **79**:2194.

Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla-Favera, R., Papas, T. S., Lautenberger, J. A., Eva, A., Reddy, E. P., Tronick, S. R., Aaronson, S. A., and Gallo, R. C., 1982b, Expression of cellular homologues of retroviral onc genes in human hematopoietic cells, *Proc. Natl. Acad. Sci. USA* **79**:2490.

Wittelsberger, S. C., Kleene, K., and Penman, S., 1981, Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype, *Cell* **24**:859.

Yamada, K. M., Yamada, S. S., and Pastan, I., 1976, Cell surface protein partially restores morphology, adhesiveness, and contact inhibition of movement to transformed fibroblasts, *Proc. Natl. Acad. Sci. USA* **73**:1217.

7

The Actin Genes in Caenorhabditis elegans

Michael Krause and David Hirsh

1. Introduction

The proliferation of research on *Caenorhabditis elegans* in 1974 included, as a major point of focus, work related to myogenesis. *C. elegans* is an attractive organism for such studies because the worm is amenable to both genetic and biochemical dissection. As a result, there exists today a detailed description of *C. elegans* muscle lineage and ultrastructure, a collection of mutants affecting the ultrastructural organization of muscle, and numerous characterized clones of genes encoding contractile proteins.

Motility in *C. elegans* is achieved by a striated body wall musculature that runs the length of the worm just below the hypodermis (Sulston and Horvitz, 1977; Gossett *et al.*, 1982). The embryonic and postembryonic lineages of all 95 adult body wall muscle cells are known (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Although the body wall musculature constitutes the predominant muscle cell type, other muscle cells are found in *C. elegans*. Pharyngeal pumping, which allows food to enter the gut, is due to 20 radially arranged muscle cells surrounding the lumen. Additional muscles are involved in defecation, peristalsis, hermaphrodite egg laying, and male copulation (Albertson and Thomson, 1976; Sulston and Horvitz, 1977).

More than 22 genes, distributed on all six linkage groups, have been identified by mutations that affect movement and muscle ultrastructure (Waterston *et al.*, 1980). The ultrastructural defects include misaligned thick and thin filaments, filament paracrystals, irregular sarcomeric spacing, and abnor-

Michael Krause and David Hirsh • Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309.

mal thin and thick filament interdigitation (Mackenzie *et al.*, 1978; Zengel and Epstein, 1980a). The products of three of the genes have been identified. The *unc-15 I* product is most likely paramyosin (Waterston *et al.*, 1977). The *unc-54 I* product, representing the most thoroughly studied muscle locus in the worm, is the major body wall muscle myosin heavy chain (MacLeod *et al.*, 1977a,b; Karn *et al.*, 1984). The third locus represents an actin gene cluster and is the focus of this chapter.

2. Genomic Organization

The cloning of the worm actin genes was simplified by the work of Kindle and Firtel (1978), who had isolated a *Dictyostelium discoidium* actin cDNA clone. This *Dictyostelium* clone, pDB1, could be used to isolate complementary sequences from a Lambda Charon-10–*C. elegans* genomic library because of the evolutionary conservation of actin. Restriction maps of several recombinant clones revealed four *C. elegans* actin genes in agreement with hybridization data collected from genomic Southern blots.

Three of the actin genes are tightly clustered in a 12-kb segment of the genome. The fourth actin gene is unlinked to the cluster (Fig. 1). A DNA polymorphism exists adjacent to the three linked genes in *C. elegans* var. Bristol and var. Bergerac, two interbreeding strains. The polymorphism, a 1600-bp insertion in the Bergerac strain, is due to the *C. elegans* transposable element Tc1 (Files *et al.*, 1983; Rosenzweig *et al.*, 1983; Liao *et al.*, 1983). This linked polymorphism was used as a phenotypic marker at the DNA level in standard genetic crosses. The results of those crosses placed the cluster in a 2-centimorgan region of linkage group V (Files *et al.*, 1983). The DNA flanking gene-4 was analyzed for polymorphisms in an attempt to genetically map the fourth actin gene. No polymorphism was detected although the search extended approximately 40 kb to either side of gene-4.

Concurrent with the search for a gene-4-associated polymorphism was

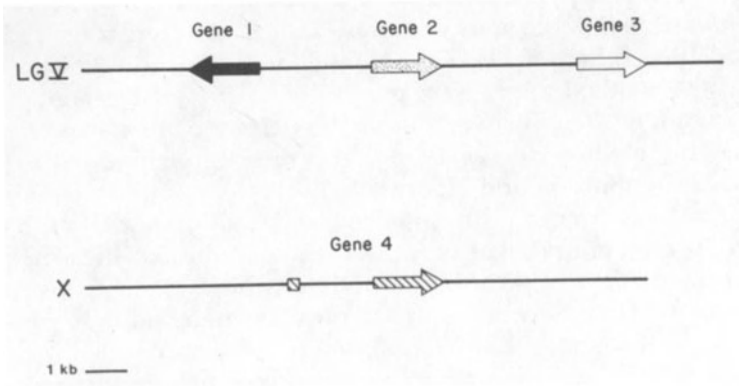


Figure 1. Genomic organization of the *C. elegans* actin genes. The direction of the arrow designating each gene indicates the direction of transcription.

the development of a chromosomal *in situ* hybridization technique by Albertson (D. Albertson, personal communication). Cloned unique DNA sequences flanking actin gene-4 were used in this technique to localize the gene to the X chromosome (D. Albertson, personal communication). Data from Southern blots comparing hermaphrodite (X/X) and male (X/O) DNA agree with this assignment; hermaphrodites have twice as many gene-4 sequences compared to males when normalized against the autosomal actin genes (L. Donahue, personal communication).

The genomic organization of the *C. elegans* actin genes is unusual in that clustered actin genes have been reported for only one other organism, *Strongylocentrotus purpuratus* (Scheller *et al.*, 1981). Of the 11 sea urchin actin genes, two are within a 12-kb region of the genome and three are within a 30-kb region. The remaining organisms with actin multigene families have dispersed genes. *Drosophila*, for example, has six actin genes distributed on three of the four linkage groups (Fyrberg *et al.*, 1981).

It is tempting to speculate that the clustering of the actin genes in the worm reflects a coordinate regulation of these genes either temporally, spatially, or both. Several instances of coordinately expressed gene clusters exist, such as the chorion genes in the silkmoth and in *Drosophila* and the histone genes in the sea urchin (Cohn *et al.*, 1976; Spradling *et al.*, 1980; Jones and Kafatos, 1980). On the other hand, cases have been found in which tightly clustered genes are not coordinately expressed. Examples include the human globin genes and the *Dictyostelium* M-4 region genes (Kimmel, 1984).

No differences in the temporal expression of the four *C. elegans* have yet been detected. There is, however, evidence (discussed in Section 8) suggesting a coordinate regulation of gene-1 and gene-3. ln addition, there appear to be spatial differences in the expression of at least one gene of the cluster and of actin gene-4. It remains to be seen whether these differences are a consequence of the cluster–singlet arrangement of the genes rather than simply coincidence.

Although the actin genes of *Drosophila* are dispersed in the genome, a cluster of muscle-related genes has been described (Karlik *et al.*, 1984). The 88F subdivision of the *Drosophila* chromosome includes an actin gene, two tropomyosin genes, and three as yet unidentified myofibrillar protein-coding regions. Apropos to this, the *myo-3* gene of *C. elegans*, which encodes a body wall muscle myosin, has been mapped to a region adjacent to the actin gene cluster (Miller, 1985). This region also contains the *sup-3* locus, an allele-specific suppressor of the major body wall myosin gene, *unc-54*. It may be that the clustering of this group of *C. elegans* muscle genes is related to a coordination of their expression.

3. Sequence Analysis

Coding and flanking regions of a gene are helpful in understanding gene regulation. For actin, sequence data are also valuable for making comparisons

to the numerous actins or actin gene sequences from other organisms. Such comparisons address questions pertaining not only to expression but to actin function and evolution as well.

The dogma that emerged from studies on vertebrate actins is that there are cell-specific actin isoforms and, therefore, a cell-specific expression of actin genes (Vandekerckhove and Weber, 1978a,b). The most general distinction of actin isoforms is the differentiation between muscle and cytoplasmic actins. Each of these two actins has specific amino acid residues at several positions characteristic of the isoform. It was of interest to see whether *C. elegans*, comprised of both muscle and nonmuscle cells, shared these sequence distinctions with the vertebrates. As many of the diagnostic residues are located in the amino-terminus of the protein, the initial sequencing efforts focused on the 5′ ends of each gene (Files *et al.*, 1983). Translation of the coding sequence showed that, like *Drosophila*, worm muscle and nonmuscle actin isoforms could not be distinguished by the diagnostic amino acid residues found in vertebrates (Fyrberg *et al.*, 1981). Subsequently, all four *C. elegans* actin genes were sequenced in their entirety. Rather than reiterate the sequence reported elsewhere (Wild and Hirsh, 1985), the gene sequences are compared schematically (Fig. 2).

Comparison of the nucleotide sequences from the four genes reveals a remarkable conservation. Genes-1 and -3 are identical within the coding region as well as in 26 of 27 bp preceding the translational start codon. The

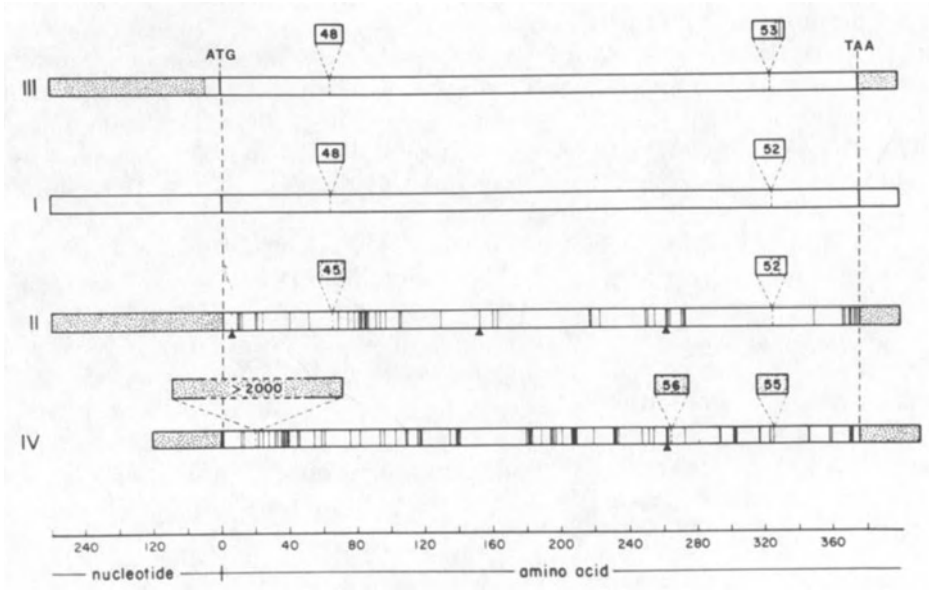


Figure 2. Nucleotide sequence comparison of *C. elegans* actin genes. All genes are compared to gene-1. Open boxes indicate regions of complete homology; vertical lines indicate single nucleotide differences; stippled boxes indicate regions of nonhomology. Positions in which nucleotide changes result in amino acid substitutions are indicated by solid triangles. Introns are shown in boxes above each gene with the intron length indicated in bp.

nucleotide sequence of gene-2 is 96% homologous to that of gene-1 or -3; gene-4 is 94% homologous to gene-1 or -3 (Wild and Hirsh, 1985).

The homology among the four genes is even greater at the amino acid level. Only 3 of the 35 nucleotide substitutions in gene-2 relative to gene-1 or -3 result in amino acid changes (Table 1). All three amino acid changes are conservative. Although gene-4 shows the least homology to gene-1 or -3 at the nucleotide level, only one nucleotide difference results in an amino acid substitution.

All four actin genes contain introns (Fig. 2) (Wild and Hirsh, 1985). Genes-1, -2, and -3 share introns interrupting amino acid codon 64, while all four genes have an intron interrupting codon 324. The introns at both positions are small, ranging in size from 48 bp to 55 bp. In addition to the conservation of intron position, genes-1 and -3 have homologous intron sequences with two exceptions, a 1-bp insertion and a single base change, both in the 3′ intron of gene-3. Gene-4 has two uniquely positioned introns, one 2081-bp intron between codons 19 and 20 and another 45-bp intron between codons 264 and 265.

There are two other conspicuous intron-related sequences associated with the *C. elegans* actin genes. Both genes-1 and -3 have consensus 3′ intron splice sequences just upstream (-28) of the initiator AUG. If these sequences demark a bona fide intron, then the putative promoter sequences of these two genes (Table 2) would be spliced out. Each of these two genes have been sequenced for at least 800 bp 5′ to the translational start site. Introns in the 5′ untranslated region have been reported for many actin genes, including those of *Drosophila*, sea urchins, and chickens (Fyrberg *et al.*, 1981; Shott-Akhurst *et al.*, 1984; Patterson *et al.*, 1984). In addition, alternative splicing of 5′ introns has been demonstrated to juxtapose different promoter sequences upstream of a myosin light-chain coding region (Nabeshima *et al.*, 1984). Although 5′ consensus intron splice sites can be found upstream of genes-1 and -3, their use in intron excision would not result in the juxtaposition of consensus promoter sequences upstream of the initiator methonine for either gene. There is no evidence to suggest that alternative splicing is involved in the expression of the *C. elegans* actin genes.

Typical eukaryotic promoter sequences (CAAT and TATA) are found upstream of most of the *C. elegans* genes sequenced to date (Benoist and

Table 1. Amino Acid Substitutions in C. elegans Actin Genes

	Amino acid position		
	4	152	262
Gene-1 and -3	Glu	Val	Met
Gene-2	Asp	Ile	Leu
Gene-4	Glu	Val	Leu

Table 2. Regulatory Sequences Flanking C. elegans Actin Genes

	5′ Control sequences		Start site			Polyadenylation signal	
Gene-1	GCTCAATac.....	TATATtA	CA[a].....	ATG // TAA.....		AATAAA.....	AATAAA
	−82	−45	−24	0	0	117	172
Gene-2	caTCAATtT.....	TAatTAT	—...	ATG // TAA.....		tATAAA	AATAAt
	−109	−41		0	0	95	144
Gene-3	ttCCAATAT	TATATcT	CA[a].....	ATG // TAA.....		AATAAA.....	AATAAA
	−104	−71	−24	0	0	90	257
Gene-4	GaCCAATgg.....	TATAAAT.....	CA	ATG // TAA.....		AATAAA.....	AATAAA
	−109	−71	−41	0	0	409	463
Consensus	G$^{GC}_{CT}$CAATAT	TATA$^{A}_{T}$A$^{A}_{T}$				AATAAA	

[a]See text.

Chambon, 1981; Corden *et al.*, 1980; Kramer *et al.*, 1985; Blumenthal *et al.*, 1984). Three of the four actin genes have such sequences at the appropriate spacing 5′ to the start of translation (Table 2). Actin gene-2 is the exception. A TATA box with poor homology to the consensus sequence is found at position -41. The absence of a consensus TATA sequence in front of gene-2 may be significant in that gene-2 transcripts are relatively low in abundance (discussed in Section 4).

Genes-1, -3, and -4 have one or more polyadenylation signal sequences (AATAAA) at variable positions downstream of the termination of translation (Proudfoot and Brownlee, 1976; Wild and Hirsh, 1985). Again, gene-2 is the exception (Table 2). No such sequence is found in the 420 bp examined 3′ to the gene-2 termination codon. A search of this region failed to reveal any putative 5′ intron splice junctions, suggesting that an intron-mediated juxtaposition of a polyadenylation signal is unlikely. The sequence AATAA does occur 145 nucleiotides downstream from the ochre codon and may serve as the polyadenylation signal for this gene. Although rare, AATAA sequences have been implicated as signal sequences for both an *Oxytricha* and yeast actin gene (Kaine and Spear, 1982; Ng and Abelson, 1980). This sequence is also present downstream from the open reading frame of the *C. elegans* transposon, Tc1 (Rosenzweig *et al.*, 1983).

In *Drosophila* and mammals the differential use of 3′ polyadenylation sites downstream of some myofibrillar protein coding regions results in multiple transcripts from a single gene (Falkenthal *et al.*, 1985; Nadal-Ginard *et al.*, 1985). Although we are unsure which polyadenylation signal is recognized downstream of the *C. elegans* actin genes, the homogenous transcript lengths suggest the use of only one.

The sequence identity of genes-1 and -3 points to a recent duplication or gene conversion event involving these two genes. The phenomena of meiotic recombination and gene conversion in tandemly repeated genes have been implicated as important mechanisms leading to both convergent and divergent evolution of gene families (Nagylaki and Petes, 1982; Klein and Petes, 1981). It is unclear how applicable these arguments are to the *C. elegans* actin genes. The extreme evolutionary conservation of actin gene sequences across species suggests that there are severe enough constraints on the protein product that cluster-enhanced gene conversion would be an insignificant mechanism for maintaining homogeneity. Similarly, the multifunctional domains of actin might limit the degree of tolerable divergence. The fusion of two or more slightly diverged actin sequences via gene conversion or recombination to generate a widely different gene product might be lethal. This consideration makes it difficult to interpret the importance of clustering on actin gene sequences in general.

Gene-2 has 96% nucleotide homology to genes-1 and -3. The 35-bp substitutions are predominantly in the third base (wobble) positions and distributed throughout the gene. This suggests that divergence of gene-2 relative to genes-1 and -3 is due to genetic drift rather than recombination-based mechanisms. This is somewhat surprising in view of the location of gene-2

within the cluster. It is interesting that approximately 10% of the nucleotide substitutions in gene-2 result in amino acid changes. Such a high rate of amino acid substitutions relative to gene-4 could be explained if gene-2 represents an insignificant member of the *C. elegans* actin gene family. The selective constraints on an insignificant protein product might be reduced. Recall that gene-2 lacks conventional 5′ and 3′ control sequences (Wild and Hirsh, 1985) in addition to giving rise to the least abundant actin transcript.

Gene-4 is the least related of the four actin genes by the criteria of nucleotide sequence and intron position. This implies that the separation of gene-4 from the cluster primordium was a relatively ancient event and that interchromosomal conversion events are rare. Since both genes-2 and -4 share a leu codon for amino acid 262, it would appear that the ancestral lineage of these two genes is closer than that of genes-1 or -3 and gene-4.

The relationship of gene organization, sequence, and intron position among actin genes, in the context of actin gene evolution, has been well reviewed by Buckingham and Minty (1983). A few points made by these authors are worth reiterating in considering the evolution of *C. elegans* actin genes. First, the size of actin gene families tends to expand with increasing organism complexity. Yeast has one actin gene, *Drosophila* six, and chickens more than seven (Ng and Abelson, 1980; Fyrberg *et al.*, 1981; Cleveland *et al.*, 1980). *C. elegans*, with four actin genes, fits this pattern. Second, *C. elegans* and *Drosophila* both have striated muscle, yet neither organism has the characteristic vertebrate muscle and cytoplasmic actin isoform distinctions. These distinctions may, therefore, reflect an evolutionary split between the protostomes and deuterostomes unrelated to functional differences in the protein itself. Finally, the *C. elegans* actin genes have a relatively small number of introns. This, in conjunction with the shared position of one of the introns with soybeans, supports the notion that the evolution of genes is accompanied by a net loss of introns (Fyrberg *et al.*, 1981).

4. Transcription

There are three size classes of actin transcripts in *C. elegans* regardless of whether total or polyA RNA is analyzed. The sizes of the transcripts, based on glyoxal gels, are 1450, 1550, and 1650 nucleotides. Gene-specific probes subcloned from the 3′ untranslated region of each gene have been used to distinguish the transcripts (Fig. 3).

Each gene gives rise to a unique size of transcript. The 1450 class is comprised of transcripts from both genes-1 and -2; the 1550 class is comprised of gene-3 transcripts; the 1650 class is comprised of gene-4 transcripts. These sizes are in agreement with the relative sizes predicted from the sequencing data.

It is difficult to quantitate accurately the relative abundance of each transcript. The small and variable lengths of the gene-specific probes (Fig. 3) result in different and variable efficiencies of hybridization and cannot be

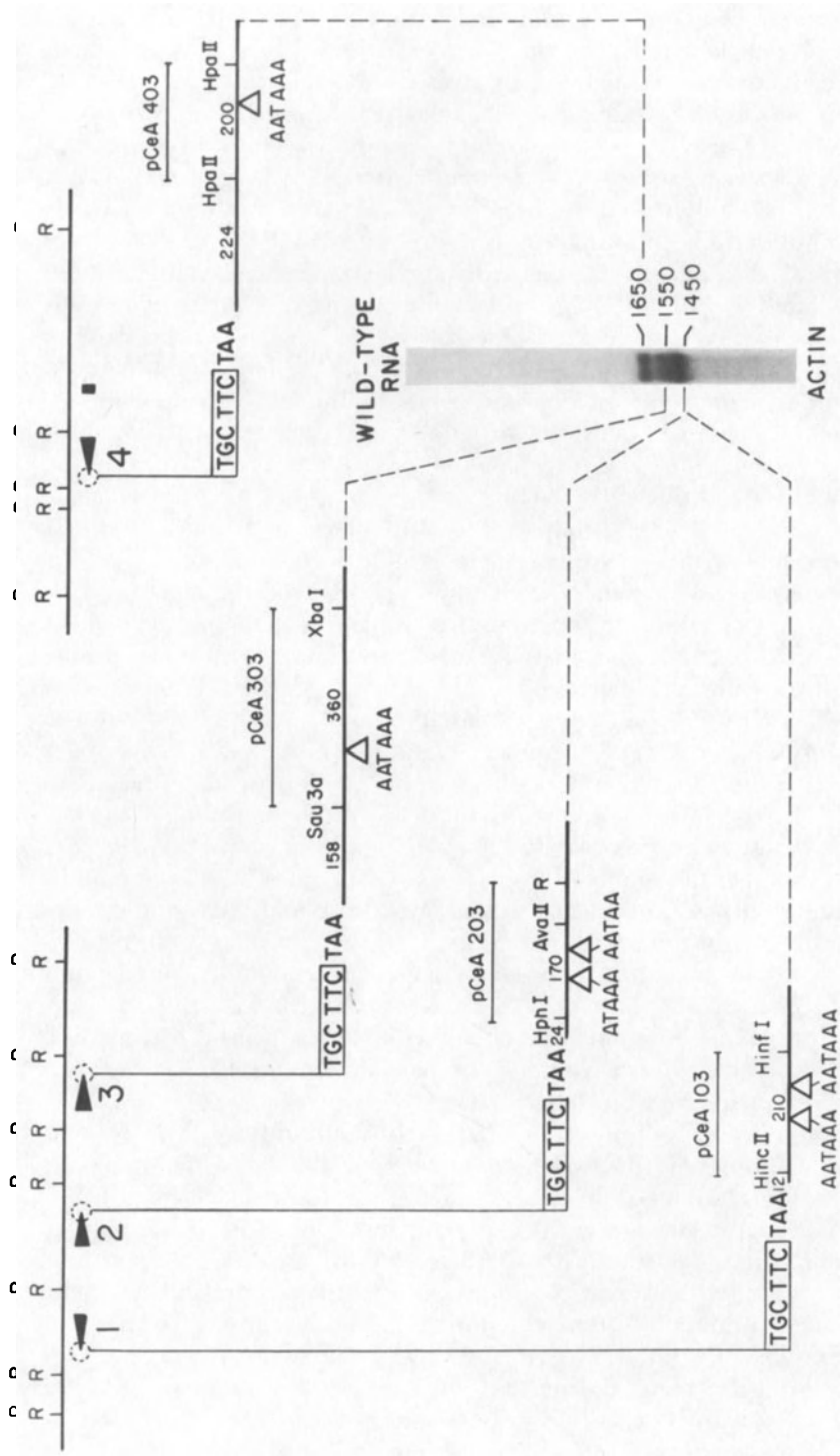


Figure 3. Actin gene-specific probes. The last two codons of each gene are boxed and the translational termination codon indicated. The gene-specific probes were subcloned from the 3′ untranslated region of each gene. The region covered by each probe is designated by lines labeled pCeA103, 203, 303, and 403, respectively. Possible polyadenylation signal sequences are indicated with distances in bp. Each gene-specific probe hybridizes to a unique-sized message of the actin transcript triplet.

used to accurately reflect relative amounts. Estimates of transcript levels must rely, therefore, on whole gene probes. For genes-3 and -4 this is not a problem as each gene gives rise to a unique-sized transcript. Gene-1 and -2 transcripts, however, are the same size, making it difficult to determine the relative contribution of each of these genes to the 1450-nucleotide-size class of actin transcripts. Given these caveats, we estimate that transcripts from genes-1, -3, and -4 are all approximately equal in abundance. Gene-2 transcripts are clearly the least abundant. These estimates are based on total RNA isolated from whole worms and do not reflect spatial differences in transcript distribution.

A convenient way of mapping transcriptional start sites for most genes is to assay S1 nuclease sensitivity of 5′ gene restriction fragments hybridized to complementary transcripts. The high degree of sequence conservation among the four actin genes of the worm, in conjunction with the short 5′ leader sequences, makes S1 mapping difficult. A unique 5′ gene fragment was available for gene-4. The results of the gene-4 S1 experiments placed on the transcriptional start at nucleotide -41.

An attempt was made to map the 5′ ends of genes-1 and -3 with S1 nuclease. Recall that these two genes are identical in sequence within the coding region as well as in 26 of 27 uncleotides upstream of the initiator AUG. The result of the S1 mapping for these two genes was a unique start site at nucleotide -24. This could indicate that both genes share a common start site even though they differ with respect to the locations of CAAT and TATA sequences (Table 2). An alternative explanation exists. Both genes-1 and -3 have a consensus 3′ intron splice sequence ending at position -22. If this splice site is used, and the intron is bigger than 800 bp, the 5′ restriction fragment used for the S1 experiment would not span the intron sequence, resulting in a fortuitous start site at approximately -24.

The temporal expression of the *C. elegans* actin genes was examined by Northern blot analysis. Total RNA was isolated and analzyed from mixed-stage embryos, gravid adults, and at 12-hr intervals between these two extremes. No fluctuations were detected in the relative amounts of the four actin transcripts. As the embryo population was asynchronous, temporal fluctuations of transcript abundance associated with early aspects of myogenesis would not be detected. Total worm RNA preparations also fail to address spatial differences in transcript abundances.

One method of examining the spatial distribution of transcripts is tissue dissection and subsequent RNA isolation. *C. elegans,* because of its small size, makes tissue dissection a tedious task. Nonetheless, Blumenthal *et al.* (1984) were able to separate intestines, gonads, and body walls in an attempt to localize vitellogenin gene transcripts. As a control for the dissection, an actin cDNA clone was used to probe Northern blots of the isolated RNAs. Actin transcripts were abundant in both the gonad and body wall preparations, as expected. Surprisingly, intestinal preparations had a paucity of actin message despite a well-defined brush border (N. Wolf, personal communication). The localization of individual actin transcripts could not be determined since the cDNA clone cross-hybridized to all four actin transcripts.

In situ hybridization is another approach to the study of tissue-specific expression. Although the resolution of current methods is limited in *C. elegans*, available techniques have yielded information on the spatial distribution of actin transcripts. Using a cross-hybridizing probe from the coding region of actin gene-3, Edwards and Wood (1983) demonstrated a quantitative difference in expression. The relative amounts of actin message in different tissues were, in decreasing abundance; body wall, pharynx, gonad, and intestine. The low level of actin transcripts in the intestine is in agreement with the dissection experiments discussed in this section.

Embryonic actin gene expression was also examined using squashed zygotes to circumvent eggshell impermeability (Edwards and Wood, 1983). A threefold increase in actin message was observed between 2 and 6 hr of development. This time period encompasses most of embryonic development up to the 550-cell stage. The first stages of myogenesis occur at about the 400-cell stage (Gossett *et al.*, 1982).

We have also used *in situ* hybridization to whole worm squashes in an attempt to examine the spatial distribution of the actin transcripts. The method used is a modification of several previously described procedures but is based on that reported by Edwards and Wood (1983). Hybridization patterns observed with nonspecific actin gene probes are shown in Fig. 4. The pattern of hybridization is the same for probes derived from the coding region of each of the four actin genes. Each probe hybridizes intensely to lateral regions of the animal which is coincident with body wall muscle. Hybridization to the gonad is also detected with the heaviest distribution of grains being in the distal arm. Only slight hybridization is detected in the intestine. These results are expected and in agreement with the earlier work cited above in this section.

Although the predominant hybridization of the actin probes is seen in the position expected for body wall muscle, the optics of *in situ* preparations do not allow a direct correlation to be made. To demonstrate that lateral bands result from body wall muscle cell hybridization, a λ-phage clone of part of the *unc-54* gene, which encodes a body wall muscle specific myosin heavy chain, was used (Karn *et al.*, 1984). Hybridization with this probe was localized specifically to the lateral regions of the animal analogous to that seen for the actin probes.

Individual members of actin multigene families are usually differentially expressed, either temporally, spatially, or both. At least 4 of the 17 *Dictyostelium discoidium* actin genes show differential expression during the lifecycle (McKeown and Firtel, 1981). All six of the actin genes of *Drosophila* show a distinct temporal or spatial pattern of expression (Fyrberg *et al.*, 1983). The tissue specificity of vertebrate actin isoforms implies that there is at least a spatial regulation of actin gene expression (Vandekerckhove and Weber, 1978a,b). It seems that the actin genes of *C. elegans* are also differentially expressed, at least spatially.

In quantitative terms, there are different levels of actin message in different tissues. For example, the level of actin message in adult intestinal cells is

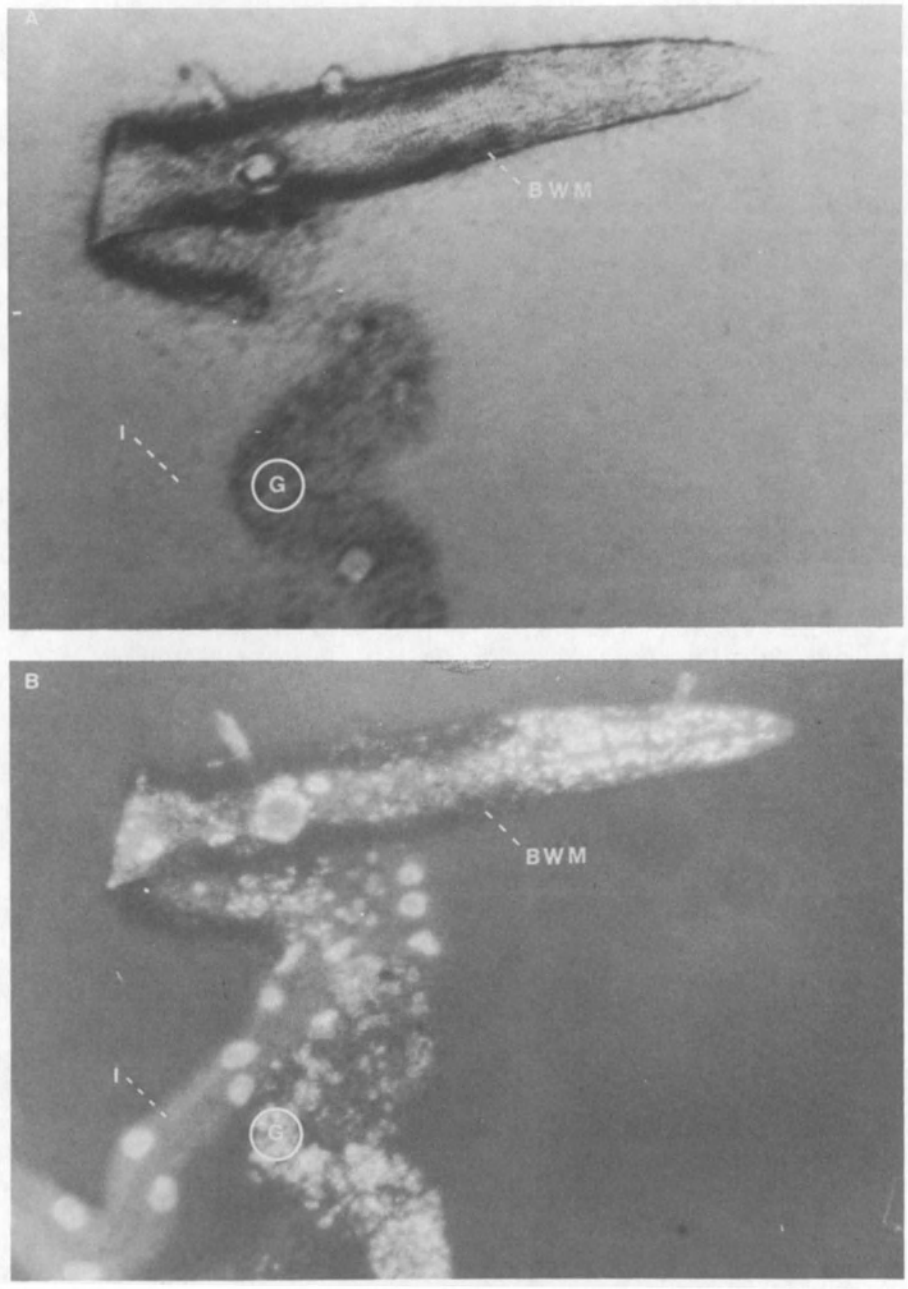


Figure 4. *In situ* hybridization. An S35-labeled probe subcloned from the coding region of actin gene-1 was hybridized to actin message in a partially dissected worm preparation. The specimen was viewed with bright field (A) and epifluorescence (B) to visualize autoradiographic grain distribution and DAPI staining, respectively. Tissue designations are BWM, body wall muscle; G, gonad; I, intestine. Autoradiographic exposure was for 3 days.

very low relative to body wall muscle cells. Moreover, actin gene-2 transcripts are present in low abundance relative to the three other actin messages in total RNA preparations. This low abundance might reflect tissue-specific expression. Alternatively, the low abundance may result from the absence of conventional 5′ controlling sequences (Table 2). Certainly, the former possibility is the more interesting. It is possible that gene-2 encodes a pharyngeal-specific actin isoform. A pharyngeal-specific myosin has been demonstrated in *C. elegans* (Zengel and Epstein, 1980b).

There is genetic evidence that gene-1 and gene-3 encode body wall muscle actins (see Section 7). If separate muscle and cytoplasmic actin isoforms are encoded for in *C. elegans*, then gene-2 and/or gene-4 must be cytoplasmic actin genes. One would expect the cytoplasmic actin message to be relatively abundant suggesting that gene-4 is a more likely candidate than gene-3 for the cytoplasmic actin gene. If genes-1 and -3 encode body wall muscle actin and gene-4 encodes cytoplasmic actin, the implications on tissue-specific isoforms is significant. The amino acid sequences derived from *C. elegans* actin gene-1 and -4 differ by only one residue at position 262 (Wild and Hirsh, 1985). This amino acid substitution (met to leu) is conservative and seems, *a priori*, to be too subtle to account for functional differences between muscle and cytoplasmic actin. This implies that tissue-specific actin isoforms may be functionally interchangeable. Evidence for the incorporation of a chicken cardiac muscle actin into the cytoskeleton of transformed fibroblasts (Gunning *et al.*, 1984) as well as incorporation of injected brain actin into sarcomeres (McKenna *et al.*, 1985) suggests that vertebrate isoform substitutions are possible.

5. *Translational Products*

One advantage of studying actin gene expression is that actin is an abundant cellular constituent and therefore amenable to biochemical analysis. Although the study of *C. elegans* actin isoforms is not extensive, information does exist about the translational products of the actin genes (Landel, 1983).

Depending on the method of solubilization, *C. elegans* has two or three actin isoforms. These proteins have been identified as actin because of their molecular weight, acidic pI, and DNAse affinity. Homogenization of whole worms by sonication results in two actin isoforms detected by two-dimensional gel electrophoresis, termed A and C in order of descending pI (Fig. 5). A third isoform of intermediate pI, termed B, can be resolved when homogenization is accomplished by boiling whole worms in SDS.

In order to resolve the discrepancy in isoform number, actin mRNA was hybrid-selected by DNA–cellulose affinity chromotography (Noyes and Stark, 1975; Childs *et al.*, 1979) and translated *in vitro* (C. Landel, Ph.D. dissertation). Three isoforms, with properties identical to isoforms A, B, and C, are observed (Fig. 5). This suggests that the absence of isoform B from sonicated worm preparations is due to insolubility of isoform B-dependent cytostruc-

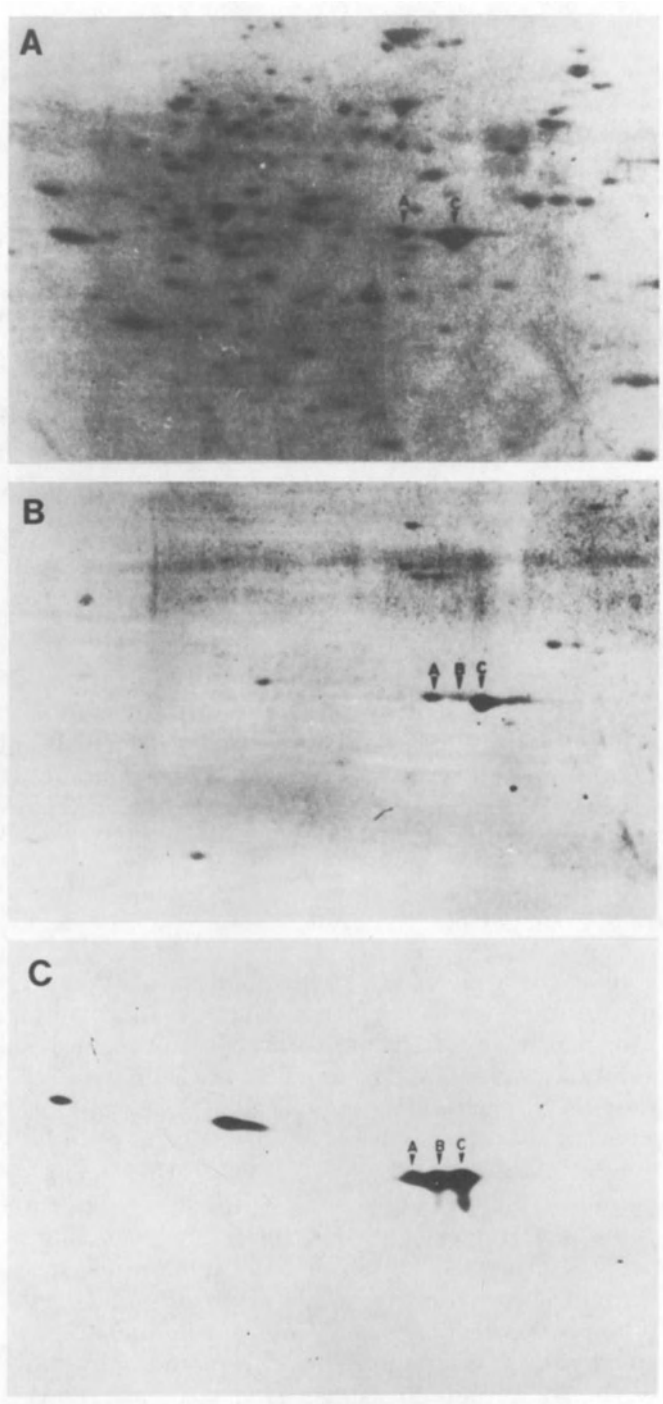


Figure 5. *C. elegans* actin isoforms. Two-dimensional gel electrophoresis was used to separate actin isoforms. Sonication extracts (A) yield isoforms A and C: boiling SDS extracts (B) yield isoforms A, B, and C. Panel (C) is an autoradiograph of *in vitro* translation products of hybrid selected actin message; isoforms A, B, and C are detected.

tures in this method of extraction. A correlation has not yet been made between the three actin isoforms and the four actin genes.

It was of interest to determine which of the three isoforms represented cytoplasmic actin. Presumably the predominant actin isoform in unfertilized eggs is cytoplasmic. The availability of temperature-sensitive spermatogenesis mutants in *C. elegans* makes oocyte isolation feasible. Only isoform C could be detected when oocytes, from a spermatogenesis mutant, were labeled *in vivo* with S35 and analyzed for actin isoform content (Fig. 6). Presumably, isoform C represents a cytoplasmic actin although this classification need not exclude the participation of isoform C in muscle structures as well. If gene-4 encodes cytoplasmic actin, then isoform C must be comprised, at least in part, of the product of gene-4.

6. *Identification of Actin Mutants*

Mutations are valuable for understanding both gene regulation and gene product function *in vivo*. For this reason, we were interested in characterizing *C. elegans* actin mutants. The search for actin mutants was simplified by the genetic mapping of the actin gene cluster. This allowed efforts to be concen-

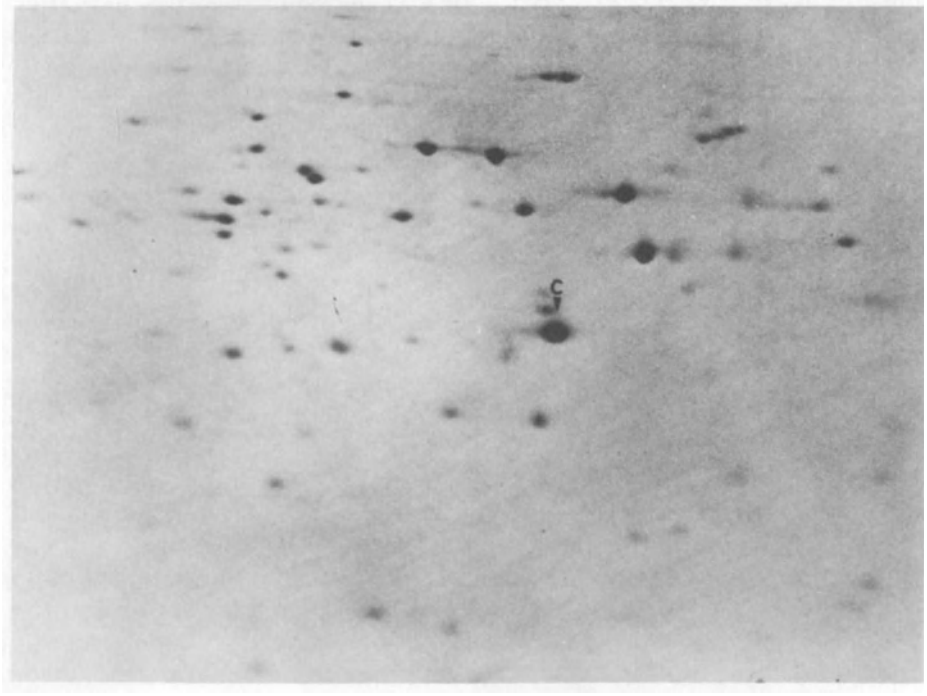


Figure 6. *C. elegans* oocyte actin isoforms. S35-labeled proteins were isolated from oocytes from a temperature-sensitive spermatogenesis mutant and separated by two-dimensional gel electrophoresis. Only actin isoform C is detected.

trated on only 1 of the 20 available genetic loci that were actin candidates because of their effect on muscle cytoarchitecture.

Adjacent to the three clustered actin genes is a site of insertion of the transposon Tc1 in the Bergerac, but not the Bristol, strain of *C. elegans* (Files *et al.*, 1983). This strain-specific insertion results in a polymorphic actin restriction fragment pattern when comparing Southern blots of genomic DNAs cut with a variety of restriction enzymes. We were able to determine the approximate map position of the actin gene cluster by following the segregation of the polymorphism in two and three factor crosses (Hirsh *et al.*, 1982).

The 2-centimorgan region containing the cluster of actin genes was also the interval containing a locus in which mutations caused an uncoordinated phenotype. The locus was defined by five mutations (*st15, st22, st94, st119, st120*) isolated and characterized by Waterston *et al.* (1984) in a search for dominant mutations affecting movement. In addition to dominance, these mutants share a similar disorganization of body wall muscle thin filaments and a high intragenic reversion frequency.

All five dominant mutations show the same pattern of body wall muscle disorganization with the degree of severity increasing in homozygous compared to heterozygous animals (Waterston *et al.*, 1984). The nomally ordered sarcomeric arrays are only occasionally discernible in the mutants. In addition, there is a characteristically irregular distribution of large patches of thin filaments in each muscle cell. Polarization and electron microscopy have been used to confirm the filament disorganization and reveal that there are also thin filaments at oblique angles to the normal muscle lattice (Waterston *et al.*, 1984).

Although all five dominant mutations cause severe body wall muscle defects, only one, *st15*, has an effect on pharyngeal muscle organization. In *st15* homozygotes, the radially oriented myofilaments of the posterior pharyngeal bulb are interrupted by circumferential thick and thin filaments (Waterston *et al.*, 1984). This abnormality causes an inefficient maceration of ingested bacteria resulting in intact bacteria in the intestinal lumen. The slow growth rate of these animals may result from this feeding abnormality (Waterston *et al.*, 1984).

Only three of the five mutations are homozygous viable; *st119* and *st120* abort as first-stage larvae when homozygous. All three homozygous viable mutants have high intragenic reversion frequencies when compared to other *C. elegans* genes in which reversion has been studied (Waterston *et al.*, 1984). A high intragenic reversion frequency is indicative of loss of function mutations implying that the original dominant mutations were the result of a toxic gene product. The coincident map position of the actin gene cluster and the five dominant mutations suggested a mechanism by which a high-reversion frequency could be explained. Namely, if the dominant mutations were actin gene lesions, unequal recombination or gene conversion between the tandem, nearly homologous genes could lead to repair or elimination of a toxic product.

Relatively large (greater than 200 bp) genomic rearrangements resulting

from these corrective mechanisms can be detected on Southern blots. Assuming that the dominant mutations were in fact actin mutations, revertants of the three homozygous viable mutants were screened for rearrangements in the actin gene cluster. The initial screen of six *st15* revertants identified one revertant, RW2246, which had an abnormal actin gene restriction fragment pattern (Landel, 1983). The subsequent screening of 67 additional revertants yielded three more events associated with DNA rearrangements. One, RW2580, is a revertant of *st22*. The remaining two, RW2458 and RW2487, are revertants of *st94*.

We assume that the five dominant mutations are indeed actin gene defects resulting in toxic gene product production because actin gene cluster rearrangements are associated with 4 of the 73 reversion events examined. The basis for reversion in the 69 revertants lacking detectable actin gene rearrangements is unclear. It seems likely that many are the result of nonsense mutations or small deletions or insertions that eliminate the toxic gene product. Alternatively, correction of the gene lesion could be accomplished by gene conversion. The high degree of sequence homology among the three clustered genes would make conversion events difficult to detect. It is also possible that reversion could occur by second-site events. The lack of phenotype associated with the revertants precludes the opportunity to map these events genetically (Waterston *et al.*, 1984).

7. *Actin Gene Organization in Revertants of Actin Mutants*

The actin gene cluster of the four revertants associated with DNA rearrangements is diagramatically represented in Fig. 7. The schematic results

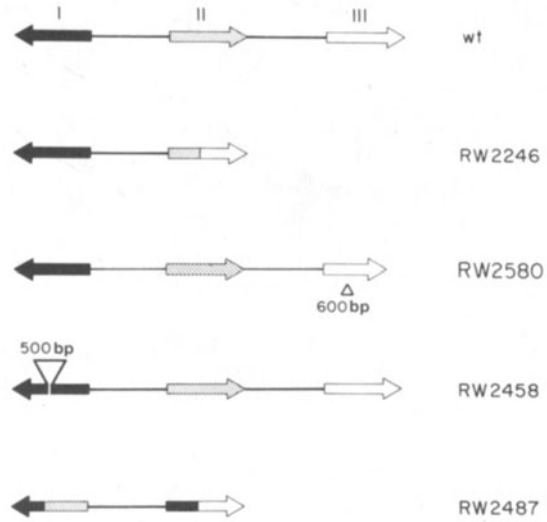


Figure 7. Genomic organization of the actin gene cluster in wild-type (N2) and four revertant animals. Break points for insertion, deletion, and recombination events are approximate and are only meant to indicate the regions involved in the rearrangements.

from restriction maps of the revertant DNAs in conjunction with hybridization data using a variety of small DNA probes from the unique sequences which are interspersed among the actin genes. The resolution of this approach is limited to the available restriction sites and probes for any given region, and therefore, the break points indicated for the recombination events are only approximate. For simplicity, the hybrid genes have been designated in the following discussions by the numbers involved in the fusion. The gene contributing the 5′ most sequences is listed first.

Two of the revertants, RW2458 and RW2580, are accompanied by an insertion and a deletion respectively (Fig. 7) (Landel *et al.*, 1984). RW2458 has a 500-bp insertion in the gene-1 region. Although the exact point of insertion is unknown, it has been localized to the 3′ region of the gene. It is assumed that the insertion leads to reversion by reducing or eliminating a mutant toxic gene-1 product. This implies that the original mutant, *st94*, is an allele of gene-1. Likewise, the 600-bp deletion in RW2580 affects only gene-3, and by the same argument, *st22* is an allele of gene-3.

The remaining two revertants are accompanied by rearrangements that involve more than one gene (Landel *et al.*, 1984). RW2246 contains a deletion that fuses the 5′ end of gene-2 with the 3′ end of gene-3 (Fig. 7). The 2/3 hybrid gene would be expected to be regulated as gene-2 because it retains the gene-2 promoter region. Presumably the original mutation was within the deleted region, making it impossible to determine whether the original mutation, *st15*, is an allele of gene-2 or -3.

Gene-2 encodes a protein with three amino acid substitutions when compared with either gene-1 or -3. All three codons for these substitutions are 5′ to a unique gene-2 restriction site. Preliminary evidence suggests that the unique restriction site is retained in the 2/3 hybrid gene. That is, the protein product encoded by the 2/3 hybrid gene is identical to the normal gene-2 product. The maintenance of a gene-2 product may reflect a vital and functionally significant role for the three amino acid differences.

The fourth revertant, RW2487, is associated with a complex rearrangement of the actin gene cluster (Fig. 7). The 5′ ends of genes-1 and -2 have been inverted in addition to a fusion between gene-3 and the gene occupying the former gene-2 site (Landel *et al.*, 1984). The result is a pair of hybrid genes: a 2/1 hybrid in the normal gene-1 position and a 1/3 hybrid in the normal gene 3 position. It has not been determined whether the 2/1 hybrid retains all three of the gene-2-specific amino acid codons.

Two of the four revertants, RW2246 and RW2487, result in a physical reduction of the actin gene cluster to two rather than three genes. The other two revertants, RW2458 and RW2580, presumably result in a functional reduction to two genes. The phenotypic differences between all four revertants and wild-type animals are subtle (Waterson *et al.*, 1984). This implies that there is a degree of reiteration of actin gene function in *C. elegans*. This is not surprising in light of the identical nucleotide sequence for genes-1 and -3 (Wild and Hirsh, 1985). In each revertant, the remaining functional gene pair that is retained is comprised of a gene-2-like copy and either gene-1 or -3.

8. Transcription in Revertants

The four revertants associated with actin gene cluster rearrangements provide an excellent opportunity to study gene regulation at the transcriptional level. Northern blot analysis of RNA isolated from the four revertants, when hybridized with a nonspecific actin probe, shows that each revertant has an abnormal transcriptional pattern (Figs. 8 and 9). Gene-specific probes (Fig. 3) were used to identify individual transcripts and correlate the genomic rearrangements with the abnormal transcriptional patterns. The results of the revertant transcriptional patterns are summarized in Table 3. It should be noted that the transcriptional pattern seen in gravid adults is probably due to the embryonic content of these animals rather than a reflection of changes in adult steady-state levels of actin transcripts. If this is true, then the changes in transcript abundance are indicative of regulatory difference between embryonic and postembryonic stages. Although the nature of this difference is unknown, the independent fluctations in transcript levels derived from the actin gene cluster suggest that these genes need not not be uniformly expressed.

The revertant RW2458 contains a 500-bp insertion in the gene-1 region (Fig. 7). This insertion is derived partially, if not entirely, from the 3′ end of actin gene-3 based on gene-3-specific probe hybridization patterns. The anticipated effect of an intragenic insertion would be an abnormally sized gene-1 transcript, yet a transcript of normal gene-1 size is detected in this revertant. The abundance of this gene-1 transcript, however, fluctuates during development (Fig. 10). RW2458 mixed-staged embryos and gravid adults have near-wild-type levels of gene-1 transcripts relative to the other three actin transcripts. RW2458 larval stages show a dramatic decrease in gene-1 transcript abundance. As Northern blots reflect steady-state levels of transcripts, it is unclear whether the temporal fluctuation is due to a change in the rate of gene-1 transcription or a change in the stability of the transcript. The size and abundance of gene-2 and -4 transcripts appear normal.

Two other aspects of the revertant RW2458 are noteworthy. First, the abundance of gene-3 transcripts is elevated above wild-type levels throughout development (Fig. 10). Second, RW2458 animals have a fifth, low abundant, actin transcript of about 900 nucleotides in length (Fig. 11). This transcript hybridizes to the gene-3-specific probe, suggesting that the insertion of gene-3 3′ sequences has resulted in a truncated hybrid gene transcript.

The transcriptional pattern of RW2580, which has a 600-bp deletion covering part of the 3′ end of gene-3, is predictable. The 1550-size class, corresponding to gene-3, is absent (Fig. 9). A truncated gene-3 transcript has not been detected. The remaining transcripts all appear normal.

The revertant RW2246 is associated with a fusion of genes-2 and -3 (Landel *et al.*, 1984). The 2/3 hybrid gene, having gene-2 5′ sequences, would be expected to be regulated as gene-2. This is indeed the case. The level of transcript abundance of the 2/3 hybrid gene is comparable to wild-type gene-2 levels. The 2/3 hybrid transcript size is indistinguishable from that of

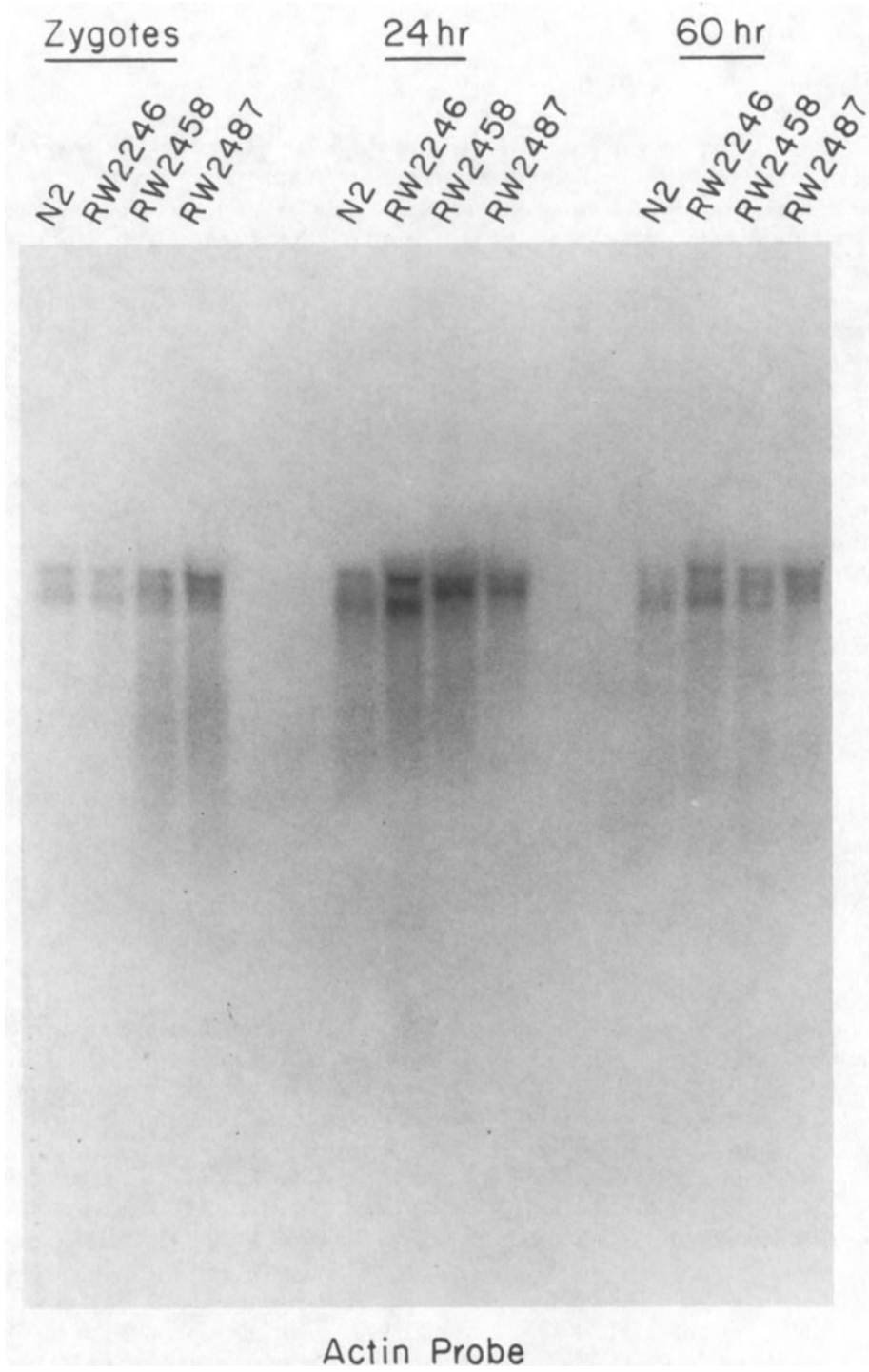


Figure 8. Comparison of wild-type (N2) and revertant (RW2246, RW2458, RW2487) actin transcripts. Total RNA was isolated from zygotes, larvae (24 hr), and gravid adults (60 hr). The RNA was separated by glyoxal gel electrophoresis, transferred to nitrocellulose (Northern blot), and hybridized with an actin gene-coding region subclone that cross-hybridizes to all four actin gene sequences.

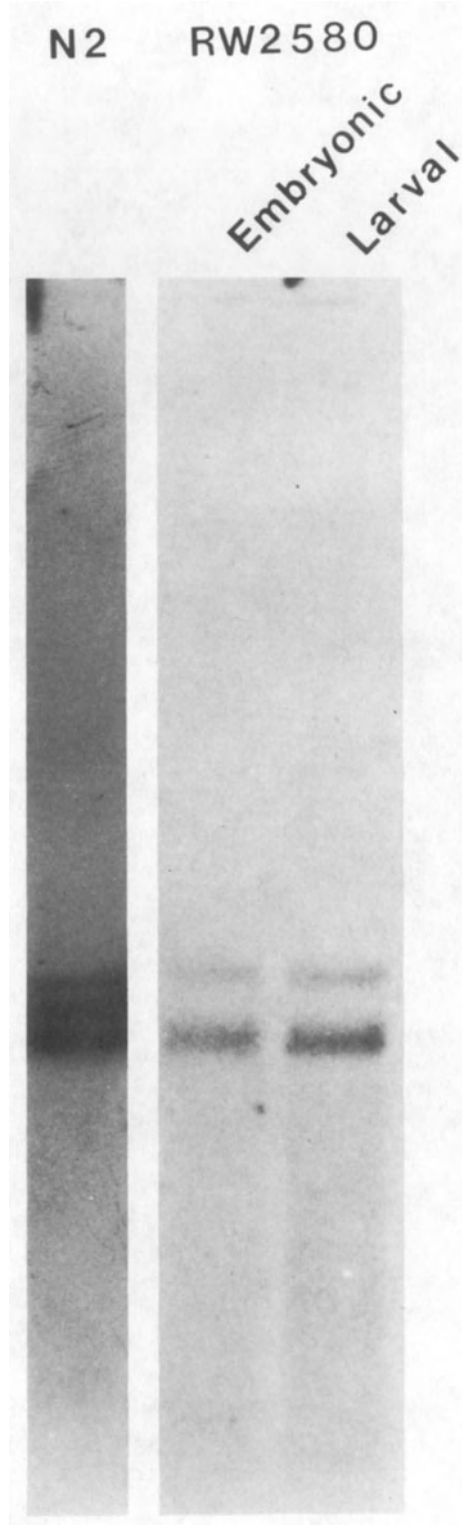


Figure 9. Comparison of wild-type (N2) and RW2580 actin transcripts. Total RNA was isolated from mixed-stage N2 animals and embryonic and midlarval-stage RW2580 animals. Northern blots of the RNA samples were probed with a cross-hybridizing actin gene clone.

Table 3. Wild-Type (N2) and Revertant Transcript Levels[a,b]

		Probe			
		Gene-1	Gene-2	Gene-3	Gene-4
N2	E	+++	+	+++	+++
	L	+++	+	+++	+++
	A	+++	+	+++	+++
RW2458	E	++	+	++++	+++
	L	+	+	++++	+++
	A	++	+	++++	+++
RW2580	E	+++	+	0	+++
	L	+++	+	0	+++
	A	ND	ND	ND	ND
RW2246	E	+++	0	+	+++
	L	+++	0	+	+++
	A	+++	0	+	+++
RW2487	E	++	ND	++++	+++
	L	+	ND	++++	+++
	A	++	ND	++++	+++

[a]E, embryos; L, larvae; A, adult.
[b]ND, not determined; 0, not detected.

wild-type gene-3. This is as expected since the variation in size of the four actin transcripts is due principally to differences in the lengths of the 3′ untranslated regions of each gene (Wild and Hirsh, 1985). No transcript is detected with the gene-2-specific probe as this region is deleted in RW2246.

The sizes of the transcripts from genes-1 and -4 are normal in RW2246, but the amounts are not. It seems, by circumstantial evidence, that both gene-1 and gene-4 transcript levels are elevated in this revertant throughout embryonic and postembryonic stages. The implications of such an event for regulation of the four actin genes are significant, and their discussion, therefore, should await further proof.

The actin gene cluster rearrangement associated with RW2487 is complicated and results in two hybrid genes. The development pattern of actin transcripts, however, is indistinguishable from that of RW2458. The 2/1 hybrid gene shows variable levels of transcript during development (Fig. 8). The RW2487 embryonic and gravid adult levels resemble wild-type gene-1 abundance, whereas RW2487 midlarval levels show a reduction to wild-type gene-2 levels. As in RW2458, this temporal fluctuation may reflect changes in either transcriptional rate or transcript stability. Gene-4 transcript levels appear unaffected.

The actin gene transcription patterns of the revertants, although complex, are informative. Two revertants, RW2246 and RW2487, result in hybrid genes under the presumptive control of a possibly inefficient gene-2 promoter (Table 2). In both revertants there is a reduction of the 2/X hybrid gene transcript abundance to wild-type gene-2 levels at all postembryonic stages of

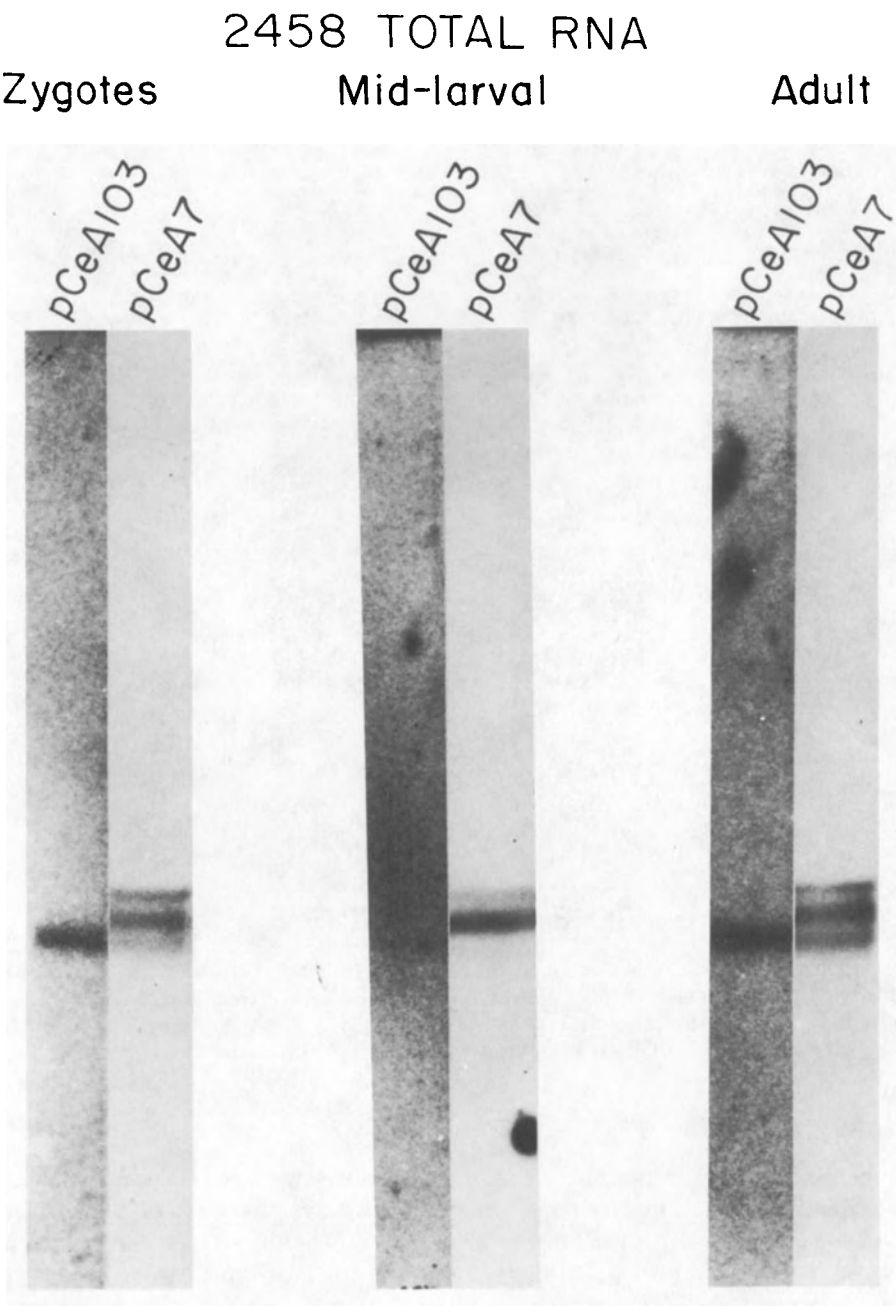


Figure 10. Temporal fluctuations of actin gene-1 transcripts in RW2458. Total RNA was isolated from either zygotic, midlarval, or adult-stage RW2458 animals. Northern blot lanes of the RNA samples were bissected. Each half was probed with either a gene-1-specific probe (pCeA103) or a cross-hybridizing actin gene clone (pCeA7).

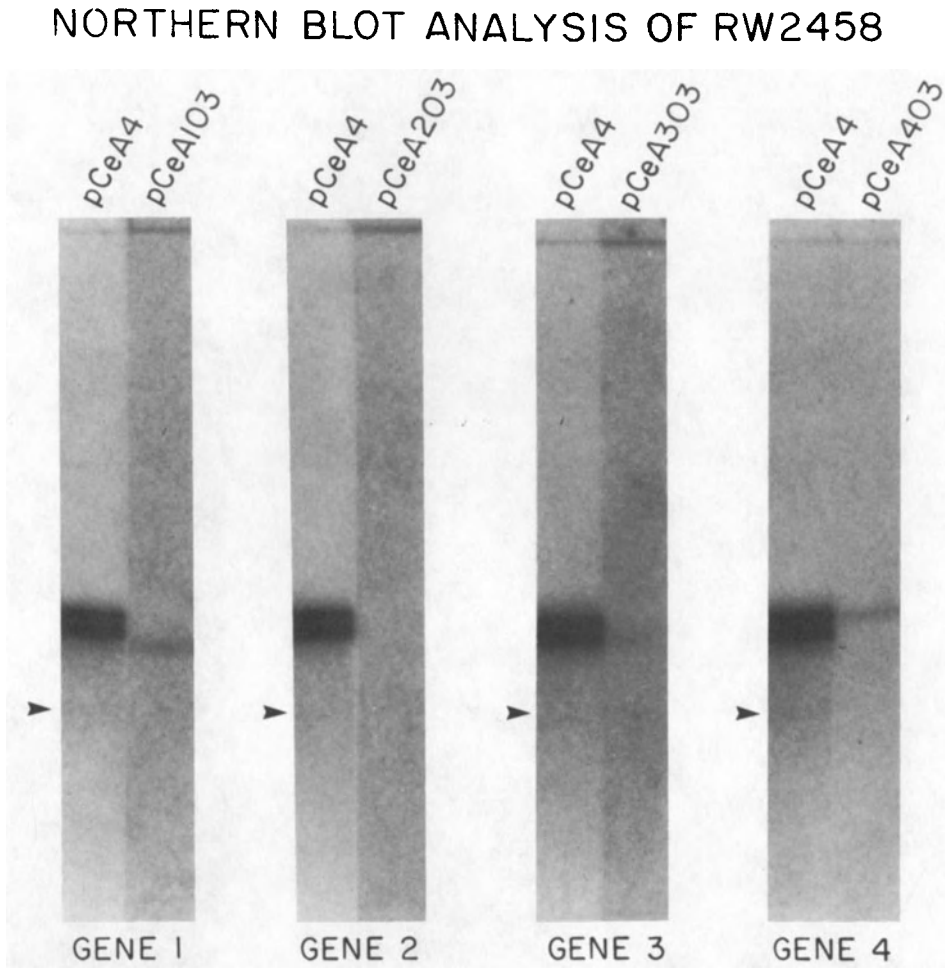


Figure 11. Actin transcript composition of midlarval RW2458 RNA. Total RNA was isolated from midlarval RW2458 animals. Replica Northern blot lanes were bissected. Each half was probed with either a gene-specific probe (pCeA103, 203, 303, 403) or a cross-hybridizing actin gene clone (pCeA4). Arrows indicate the size of a novel actin transcript in this revertant.

development (assuming that gravid adult patterns are a reiteration of embryonic steady states). The two revertants differ with regard to hybrid transcript levels in embryos. In RW2246 the abundance is again reduced to wild-type gene-2 levels. In contrast, RW2487 embryos have a hybrid gene transcript abundance comparable to that of a normal gene-1. A simple model of transcript regulation to explain these results is difficult to construct. It may be that the 3′ sequence differences between the two hybrid genes are responsible for the temporal differences in their expression. Two revertants, RW2458 and RW2487, show a clear increase in the abundance of gene-3 (RW2458) and

-1/3 (RW2487) transcripts. There is also evidence that RW2246 increases gene-1 transcript abundance in conjunction with a decrease in gene-2/3 levels. Recall that genes-1 and -3 encode identical protein products. These results suggest that genes-1 and -3 can reciprocally compensate for each other transcriptionally. Because the increase in the compensatory transcript abundance is not temporally coincident with the deficient transcript (RW2458 and RW2487 embryos), it seems that regulation between these two genes is dependent on factors other than transcript abundance. The control of tubulin gene transcription has been shown to be regulated by tubulin monomer concentrations in a variety of systems (Cleveland *et al.*, 1981; Ben-Ze'ev *et al.*, 1979; Weeks *et al.*, 1977). It may be that a similar control mechanism is involved in the regulation of the *C. elegans* actin genes.

The preliminary results of RW2580 are an apparent contradiction to this conclusion. Gene-3 contains a 600-bp deletion in gene-3. One would expect that gene-1 transcripts levels would increase to compensate for the absence of an intact gene-3. The abundance of gene-1 transcripts, relative to gene 4, is not elevated to RW2580. This raises an important point about the revertant transcriptional analysis. The internal standard for transcript abundance is gene-4 as it is unlinked to the rearranged cluster. This does not mean that gene-4 is unaffected, transcriptionally, by the rearrangement. In fact, the level of gene-4 transcripts may be elevated in the revertant RW2246. A similar situation may exist in RW2580; that is, both genes-1 and -4 might be elevated in abundance so that their relative amounts appear unchanged.

The other possibility is that both revertants, RW2246 and RW2580, have normal gene-1 and -4 transcript levels. This would imply that the compensatory regulation between genes-1 and -3 is unidirectional rather than reciprocal. That is, gene-3 transcript abundance can be increased in response to deficient or defective gene-1 transcripts, but the inverse is not true. If this is the case, then gene-1 transcripts would presumably have an increased translatability to compensate for the lack of a gene-3 protein product. Such a mechanism of interactive regulation among the two genes seems overly complicated and therefore unlikely.

9. Future Prospects

The revertants of actin mutants have provided, and will continue to provide, information on the mechanism underlying the developmental regulation of the *C. elegans* actin genes. One would also like to know the cause of the original mutations. As the resolution of the genetic mapping for the mutants does not distinguish between the three actin genes in the cluster, the search for the site of the mutations becomes a formidable task. The revertants can circumvent this problem by limiting the search to a small region or single gene. Sequencing these defined regions will allow us to correlate directly specific DNA lesions with their phenotypic defects. This approach can provide insight into aspects of both gene regulation and protein function. Hope-

fully, we will be able to expand and expedite this approach by combining *in vitro* site-directed mutagenesis with transformation to reintroduce mutant gene copies and study their effects *in vivo*. The advantage of *C. elegans* for such studies is that severe body wall muscle defects can be tolerated and inherited thus allowing one to study a broad range of muscle component mutations.

ACKNOWLEDGMENTS. We thank Bob Waterston for making available the actin mutants and revertants on which much of this work was based. This work was supported by grants GM19851 and GM26515 from the Public Health Service. M. K. received support from a Public Health Service training grant (GM07135).

References

Albertson, D. G., and Thomson, J. N., 1976, The Pharynx of *Caenorhabditis elegans*, *Philos. Trans. Roy. Soc. London, Sec. B*, **275:**299–325.

Benoist, C., and Chambon, P., 1981, *In vivo* sequence requirements of the SV40 early promoter region, *Nature* **290:**304–310.

Ben-Ze'ev, A., Farmer, S. R., and Pennman, S., 1979, Mechanisms of regulating tubulin synthesis in cultured mammalian cells, *Cell* **17:**319–325.

Blumenthal, T., Squire, M., Kirtland, S., Cane, J., Donegan, M., Spieth, J., and Sharrock, W., 1984, Cloning of a yolk protein gene family from *Caenorhabditis elegans*, *J. Mol. Biol.* **174:**1–18.

Buckingham, M., and Minty, A., 1983, Contractile proteins genes, in: *Eukaryotic Genes: Their Structure, Activity and Regulation* (N. Machean, S. Gregory, and R. Flavell, eds.), Butterworths, London.

Childs, G., Levy, S., and Kedes, L. H., 1979, Rapid purification of biologically active individual histone messenger RNA by hybridization to cloned DNA linked to cellulose, *Biochemistry* **18:**208–213.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirschner, M. W., 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes, *Cell* **20:**95–105.

Cleveland, D. W., Lopata, M. A., Sherline, P., and Kirschner, M. W., 1981, Unpolimerized tubulin modulates the level of tubulin RNAs, *Cell* **25:**537–546.

Cohn, N. H., Lowry, J. C., and Kedes, L. H., 1976, Histone genes of the Sea Urchin (*S. purpuratus*) cloned in *E. coli:* Order, polarity, and strandedness of the five histone-coding and spacer regions, *Cell* **9:**147–161.

Corden, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C., and Chambon, P., 1980, Promoter sequences of eucaryotic protein-coding genes, *Science* **209:**1406–1414.

Edwards, M. K., and Wood, W. B., 1983, Location of specific messenger RNAs in *Caenorhabditis elegans* by cytological hybridization, *Dev. Biol.* **97:**375–390.

Falkenthal, S., Parker, V. P., and Davidson, N., 1985, Developmental variations in the splicing pattern of transcripts from the *Drosophila* gene encoding myosin alkali light chain result in different carboxyl-terminal amino acid sequences, *Proc. Natl. Acad. Sci. U.S.A.* **82:**449–453.

Files, J. G., Carr, S., and Hirsh, D., 1983, Actin gene family of *Caenorhabditis elegans*, *J. Mol. Biol.* **164:**355–375.

Fyrberg, E. A., Bond, B. J., Hershey, N. D., Mixter, M. S., and Davidson, N., 1981, The actin genes of *Drosophila:* Protein coding regions are highly conserved but intron positions are not, *Cell* **24:**107–116.

Fyrberg, E. A., Mahaffey, J. W., Bond, B. J., and Davidson, N., 1983, Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner, *Cell* **33:**115–123.

Gossett, L. A., Hecht, R. M., and Epstein, H. E., 1982, Muscle differentiation in normal and cleavage-arrested mutant embryos of *Caenorhabditis elegans*, *Cell* **30:**193–204.

Gunning, P., Ponte, P., Kedes, L., Hickey, R. J., and Skoultchi, A. I., 1984, Expression of human cardiac actin in mouse L cells: A sarcomeric actin associates with a nonmuscle cytoskeleton, *Cell* **36:**709–715.

Hirsh, D., Files, J. G., and Carr, S. H., 1982, Isolation and genetic mapping of the actin genes of *Caenorhabditis elegans*, in: *Muscle Development: Molecular and Cellular Control* (M. L. Pearson and H. E. Epstein, eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Jones, C. W., and Kafatos, F. C., 1980, Coordinately expressed members of two chorion multigene families are clustered, alternating and divergently oriented, *Nature (London)* **284:**635–638.

Kaine, B. P., and Spear, B. B., 1982, Nucleotide sequence of a macronuclear gene for actin in *Oxytricha fallax*, *Nature* **295:**430–432.

Karlik, C. C., Mahaffey, J. W., Couto, M. D., and Fyrberg, E. A., 1984, Organization of contractile protein genes within the 88F subdivision of the *D. melanogaster* third chromosome, *Cell* **37:**469–481.

Karn, J., Dibb, N. J., and Miller, D. M., 1985, Cloning nematode myosin genes, in: *Cell and Muscle Motility*, Vol. 6 (J. Shay, ed.), pp. 185–237, Plenum Press, New York.

Kimmel, A., 1984, Tightly linked genes with different modes of developmental regulation in *Dictyostelium*, in: *Molecular Biology of Development* (E. H. Davidson and R. A. Firtel, eds.), pp. 373–382, Alan R. Liss, New York.

Kindle, K. L., and Firtel, R. A., 1978, Identification and analysis of *Dictyostelioum* actin genes, a family of moderately repeated genes, *Cell* **15:**763–778.

Klein, H. L., and Petes, T. D., 1981, Intrachromosomal gene conversion in yeast, *Nature* **289:**144–148.

Kramer, J. M., Cox, G. N., and Hirsh, D., 1985, Expression of the *Caenorhabditis elegans* collagen genes *col-1* and *col-2* is developmentally regulated, *J. Biol. Chem.* **260:**1945–1951.

Landel, C. P., 1983, Identification of the genetic locus of an actin gene in the nematode *Caenorhabditis elegans*, Ph.D. dissertation, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado.

Landel, C. P., Krause, M., Waterston, R., and Hirsh, D., 1984, DNA rearrangements of the actin gene cluster in *C. elegans* accompany reversion of three muscle mutants, *J. Mol. Biol.* **180:**487–513.

Liao, L. W., Rosenzweig, B., and Hirsh, D., 1983, Analysis of a transposable element in *Caenorhabditis elegans*, *Proc. Natl. Acad. Sci. U.S.A.* **80:**3585–3589.

Mackenzie, J. M., Garcea, R. L., Zengel, J. M., and Epstein, H. F., 1978, Muscle development in *Caenorhabditis elegans:* Mutants exhibiting retarded sarcomere construction, *Cell* **15:**751–762.

MacLeod, A. R., Waterston, R. H., and Brenner, S., 1977a, An internal deletion mutant of a myosin heavy chain in *Caenorhabditis elegans*, *Proc. Natl. Acad. Sci. U.S.A.* **74:**5336–5340.

MacLeod, A. R., Waterston, R. H., Fishpool, R. M., and Brenner, S., 1977b, Identification of the structural gene for a myosin heavy chain in *Caenorhabditis elegans*, *J. Mol. Biol.* **114:**133–140.

McKenna, N., Meigs, J. B., and Wang, Y.-L., 1985, Identical distribution of fluorescently labeled brain and muscle actins in living cardiac fibroblasts and myocytes, *J. Cell Biol.* **100:**292–296.

McKeown, M., and Firtel, R. A., 1981, Differential expression and 5′ end mapping of actin genes in *Dictyostelium*, *Cell* **24:**799–807.

Miller, D. M., 1985, *Sup-3:* A mutation which elevates myosin levels in nematode muscle is closely linked to a myosin heavy chain gene, *J. Cell. Biochem.* **Suppl. 9B:**50.

Nabeshima, Y., Fojii-Kuriyama, Y., Muramatsu, M., and Ogata, K., 1984, Alternative transcription and two modes of splicing result in two myosin light chains from one gene, *Nature* **308:**333–338.

Nadal-Ginard, B., Breitbart, R. E., Strehler, E. E., Periasamy, M., and Ruiz-Opazo, N., 1985, Alternative splicing: A common mechanism for the generation of contractile protein diversity from single genes, *J. Cellular Biochem.* **Suppl. 9B:**76.

Nagylaki, T., and Petes, T. D., 1982, Intrachromosomal gene conversion and the maintenance of sequence homogeneity among reported genes, *Genetics* **100**:315–337.

Ng, R., and Abelson, J., 1980, Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*, *Proc. Natl. Acad. Sci. U.S.A.* **77**:3912–3916.

Noyes, B. E., and Stark, G. R., 1975, Nucleic acid hybridization using DNA covalently coupled to cellulose, *Cell* **5**:301–310.

Patterson, B. M., Seiler-Tuyns, A., and Eldridge, J. D., 1984, Expression and regulation of chicken actin genes in avian and murine myogenic cells, in: *Molecular Biology of Development* (E. H. Davidson and R. A. Firtel, eds.), pp. 383–394, Alan R. Liss, New York.

Proudfoot, N. J., and Brownlee, G. G., 1976, 3′ Noncoding region sequences in eucaryotic messenger RNA, *Nature* **263**:211–214.

Rosenzweig, B., Liao, L. W., and Hirsh, D., 1983, Target sequences for the *C. elegans* transposable element Tc1, *Nucl. Acid Res.* **11**:7137–7140.

Sheller, R. H., McAllister, L. B., Cruin, W. R., Jr., Durica, D. S., Posakony, J. W., Thomas, T. L., Britten, R. J., and Davidson, E. H., 1981, Organization and expression of multiple actin genes in the sea urchin, *Mol. Cell. Biol.* **1**:609–628.

Shott-Akhurst, R. J., Calzone, F. S., Britten, R. J., and Davidson, E. H., 1984, Isolation and characterization of a cell lineage-specific cytoskeletal actin gene family of *Strongylocentrotus purpurtos*, in: *Molecular Biology of Development* (E. H. Davidson and R. A. Firtel, eds.), pp. 119–129, Alan R. Liss, New York.

Spradling, A. C., Digan, M. E., Mahowald, A. P., Scott, M., and Craig, E. A., 1980, Two clusters of genes for major chorion proteins of *Drosophila melanogaster*, *Cell* **19**:905–914.

Sulston, J. E., and Horvitz, H. R., 1977, Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*, *Dev. Biol.* **56**:110–156.

Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N., 1983, The embryonic cell lineage of the nematode *Caenorhabditis elegans*, *Dev. Biol.* **100**:64–119.

Vandekerckhove, J., and Weber, K., 1978a, At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide, *J. Mol. Biol.* **126**:783–802.

Vandekerckhove, J., and Weber, K., 1978b, Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins, *Proc. Natl. Acad. Sci. U.S.A.* **75**:1106–1110.

Waterston, R. H., Fishpool, R. M., and Brenner, S., 1977, Mutants affecting paramyosin in *Caenorhbditis elegans*, *J. Mol. Biol.* **117**:679–697.

Waterston, R. H., Thomson, J. N., and Brenner, S., 1980, Mutants with altered muscle structure in *Caenorhabditis elegans*, *Dev. Biol.* **77**:271–302.

Waterston, R. H., Hirsh, D. I., and Lane, T. R., 1984, Dominant mutations affecting muscle structure in *Caenorhabditis elegans* that map near the actin gene cluster, *J. Mol. Biol.* **180**:473–496.

Weeks, D., Collis, P. S., and Gealt, M., 1977, Control of induction of tubulin synthesis in *Chlamydomonas reinhardii*, *Nature* **268**:667–668.

Wild, M. A., and Hirsh, D., 1986, Sequence analysis of *Caenorhabditis elegans* actin genes (in preparation).

Zengel, J. M., and Epstein, H. F., 1980a, Identification of genetic elements associated with muscle structure in the nematode *Caenorhabditis elegans*, *Cell Motil.* **1**:73–97.

Zengel, J. M., and Epstein, H. F., 1980b, Mutants altering coordinate synthesis of specific myosins during nematode muscle development, *Proc. Natl. Acad. Sci. USA* **77**:852–856.

8

Implications of Microtubule Polarity for Microtubule Function

Ursula Euteneuer

1. Introduction

Microtubules (MTs) and actin filaments have an intrinsic molecular polarity based on the unipolar stacking of their asymmetrical subunits. Filament polarity is believed to be important for many cellular activities. For example, the interaction of actin and myosin that produces shortening of the sarcomere in striated muscle depends on the correct vectorial orientation of actin filaments relative to myosin. While a convenient polarity marker for actin has been available for over 20 years, similar biochemical tools became available for MTs only a few years ago. The purpose of this paper is to summarize some observations on the relation between MT polarity and MT involvement in cellular motility. For more complete accounts of MT biochemistry and physiology, the reader is referred to recent overviews and monographs (Roberts and Hyams, 1979; DeBrabander and DeMey, 1980; Gunning and Hardham, 1982; Sakai *et al.*, 1982; Dustin, 1984; McKeithan and Rosenbaum, 1984).

2. Background and Methods for Determining MT Polarity

MTs are cylindrical cell organelles with a diameter of about 25 nm. They are assembled from asymmetrical subunits, the αβ-tubulin dimer which is the functional subunit in MT assembly (Stephens, 1970; Fine, 1971; Everhart, 1971; Feit *et al.*, 1971). The globular monomers that form the dimer have a molecular weight of about 50,000 daltons each (based on amino acid composi-

Ursula Euteneuer • Department of Zoology, University of California, Berkeley, California 94720.

tion; Valenzuela *et al.*, 1981; Lemischka and Sharp, 1982) and a diameter of approximately 4 nm. The organization of subunits within the MT wall can be seen in electron microscope images of negatively stained preparations (Fig. 1; Amos and Klug, 1974). Within a MT, the dimers are aligned into protofilaments, 13 of which are required to form a MT in most cells (Ledbetter and Porter, 1964; Tilney *et al.*, 1973), but exceptions to this rule are known (Burton *et al.*, 1975; Chalfie and Thomson, 1982). The precise arrangement of the αβ-dimers in the MT can be deduced from optical diffraction patterns, which show all dimers to be oriented unidirectionally (Fig. 2; Amos and Klug, 1974; Amos, 1979). The structural polarity of MTs is believed to be important for

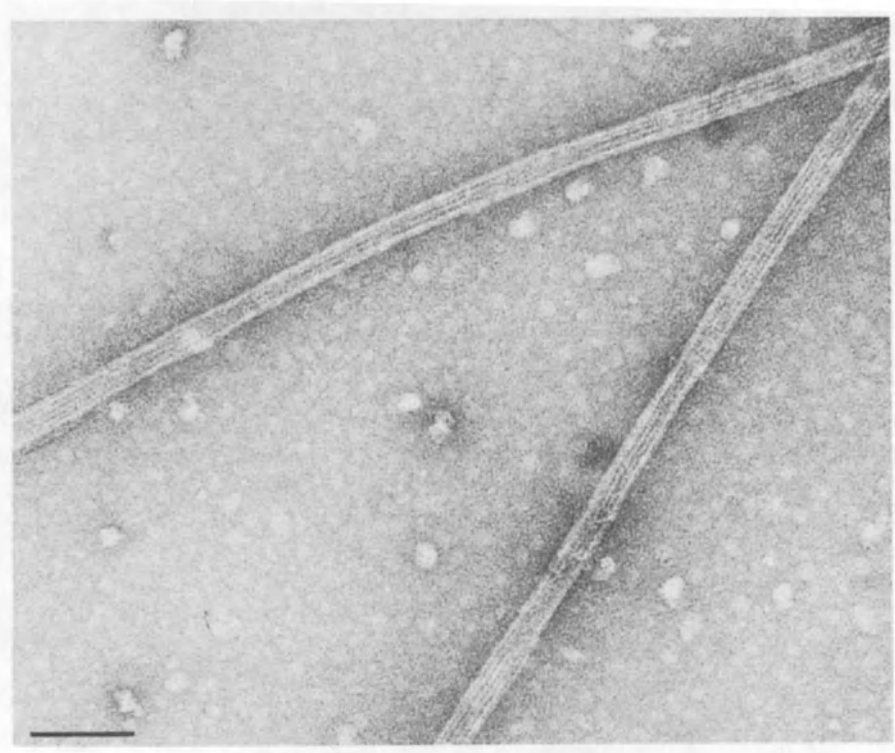
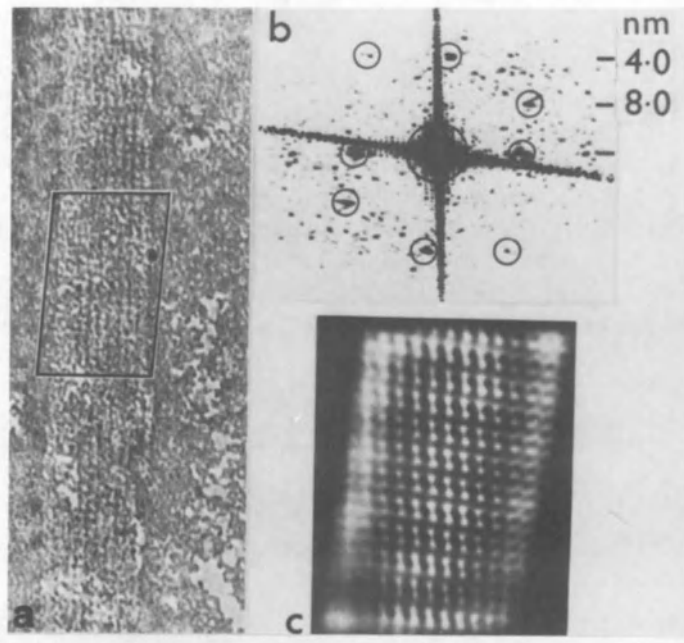
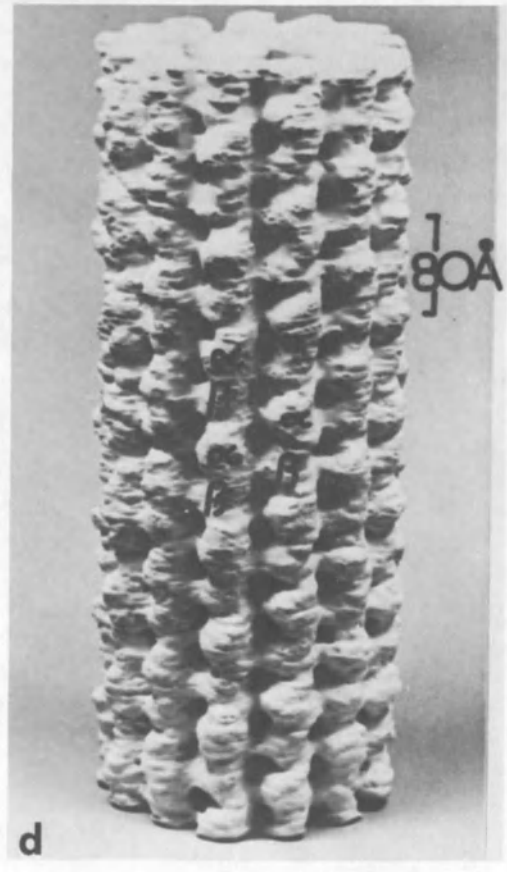


Figure 1. Bovine brain tubulin assembled into microtubules in the presence of 2 μg/ml taxol and negatively stained with uranyl acetate. Beaded protofilaments can be recognized in most parts of the microtubules. Bar, 0.1 μm. (Micrograph kindly provided by M. Schliwa.)

Figure 2. (a) Electron micrograph (taken by H. P. Erickson) of a negatively stained microtubule. The flagellar B tubule has formed a flat sheet on the carbon substrate, revealing its 10 protofilaments. ×300,000. (b) Diffraction pattern of the framed region of the tubule shown in a. (c) Optically filtered image of a, obtained from the reflections circled in b. (d) Three-dimensional model of a flagellar A tubule, reconstructed from electron microscopic images. The identity of α- and β-subunits may be the reverse of that indicated. (Reproduced with permission from Amos and Klug, 1974.)


a
b
nm
4·0
8·0
c


80Å
d

functional properties such as rate of subunit addition, stability, and interactions with each other or other cellular components. For example, MT growth seems to be biased by MT polarity. When flagellar or ciliary axonemes are incubated with brain tubulin under conditions that will not support self-assembly, their MTs will serve as seeds for the assembly of tubulin and elongate. However, the two ends of the axoneme will grow at different rates (Allen and Borisy, 1974; Dentler *et al.*, 1974; Binder *et al.*, 1975). Because the two ends of an axoneme are morphologically different, it is possible to associate the fast-growing or plus end with the distal end of the axoneme and the slow-growing or minus end with its proximal portion (Fig. 3a). Similar biased growth is also shown by basal bodies (Fig. 3b; Binder and Rosenbaum, 1978) and MT pieces (Rosenbaum *et al.*, 1975). The property to elongate at different rates from the two ends is a reliable criterion for determining MT polarity and has often been used to determine the polarity of MTs assembled by different organizing centers. However, a drawback of this assay is that it requires isolation and partial purification of the MT organizing center (MTOC) or the MTs whose polarity is to be determined.

In looking for alternative ways to determine MT polarity, an obvious goal was to develop an assay that could be used *in situ* and that was as convenient and reliable as that used for determining the polarity of actin filaments. There, heavy meromyosin or subfragment 1 is used to decorate cellular actin filaments in the absence of ATP with arrowhead complexes that are easily visualized in the electron microscope (Huxley, 1963; Ishikawa *et al.*, 1969). Because these arrowhead complexes are unidirectional, they reveal the polarity of filaments that are viewed laterally. So far one molecule has been identified that has the properties required from a polarity marker for MTs: *dynein*. Axonemal dynein is a large, asymmetrical molecular complex that carries the ATPase activity which powers ciliary and flagellar movement. Isolated from axonemes with relative ease, dynein will rebind to axonemal or cytoplasmic MTs. Due to its uniquely oriented binding, it reveals MT polarity in transverse and longitudinal sections (Figs. 4 and 5). Its usefulness as a polarity marker was first demonstrated by Haimo *et al.* (1979), and since then it has been used to identify MT polarity in several cell types (see Section 3).

Another method developed at about the same time (Heidemann *et al.*, 1980; Heidemann and McIntosh, 1980) takes advantage of polymerization conditions that induce polymorphic tubulin assemblies (Himes *et al.*, 1977; Burton and Himes, 1978; Mandelkow and Mandelkow, 1979). Compared to conventional MT assembly, these unusual conditions enhance lateral interactions between subunits or protofilaments producing curved, incomplete protofilament sheets attached to the side of the MT wall (Fig. 6). Factors that favor these lateral interactions are high salt (0.5 M 1,4-piperazinediethanesulfonic acid) and dimethyl sulfoxide (2.5%; Heidemann and Euteneuer, 1982). In lysed cells, tubulin will add to preexisting cellular MTs under these polymerization conditions and form hook-shaped appendages. Remarkably, the direction of curvature of these hooks is specified by the molecular polarity of the MT to which they are attached. Model experiments with cell systems in

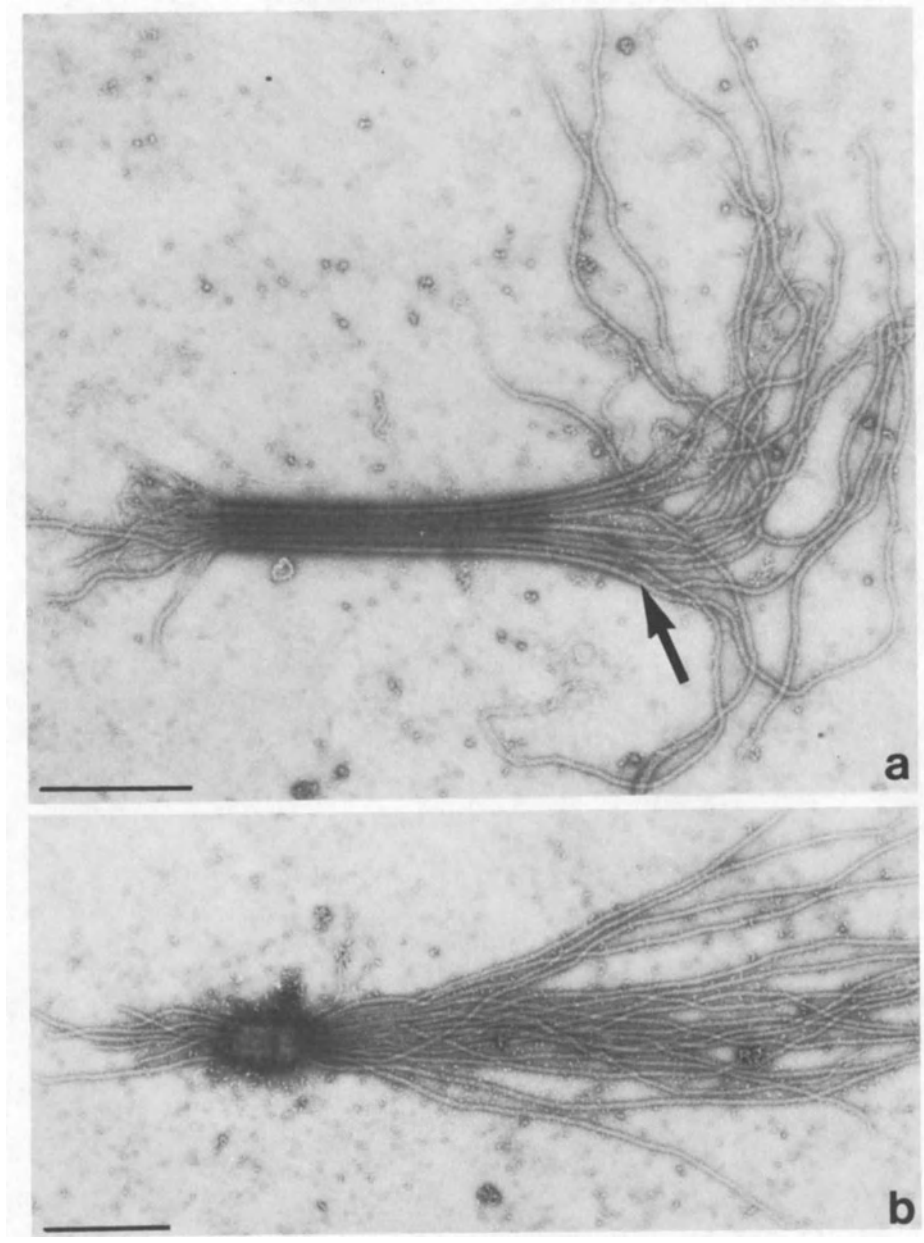


Figure 3. (a) Microtubule (MT) assembly of outer doublet tubulin onto *Chlamydomonas* axonemes. A and B subfibers as well as central pair MTs have grown. The rate of assembly is greater at the distal, splayed end of the axoneme (arrow) than at the proximal, compact end. (b) Outer doublet tubulin assembled onto a basal body from *Chlamydomonas*. As in axonemes, the growth is biased, Bar, 0.5 μm. (Reproduced with permission from Binder and Rosenbaum, 1978.)

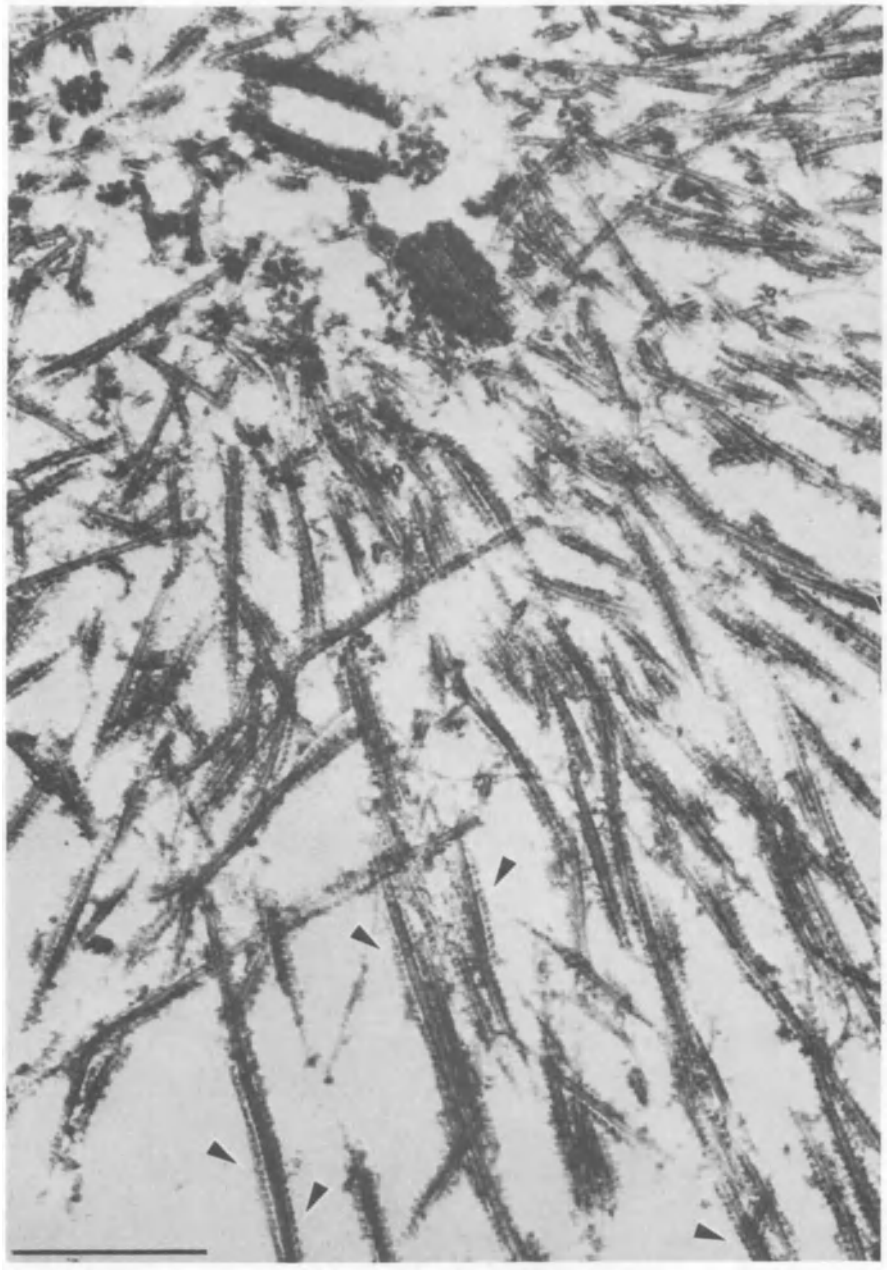


Figure 4. Longitudinal section through a polar area of a meiotic spindle of *Spisula solidissima* (surf clam). The spindle was incubated with dynein isolated from *Chlamydomonas* flagella. Most microtubules are decorated. Dynein arms tilt toward the pole (arrowheads), indicating that the plus ends of these spindle MTs are distal to the pole. Bar, 0.5 μm. (Reproduced with permission from Haimo, 1985.)

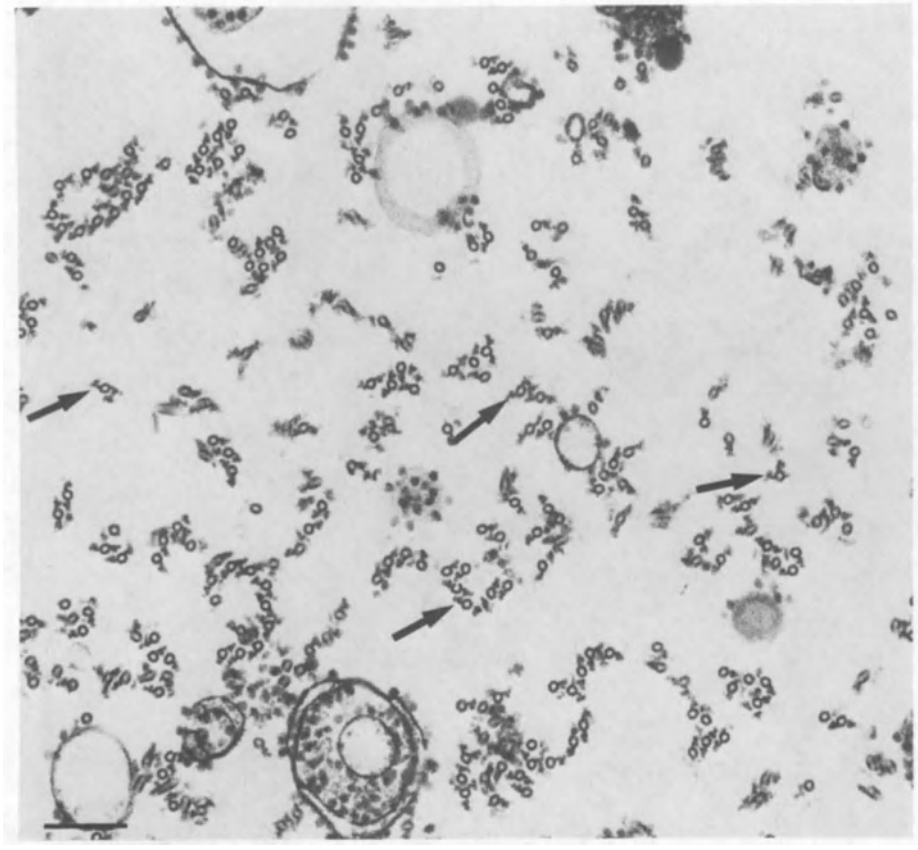


Figure 5. Cross-section through a *Strongylocentrotus purpuratus* spindle whose microtubules (MTs) are decorated with dynein. The arms curve clockwise (arrows), indicating that the plus ends of the MTs are away from the viewer. Bar, 0.2 μm. (Micrograph kindly provided by L. Haimo.)

which MT polarity is known (e.g., basal bodies or ciliary axonemes) allowed the determination of the relationship between hook curvature and polarity (Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1980). Looking along a MT from its plus to its minus end (in axonemes, for example, toward the basal body), the hooks curve clockwise (Fig. 6a). The hook decoration method is highly reliable (McIntosh and Euteneuer, 1984) and as convenient a procedure as dynein decoration.

Each of the methods used to determine MT polarity—optical diffraction, growth rate measurements, dynein or hook decoration—has its advantages and drawbacks. The optimal procedure has to be selected on the basis of the biological system studied. For example, growth rate determinations are best suited for MT systems that are easily isolated and can even be done at the light microscopic level without difficulty (Summers and Kirschner, 1979; Mitchison and Kirschner, 1984a,b, 1985a,b). Both decoration methods require that

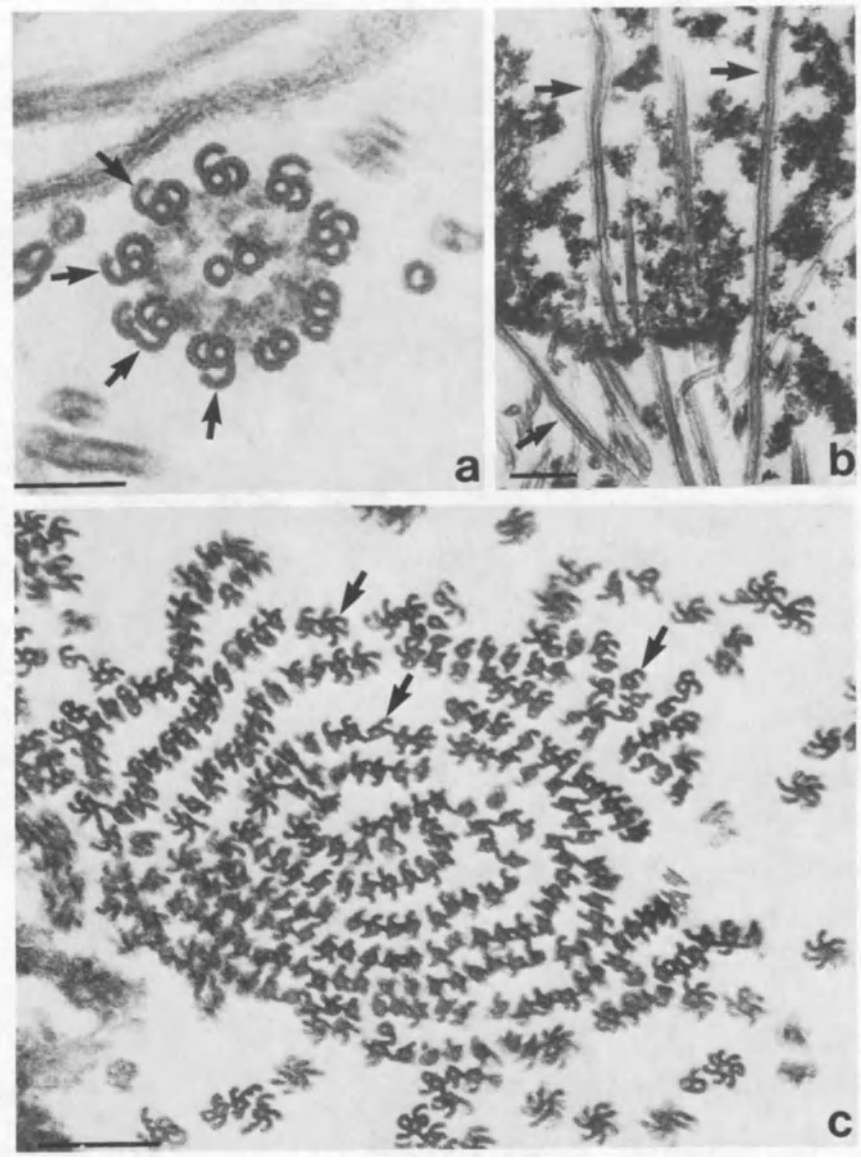


Figure 6. (a) Cross-section through an isolated, demembranated *Tetrahymena* cilium incubated with bovine brain tubulin under conditions that promote the formation of hooks. The arrangement of A and B subfibers in each doublet indicates that one is looking toward the base of the cilium (minus ends of the MTs). The eight hooks seen attached to A tubules are curving clockwise (arrows). Bar, 0.1 μm. (Reproduced with permission from Euteneuer and McIntosh, 1980.) (b) Longitudinal section through the spindle of a PtK_1 cell. The microtubules (MTs) are decorated with tubulin hooks revealed here as sheets running parallel to the dense spindle MTs (arrows). Bar, 0.2 μm. (c) Cross-section through an axopodium of *Actinosphaerium nucleofilum*. The MTs are decorated with tubulin hooks (arrows). All hooks curve clockwise, indicating that one is looking toward the minus end of the MTs. Bar, 0.2 μm.

dynein or tubulin be accessible to the MTs and therefore necessitate cell lysis; they require preparation for electron microscopy and, in many cases, skill in serial sectioning. A number of systems cannot be studied with any of the methods described; for example, fragile cells or complex tissues that either do not withstand the lysis procedure or cannot be lysed sufficiently to allow infiltration with dynein or tubulin. Visualization of MT polarity by optical diffraction and computer analysis is limited to isolated MTs (Amos and Klug, 1974; Amos, 1979).

3. MT Polarity and Spindle Function

The mitotic apparatus is an ephemeral structure assembled once in the cell cycle to mediate the separation of the genetic material. Its major structural components are MTs which are implicated in the movements of chromosomes and poles. Based on morphological criteria, the MTs forming the spindle can be subdivided into two classes: kinetochore MTs and nonkinetochore MTs. The latter are usually further subdivided into aster MTs, interzonal MTs, free MTs, and midbody or phragmoplast MTs (Fig. 7). These classes are arbitrary and therefore do not necessarily reflect the origin or the function of a particular MT. For example, kinetochore MTs may be former aster or former free MTs, and interzonal MTs may change class later and become midbody MTs.

The polarity of spindle MTs has been assigned an important role in a number of models for mitosis. Different mechanisms for chromosome and pole movement were proposed in which specific MT polarities are required for force production (Fig. 8; Subirana, 1968; McIntosh *et al.*,1969; Nicklas, 1971; Margolis *et al.*, 1978). However, it was not until 1979 that the polarity aspect of the models could be tested directly. Isolated centrosomes and chro-

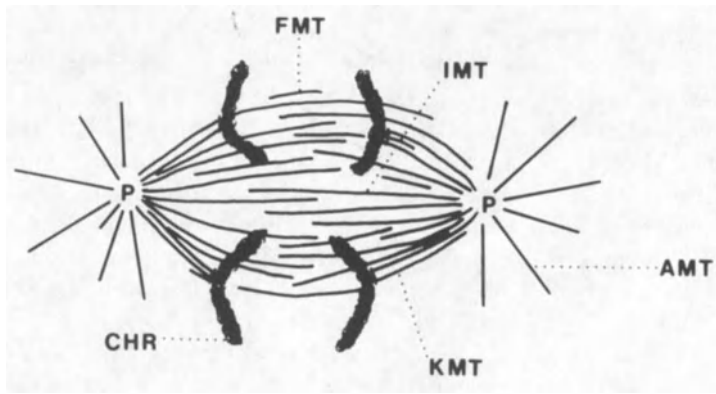


Figure 7. Schematic diagram of an animal spindle in anaphase, illustrating some of the classes of microtubules (MTs). KMT, kinetochore MTs; AMT, aster MTs; IMT, interzonal MTs; FMT, free MTs. P indicates position of poles. CHR, chromosomes.

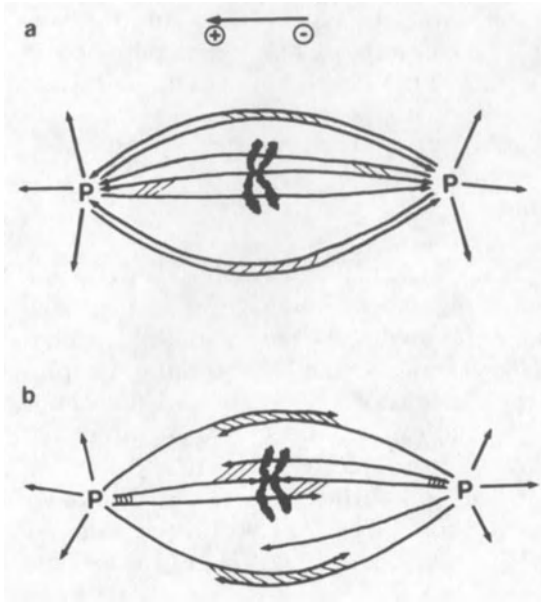


Figure 8. Schematic diagrams of models for mitosis that involve microtubule (MT) polarity. (a) MT polarity for the major spindle MT classes in the models of Subirana (1968) and McIntosh *et al.* (1969). In these models, spindle MTs of opposite polarity are thought to interact with each other via "bridge" molecules (shown as thin lines). These interactions were proposed to provide the mechanochemical force necessary to move chromosomes and poles. The model of Nicklas (1971) is a modification of the McIntosh *et al.*(1969) model. (Redrawn from McIntosh *et al.*, 1969, modified.) (b) MT polarity in the model of Margolis *et al.* (1978). This model is based on the phenomenon of treadmilling, observed for MTs at steady state *in vitro*. It also proposes an interaction between MTs of opposite polarity but, in addition, assumes that treadmilling of kinetochore and interzonal MTs takes place in the equatorial region during prometaphase, metaphase, and—for interzonal MTs only—anaphase. (Redrawn from Margolis *et al.*, 1978.)

mosomes were shown to act as organizing centers for the polymerization of MTs *in vitro* (Telzer *et al.*, 1975; McGill and Brinkley, 1975; Gould and Borisy, 1977, 1978), although the nucleating capacity of kinetochores was considerably weaker than that of centrosomes. Growth rate measurements of the MTs nucleated by these structures suggested that all MTs were growing at a "fast rate," i.e., the growth rate of the plus end of cilia or flagella (Summers and Kirschner, 1979; Bergen and Borisy, 1980). Although it was not clear from the experiments where the subunits were adding, it was assumed that it was distal. These observations supported the concept, incorporated in several models, that kinetochores are true MTOCs (Subirana, 1968; McIntosh *et al.*, 1969; Dietz, 1972; Inoue and Sato, 1967; Margolis *et al.*, 1978). Furthermore, kinetochores were demonstrated to initiate the growth of spindle MTs *in vivo* in cells recovering from treatment with colcemid or nocodazole (Witt *et al.*, 1980; DeBrabander *et al.*, 1980, 1981). In these experiments the MT depolymerizing drugs were used to arrest mitosis and accumulate cells free of MTs in order to visualize MTOC activity better.

The observations on MT polarity made by *in vitro* growth measurements were only partially confirmed when the polarity of spindle MTs was studied *in situ* using dynein or hook decoration assays (Heidemann, 1980; Telzer and Haimo, 1981; Euteneuer and McIntosh, 1981a). Whereas the polarity of polar MTs was the same as that determined for aster MTs by growth rate mea-

surements, the polarity of kinetochore MTs was found to be opposite that reported earlier. Hence the majority of the MTs in a given half-spindle have the same polarity (Figs. 4, 5, 9, and 10). These findings were established for PtK_1 cells, CHO cells, HeLa cells, BSC cells, surf clam spindles, and *Haemanthus* endosperm cells (Telzer and Haimo, 1981; Euteneuer and McIntosh,

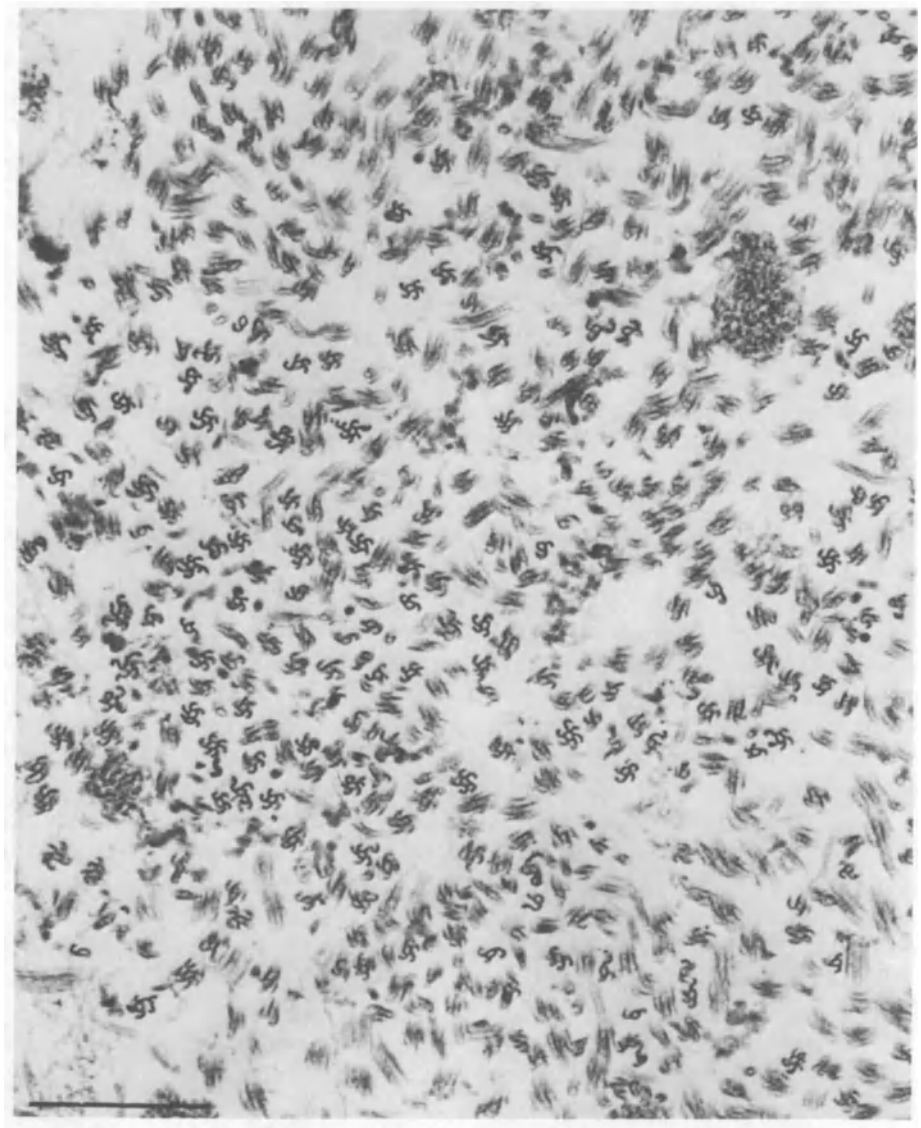


Figure 9. Cross-section through an early anaphase spindle of a PtK_1 cell. The spindle microtubules (MTs) are decorated with tubulin hooks. The section is taken between the chromatids and the second pole. The majority of MTs are decorated with clockwise-curving hooks, indicating that their minus ends are located in the polar region. Bar, 0.5 μm.

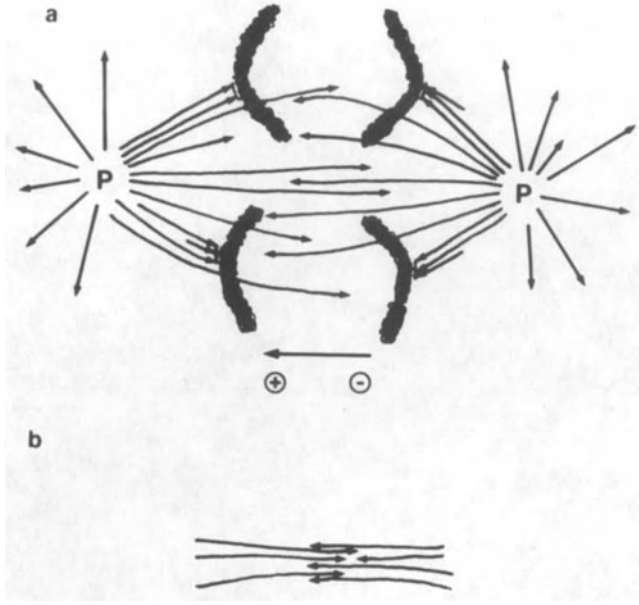


Figure 10. Schematic drawing illustrating the polarity of spindle microtubules (MTs) (a) and midbody MTs (b). All MTs in a half-spindle are of uniform polarity. P, poles.

1981a; Haimo and Telzer, 1982; Euteneuer *et al.*, 1982; Euteneuer, 1983; Telzer and Quinlan, 1984).

The observations on kinetochore MT polarity are not easily compatible with the idea that kinetochores act as nucleating sites during spindle formation. Because kinetochore MTs have their minus or slow-growing ends distal to the kinetochores, they either grow slowly compared to polar MTs or incorporate subunits at a fast rate at the kinetochore (Margolis *et al.*, 1978). In either case, the mode of incorporation of subunits would be significantly different from that established for centrosome-initiated MTs, which add subunits distal to the organizing site (Heidemann *et al.*, 1980) and grow at a fast rate. An intriguing alternative explanation for the observed polarity is that kinetochore MTs are former aster MTs that were "captured" or recruited by the kinetochore during spindle formation (Pickett-Heaps and Tippit, 1978; Rieder and Borisy, 1981; Pickett-Heaps *et al.*, 1982). This model would explain the orientation of kinetochore MTs and would solve the problem of how the connection between kinetochores and poles is established and maintained. Indeed, several light and electron microscopic observations of prometaphase and metaphase spindles are more compatible with a capture mechanism than with other modes of kinetochore MT formation. For example, during prometaphase, only kinetochores that move toward, or face, a pole appear to possess kinetochore MTs (Molè-Bajer *et al.*, 1975; Roos, 1976; Rieder and Borisy, 1981), and the absence of centrosomes from one or both poles in sea urchin egg spindles interferes with the formation of proper kinetochore MTs

(Paweletz and Mazia, 1979; Mazia *et al.*, 1981; Sluder and Rieder, 1985). From these and other observations (Ritter *et al.*, 1978; Nicklas *et al.*, 1979; Schibler and Pickett-Heaps, 1980; Tippit *et al.*, 1980; Rieder and Nowogrodzki, 1983), the concept has emerged that the majority of the kinetochore MTs in normal untreated cells are former aster MTs (Pickett-Heaps *et al.*, 1982, 1984; for an excellent detailed discussion of this issue, see Rieder, 1982).

Recently, capture of MTs by kinetochores was demonstrated *in vitro* (Mitchison and Kirschner, 1985b). When incubated with asters induced by isolated centrosomes, isolated metaphase chromosomes will attach to the asters via MTs. That these connections between isolated centrosomes and kinetochores are functionally significant was suggested by experiments in which these aster–chromosome complexes were subjected to depolymerizing conditions by lowering the tubulin concentration. Just like kinetochore MTs *in vivo*, the MTs that connect poles and kinetochores were more stable than the other MTs present in the preparation. In addition, kinetochores are able to "move" along captured free MTs upon the addition of ATP. However, this movement involves the incorporation of new subunits at the kinetochore, thus leading to elongation of the kinetochore fiber. In addition to the new observations that kinetochores can capture MTs *in vitro*, Mitchison and Kirschner (1985a) also confirmed the previous finding that under appropriate conditions, kinetochores initiate the growth of MTs. They are, however, less efficient in nucleating MTs than isolated centrosomes and, in contrast to those, initiate MTs of both polarities. Elegant experiments using biotinylated tubulin and immunofluorescence microscopy showed that subunit addition occurred distally on the MTs nucleated by the kinetochore.

Whether kinetochores can nucleate MTs *in vivo* in cells that have not been drug-treated cannot be decided on the basis of the available evidence. Caution is advised in interpreting experiments involving drug treatment; the *in vitro* data on kinetochore MTs are a good example. Thus some experiments suggest that all MTs have their fast-growing ends distal (Summers and Kirschner, 1979; Bergen *et al.*, 1980); others indicate that they have their plus ends proximal, at the kinetochore (Telzer and Quinlan, 1984); and there is evidence that kinetochores initiate MTs of both polarities (Mitchison and Kirschner, 1985a). Some of these discrepancies are probably directly related to the use of drugs like colcemid and nocodazole, which are used to accumulate mitotic cells for the isolation of chromosomes free of cellular MTs (Pickett-Heaps *et al.*, 1982). Another source of uncertainty is that tubulin (not MTs) may be present at the kinetochores of chromosomes isolated from these cells (Pepper and Brinkley, 1977; Mitchison and Kirschner, 1985a), and that kinetochores have an affinity for monomeric tubulin. The ability of the kinetochore to bind tubulin can affect nucleation assays and hence growth rate determinations. However, it is difficult to assess whether the binding of either cellular or exogenous tubulin can explain the observed discrepancies.

With respect to nonkinetochore spindle MTs, both the hook and dynein decoration procedures suggest that the spindle is composed of two sets of MTs with opposite polarity that interdigitate in the interzone (Fig. 10b; Eu-

teneuer and McIntosh, 1980; Telzer and Haimo, 1981; Euteneuer *et al.*, 1982; Euteneuer, 1983). As shown by serial thin-section reconstruction and polarity analyses in anaphase and telophase cells, the overlap decreases as the spindle elongates (Manton *et al.*, 1970; Paweletz, 1967; McIntosh *et al.*, 1975; Tippit and Pickett-Heaps, 1977; McDonald *et al.*, 1977; Tippit *et al.*, 1978; McDonald *et al.*, 1979; Euteneuer *et al.*, 1982; Euteneuer, 1983; McDonald and Euteneuer, 1983). Spindle elongation is proposed to be achieved, at least in part, by mechanochemical interaction between antiparallel MTs mediated by dyneinlike force-generating molecules (McIntosh *et al.*, 1969; Nicklas, 1971; Margolis *et al.*, 1978). The strongest evidence in support of such a model is derived from detailed studies of the structure of diatom spindles (McDonald *et al.*, 1977, 1979; Tippit *et al.*, 1978; McIntosh *et al.*, 1979) and from experiments on spindle elongation in lysed-cell models or isolated spindles: (1) In lysed-cell models of PtK_1 cells, Cande (1982a,b) showed that spindle elongation is inhibited by vanadate or erythro-9-3-(2-hydroxynonyl)adenine, (EHNA), substances that interfere with the action of dyneinlike ATPases (Gibbons *et al.*, 1978; Bouchard *et al.*, 1981). (2) In isolated spindles of the diatom *Stephanopyxis*, spindle elongation can be initiated *in vitro* by the addition of ATP (Cande and McDonald, 1985). (3) In another diatom, *Hantzschia*, ultraviolet irradiation of the overlap region of the two half-spindles will destroy part of the MTs and will interfere with spindle elongation (Leslie and Pickett-Heaps, 1984). These observations argue that the mechanism for spindle elongation involves an interaction of MTs of opposite polarity in the interzone. However, several uncertainties need to be eliminated before this mechanism can be considered likely: (1) Except for some diatoms, no fine structural evidence has been obtained for an extensive overlap of interzonal spindle MTs during prometaphase or metaphase (Molè-Bajer, 1975; Rattner and Berns, 1976; Euteneuer and Bereiter-Hahn, 1976; McDonald *et al.*, 1977; Tippit and Pickett-Heaps, 1977). (2) Observations on aster movement during prometaphase in newt spindles and dividing spermatocytes indicate that asters can move about the cell independently of one another and therefore do not depend on a connection via MTs for their motility (Dietz, 1966; Bajer, 1982). (3) Severing the MTs in the interzone during anaphase will not prevent spindle elongation in PtK_1 cells, although it seems to interfere with the interaction of the MTs in that area (Kronebusch and Borisy, 1982). (4) There are many reports in the literature of cells (predominantly lower eukaryotes) that show a greater than twofold spindle elongation (Cleveland, 1954; Inoue and Ritter, 1978; Aist and Berns, 1981; Roos and Camenzind, 1981; King *et al.*, 1982). A twofold elongation is the maximum elongation that can be achieved by two sets of completely overlapping MTs. To account for additional elongation, one has to postulate MT assembly concomitant to sliding, or a telescoping action of the sliding MTs.

Observations 1 and 4 can be explained by extending the sliding mechanism and incorporating MT assembly or a telescoping action. Observations 2 and 3 argue for a model where the site of force production is not located in the interzone. If this is true, then the interdigitation and interaction of MTs in

the interzone during anaphase and telophase might actually operate as a braking rather than a force-producing mechanism. Additional evidence is needed to decide between the two ideas.

The elucidation of spindle MT polarity has provided some insights into the details of spindle structure. In all cell types studied so far, kinetochore and nonkinetochore MTs in a half-spindle have the same polarity (Figs. 4, 5, 9, and 10). These studies have helped to evaluate several models of mitosis; some of these can now be ruled out based on these results since they predicted incorrect MT polarity arrangements (Subirana, 1968; McIntosh *et al.*, 1969). The true importance of MT polarity for spindle function will be established when more is known about the actual mechanisms of force production.

4. MT Polarity and Intracellular Motility

Another process in which MTs are believed to be intimately involved is the form of intracellular organelle transport known as saltatory particle movement (Rebhun, 1972). Saltatory transport is thought to be MT-dependent, based on the following criteria: (1) Drugs that depolymerize MTs *in vivo* interfere with movement, whereas drugs that affect actin do not (Freed and Lebowitz, 1970; Murphy and Tilney, 1974; Wang and Goldman, 1978; Obika *et al.*, 1978; Schliwa *et al.*, 1979, 1981; Beckerle and Porter, 1983; Herman and Albertini, 1984; reviews by Grafstein and Forman, 1980; Schliwa, 1984). (2) The pathways of particles or granules follow the arrangement of cellular MTs (Freed and Lebowitz, 1970; Bikle *et al.*,1966; Travis *et al.*, 1983; Koonce and Schliwa, 1985). (3) Close associations can often be seen between MTs and granules (Smith, 1971; Murphy and Tilney, 1974; Hirokawa, 1982; Kachar *et al.*, 1984). (4) Inhibitors of dynein (vanadate and EHNA) interfere with saltatory movements (Beckerle and Porter, 1982; Stearns and Ochs, 1982; Clark and Rosenbaum, 1982; Forman, 1982a; Forman *et al.*, 1983a,b). (5) Vesicular organelles or polystyrene beads move in close association with isolated single MTs (Schnapp *et al.*, 1985; Vale *et al.*, 1985).

It was speculated that two structurally and/or functionally different sets of MTs exist, each correlated with one direction of movement. In an attempt to answer that question, the hook polarity assay was applied to a number of systems that show elaborate intracellular transport: melanophores (Euteneuer and McIntosh, 1981b; McNiven *et al.*, 1984; Fig. 11a), axons (Heidemann *et al.*, 1981; Burton and Paige, 1981; Filliatreau and Di Giamberardino, 1981), heliozoan axopodia (Euteneuer and McIntosh, 1981b; Fig. 6c), nutritive tubes in insect ovarioles (Stebbings and Hunt, 1983), and sensory neurons of bullfrogs (Burton and Bickford, 1982). In all cases MT polarity was uniform, with the plus ends distal to the organizing center or cell body. These findings eliminate the possibility that bidirectional transport requires MTs with opposite polarity, but they do not rule out the possibility that different MT accessory proteins are associated with different sets of MTs. However, this

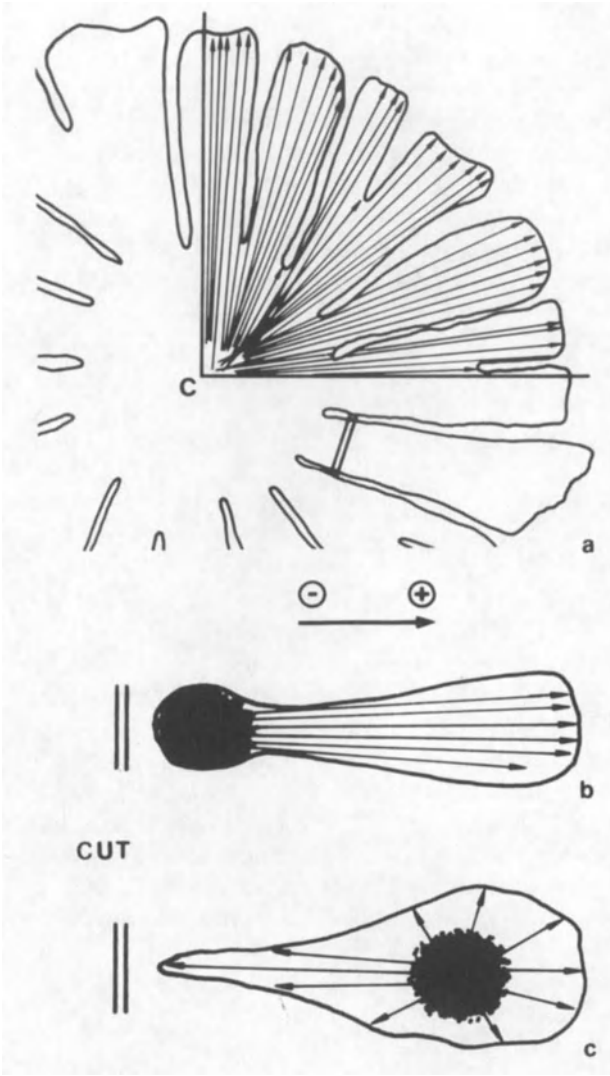


Figure 11. Schematic drawing of microtubule (MT) polarity in melanophores (a) and in severed melanophore arms (b, c). During the first 2 hr after severing, melanosomes will aggregate to the formerly proximal region of the arm (b). After 2–4 hr, melanosomes will aggregate to the geometrical center of the arm; the polarity of the MTs has changed accordingly (c).

possibility now appears unlikely in light of the observation that bidirectional transport can occur along single MTs (Hayden and Allen, 1984; Koonce and Schliwa, 1985). Therefore, the cell specifies directionality of organelle movement not by providing separate "tracks" for separate directions, but by a molecular mechanism that operates with a single MT as its substrate. The nature of that directionality marker is presently unknown.

Although these results seem to suggest that MT polarity is of no importance for intracellular saltatory movement, cells seem to prefer a distinct polarity for their MTs in order to move organelles back and forth. In teleost melanophores, all MTs have the same polarity with respect to the MTOC located close to the cell center (Fig. 11a; Euteneuer and McIntosh, 1981b; McNiven *et al.*, 1984). McNiven *et al.* (1984) discovered that cell fragments can

alter the arrangement of their MTs, including their polarity, after experimental manipulation. Immediately after a cell process is cut off from a melanophore with its pigment dispersed, the pigment in this cell fragment will aggregate toward the former proximal end upon stimulation (Fig. 11b). However, if the severed arms are incubated for 2–4 hr before stimulated to aggregate, the pigment will move toward the center of the arm rather than to its edge. When the polarity of the MTs in that arm is determined, the majority of the MTs have their plus ends distal to the new "cell center" (Fig. 11c). Furthermore, if the preparation is treated with taxol, a drug that stabilizes MTs, after the arm has been detached from the melanophore, the direction of pigment aggregation and the polarity of the MTs initially remains unchanged. Only after 8 hr in taxol do the pigment granules aggregate toward the new center, and by that time MT polarity in the proximal half of the arm has reversed (McNiven and Porter, 1984). These observations suggest that the direction of pigment transport is linked to MT polarity in ways other than through specification of directionality of movement (McNiven *et al.*, 1984). In melanophores the minus ends of the MTs are usually embedded in the MTOC area, but they will be exposed upon severing of the arm. It is not known whether MTs will easily depolymerize from a free minus end *in vivo* (Borisy and Kronebusch, 1984). As a rule MTs in melanophores are comparatively stable and treatment with even high concentrations of antimicrotubular agents will not depolymerize all MTs (Schliwa and Bereiter-Hahn, 1973; Murphy and Tilney, 1974). The observed rearrangement after 2–4 hr of incubation in medium may reflect the normal turnover time for melanophore MTs. The results on MT polarity in melanophores do not provide direct clues as to the mechanism of particle movement but suggest a close correlation between MT polarity and movement. Additional questions that are raised by these recent experiments are: Does the new center determine the polarity of the MTs or do the MTs with their new polarity specify the location of the new center? What is the exact sequence of events that lead to the new MT arrangement? The events seem to be coupled but may not occur simultaneously. Further studies should provide some answers.

Of the other systems in which the relationship of MT polarity and directionality of organelle movements were studied, axons and heliozoan axopodia exhibit bidirectional transport along MTs of one polarity (plus ends distal to the cell center), while in nutritive tubes neither the cellular origin of the MTs is known, nor has it been established whether transport is bidirectional. A consistent correlation between transport and MT polarity is therefore not unequivocally established. In heliozoan axopodia, for example, it is likely that MTs provide only the framework for transport and are not directly involved in moving organelles. In an elegant experiment, Edds (1975) supplied an *Actinosphaerium* with an artificial axopodial rod. He pushed a glass needle through the layer of cortical cytoplasm and observed that mitochondria and granules were transported along these artificial structures just as in normal axopodia. Organelle movements even persisted in the presence of colchicine concentrations that induced retraction of all endogenous axopodia.

There is ample evidence that MTs are required for fast transport of

organelles in axons (Forman, 1982b; reviewed by Grafstein and Forman, 1980). While MTs in axons are reported to have a uniform polarity, it is not clear whether uniform polarity is an absolute requirement to maintain normal transport. Under certain experimental conditions, MT polarity is transiently altered. In axons of cat neurons, the percentage of MTs with opposite polarity was considerably higher after 2 hr of recovery from cold treatment than in untreated controls (Heidemann *et al.*, 1984). Interestingly, the percentage of MTs with correct polarity correlates with the number of cold stable MTs in the cold control preparations. Because these experiments were performed on excised segments of axons, they demonstrate that MT polarity can be restored in the absence of a morphologically defined MTOC. The effect of altered MT polarity on the transport of particles was not investigated.

Essentially all cells seem to control the organization of their MT cytoskeleton with one or more MTOCs. The activity of an MTOC probably specifies spatial distribution of MTs and also their polarity. Uniform MT polarity can therefore be explained by assuming that all MTs were initiated by a centrally located organizing center. Although different polarities are not required for bidirectional movement, the cell seems to prefer a defined MT polarity with respect to a cellular reference point. This preference suggests that MT polarity and direction of saltatory organelle movement are closely linked.

ACKNOWLEDGMENTS. I am grateful to Dr. M. Schliwa and S. Spath for helpful suggestions and critical reading of the manuscript. I also thank Drs. Linda Amos, Lester Binder, Leah Haimo, and Manfred Schliwa for kindly providing illustrative material.

References

Aist, J. R., and Berns, M. W., 1981, Mechanics of chromosome separation during mitosis in *Fusarium* (fungi imperfecti): New evidence from ultrastructural and laser microbeam experiments, *J. Cell Biol.* **91**:446–458.

Allen, C., and Borisy, G. G., 1974, Structural polarity and directional growth of microtubules of *Chlamydomonas* flagella, *J. Mol. Biol.* **90**:381–402.

Amos, L. A., 1979, Structure of microtubules, in: *Microtubules* (K. Roberts and J. S. Hyams, eds.), pp. 1–64, Academic Press, London.

Amos, L. A., and Klug, A., 1974, Arrangement of subunits in flagellar microtubules, *J. Cell Sci.* **14**:523–549.

Bajer, A. S., 1982, Functional autonomy of monopolar spindle and evidence for oscillatory movement in mitosis, *J. Cell Biol.* **93**:33–48.

Beckerle, M., and Porter, K. R., 1982, Inhibitors of dynein activity block intracellular transport in erythrocytes, *Nature* **295**:701–703.

Beckerle, M. C., and Porter, K. R., 1983, Analysis of the role of microtubules and actin in erythrophore intracellular motility, *J. Cell Biol.* **96**:354–362.

Bergen, L. G., and Borisy, G. G., 1980, Head-to-tail polymerization of microtubules *in vitro*, *J. Cell. Biol.* **84**:141–150.

Bergen, L. G., Kuriyama, R., and Borisy, G. G., 1980, Polarity of microtubules nucleated by

centrosomes and chromosomes of Chinese hamster ovary cells *in vitro*, *J. Cell Biol.* **84:**151–159.

Bikle, D., Tilney, L. G., and Porter, K. R., 1966, Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus*, *Protoplasma* **61:**322–345.

Binder, L. I., and Rosenbaum, J. L., 1978, The *in vitro* assembly of flagellar outer doublet tubulin, *J. Cell Biol.* **79:**500–515.

Binder, L. I., Dentler, W. L., and Rosenbaum, J. L., 1975, Assembly of chick brain tubulin onto flagellar axonemes of Chlamydomonas and sea urchin sperm, *Proc. Natl. Acad. Sci. U.S.A.* **72:**1122–1126.

Borisy, G. G., and Kronebusch, P., 1984, Microtubule dynamics *in vivo*, in: *Molecular Biology of the Cytoskeleton* (G. G. Borisy, D. Cleveland, and D. Murphy, eds.), p. la, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Bouchard, P., Penningroth, S. M., Cheung, A., Gagnon, C., and Bardin, C. W., 1981, Erythro-9-3-(2-hydroxynonyl)adenine is an inhibitor of sperm motility that blocks dynein ATPase and protein carboxymethylase activities, *Proc. Natl. Acad. Sci. U.S.A.* **78:**1033–1036.

Burton, P. R., and Bickford, M. E., 1982, Microtubules and other cytoskeletal elements of frog olfactory neurons and some possible associations with axonal transport, *J. Cell Biol.* **95:**333a.

Burton, P. R., and Himes, R. H., 1978, Electron microscope studies of pH effects on assembly of tubulin free of associated proteins, *J. Cell Biol.* **77:**120–133.

Burton, P. R., and Paige, J. L., 1981, Polarity of axoplasmic microtubules in the olfactory nerve of the frog, *Proc. Natl. Acad. Sci. U.S.A.* **78:**3269–3273.

Burton, P. R., Hinkley, R. E., and Pierson, G. B., 1975, Tannic acid-stained microtubules with 12, 13, and 15 protofilaments, *J. Cell Biol.* **65:**227–233.

Cande, W. Z., 1982a, Inhibition of spindle elongation in permeabilized mitotic cells by erythro--9-[3-(2-hydroxynonyl)]adenine, *Nature* **295:**700–701.

Cande, W. Z., 1982b, Nucleotide requirements for anaphase chromosome movements in permeabilized mitotic cells. Anaphase B but not anaphase A requires ATP, *Cell* **28:**15–22.

Cande, W. Z., and McDonald, K. L., 1985, *In vitro* reactivation of anaphase spindle elongation using isolated diatom spindles, *Nature* **316:**168–170.

Chalfie, M., and Thomson, J. N., 1982, Structural and functional diversity in the neuronal microtubules of *Caenorhabditis elegans*, *J. Cell Biol.* **93:**15–23.

Clark, T. G., and Rosenbaum, J. L., 1982, Pigment particle translocation in detergent-permeabilized melanophores of *Fundulus heteroclitus*, *Proc. Natl. Acad. Sci. U.S.A.* **79:**4655–4659.

Cleveland, L. R., 1954, Hormon-induced sexual cycles of flagellates. XII. Meiosis in *Barbulanympha* following fertilization, autogamy, and endomitosis, *J. Morphol.* **95:**557–619.

DeBrabander, M., and DeMey, J., 1980, *Microtubules and Microtubule Inhibitors*, Elsevier, Amsterdam.

DeBrabander, M., Geuens, G., Nuydens, R., Willebrords, R., and DeMey, J., 1980, The microtubule nucleating and organizing activity of kinetochores and centrosomes in living PtK_2-cells, in: *2nd International Symposium on Microtubules and Microtubule Inhibitors* (M. DeBrabander and J. DeMey, eds.), pp. 255–268, Elsevier, Amsterdam.

DeBrabander, M., Geuens, G., deMey, J., and Jonian, M., 1981, Nucleated assembly of mitotic microtubules in living PtK_2 cells after release from nocodazole treatment, *Cell Motil.* **1:**469–483.

Dentler, W. L., Granett, S., Witman, G. B., and Rosenbaum, J. L., 1974, Directionality of brain microtubule assembly *in vitro*, *Proc. Natl. Acad. Sci. U.S.A.* **71:**1710–1714.

Dietz, R., 1966, The dispensability of the centrioles in the spermatocyte divisions of *Pales ferruginea (Nematocera)*, *Chromosomes Today* **1:**161–166.

Dietz, R., 1972, Die Assembly-Hypothese der Chromosomenbewegung und die Veränderungen der Spindellänge während der Anaphase I in Spermatocyten von Pales ferruginea (Tipulidae, Diptera), *Chromosoma* **38:**11–76.

Dustin, P., 1984, *Microtubules*, Springer, Berlin.

Edds, K. T., 1975, Motility in Echinosphaerium I. An analysis of particle motions in the axopodia and a direct test of the involvement of the axoneme, *J. Cell Biol.* **66:**145–155.

Euteneuer, U., 1983, Microtubule polarity and spindle function, *Proc. EMSA* **41**:540–543.
Euteneuer, U., and Bereiter-Hahn, J., 1976, Ring-like prometaphase chromosome arrangement in flat amphibian cells, *J. Cell Biol.* **70**:12a.
Euteneuer, U., and McIntosh, J. R., 1980, Polarity of midbody and phragmoplast microtubules, *J. Cell Biol.* **87**:509–515.
Euteneuer, U., and McIntosh, J. R., 1981a, Structural polarity of kinetochore microtubules in PtK_1 cells, *J. Cell Biol.* **89**:338–345.
Euteneuer, U., and McIntosh, J. R., 1981b, Polarity of some motility-related microtubules, *Proc. Natl. Acad. Sci. U.S.A.* **78**:372–376.
Euteneuer, U., Jackson, W. T., and McIntosh, J. R., 1982, Polarity of spindle microtubules in Haemanthus endosperm, *J. Cell Biol.* **94**:644–653.
Everhart, L. P., 1971, Heterogeneity of microtubule proteins from *Tetrahymena* cilia, *J. Mol. Biol.* **61**:745–748.
Feit, H., Slusarek, L., and Shelanski, M. L., 1971, Heterogeneity of tubulin subunits, *Proc. Natl. Acad. Sci. U.S.A.* **68**:2028–2031.
Filliatreau, G., and Di Giamberardino, L., 1981, Microtubule polarity in myelinated axons as studied after decoration with tubulin, *Biol. Cell* **42**:69–72.
Fine, R. E., 1971, Heterogeneity of tubulin, *Nature New Biol.* **233**:283–284.
Forman, D. S., 1982a, Vanadate inhibits saltatory organelle movement in a permeabilized cell model, *Exp. Cell Res.* **141**:139–147.
Forman, D. S., 1982b, Saltatory organelle movement and the mechanism of fast axonal transport, in: *Axoplasmic Transport* (D. G. Weiss, ed.), pp. 234–240, Springer, Berlin.
Forman, D. S., Brown, K. J., and Livengood, D. R., 1983a, Fast axonal transport in permeabilized lobster giant axons is inhibited by vanadate, *J. Neurosci.* **3**:1279–1288.
Forman, D. S., Brown, K. J., and Promersberger, M. E., 1983b, Selective inhibition of retrograde axonal transport by erythro-9-3-(2-hydroxynonyl)adenine, *Brain Res.* **272**:194–197.
Freed, J. J., and Lebowitz, M. M., 1970, The association of a class of saltatory movements with microtubules in cultured cells, *J. Cell Biol.* **45**:334–354.
Gibbons, I. R., Cosson, M. P., Evans, J. A., Gibbons, B. H., Houck, B., and Martinson, K., 1978, Potent inhibition of dynein adenosine triphosphatase and the motility of cilia and sperm flagella by vanadate, *Proc. Natl. Acad. Sci. U.S.A.* **75**:2220–2224.
Gould, R. R., and Borisy, G. G., 1977, The pericentriolar material in Chinese hamster cells nucleates microtubule formation, *J. Cell Biol.* **73**:601–615.
Gould, R. R., and Borisy, G. G., 1978, Quantitative initiation of microtubule assembly by chromosomes from Chinese hamster ovary cells, *Exp. Cell Res.* **113**:369–374.
Grafstein, B., and Forman, D. S., 1980, Intracellular transport in neurons, *Physiol. Rev.* **60**:1167–1283.
Gunning, B. E. S., and Hardham, A. R., 1982, Microtubules, *Ann. Rev. Plant Physiol.* **33**:651–698.
Haimo, L. T., 1985, Microtubule polarity in taxol-treated isolated spindles, *Can. J. Biochem. Cell Biol.* **63**:519–532.
Haimo, L. T., and Telzer, B. R., 1982, Dynein–microtubule interactions: ATP-sensitive dynein binding and the structural polarity of mitotic microtubules, *Cold Spring Harbor Symp. Quant. Biol.* **46**:207–217.
Haimo, L. T., Telzer, B. R., and Rosenbaum, J. L., 1979, Dynein binds to and crossbridges cytoplasmic microtubules, *Proc. Natl. Acad. Sci. U.S.A.* **76**:5759–5763.
Hayden, J. H., and Allen, R. D., 1984, Detection of single microtubules in living cells: Particle transport can occur in both directions along the same microtubule, *J. Cell Biol.* **99**:1785–1793.
Heidemann, S. R., 1980, Visualization of microtubule polarity, in: Microtubules and microtubule inhibitors 1980 (M. DeBrabander and J. DeMey, eds.), pp. 341–355, Elsevier, Amsterdam.
Heidemann, S. R., and Euteneuer, U., 1982, Microtubule polarity determination based on conditions for tubulin assembly *in vitro*, *Methods Cell Biol.* **24**:207–216.
Heidemann, S. R., and McIntosh, J. R., 1980, Visualization of the structural polarity of microtubules, *Nature* **286**:517–519.

Heidemann, S. R., Zieve, G. W., and McIntosh, J. R., 1980, Evidence for microtubule subunit addition to the distal end of mitotic structures *in vitro*, *J. Cell Biol.* **87**:152–159.

Heidemann, S. R., Landers, J. M., and Hamborg, M. A., 1981, Polarity orientation of axonal microtubules, *J. Cell Biol.* **91**:661–665.

Heidemann, S. R., Hamborg, M. A., Thomas, S. J., Song, B., Lindley, S., and Chu, D., 1984, Spatial organization of axonal microtubules, *J. Cell Biol.* **99**:1289–1295.

Herman, B., and Albertini, D. F., 1984, A time-lapse video image intensification analysis of cytoplasmic movements during endosome translocation, *J. Cell Biol.* **98**:565–576.

Himes, R. H., Burton, P. R., and Gaito, J. M., 1977, Dimethyl sulfoxide-induced self-assembly of tubulin lacking associated proteins, *J. Biol. Chem.* **252**:6222–6228.

Hirokawa, N., 1982, Cross-linker system between neurofilaments, microtubules, and membraneous organelles in frog axons revealed by the quick-freeze, deep-etching method, *J. Cell Biol.* **94**:129–142.

Huxley, H. E., 1963, Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle, *J. Mol. Biol.* **7**:281–308.

Inoue, S., and Ritter, H., 1978, Mitosis in Barbulanympha. II. Dynamics of a two-stage anaphase, nuclear morphogenesis, and cytokinesis, *J. Cell Biol.* **77**:655–684.

Inoue, S., and Sato, H., 1967, Cell motility by labile association of molecules, *J. Gen. Physiol.* **50**:259–292.

Ishikawa, H., Bischoff, R., and Holtzer, H., 1969, The formation of arrowhead complexes with heavy meromyosin in a variety of cell types, *J. Cell Biol.* **43**:312–328.

Kachar, B., Bridgman, P. C., and Reese, T. S., 1984, Structural relationship of moving cytoplasmic organelles to microtubules in the foraminifer, *Allogromia*, *J. Cell Biol.* **99**:50a.

King, S. M., Hyams, J. S., and Luba, A., 1982, Absence of microtubule sliding and an analysis of spindle formation and elongation in isolated mitotic spindles from the yeast *Saccharomyces cerevisiae*, *J. Cell Biol.* **94**:341–349.

Koonce, M. P., and Schliwa, M., 1985, Bidirectional organelle transport can occur in cell processes that contain single microtubules, *J. Cell Biol.* **100**:322–326.

Kronebusch, P. J., and Borisy, G. G., 1982, Mechanics of anaphase B movement, in: *Biological Functions of Microtubules and Related Structures* (H. Sakai, H. Mohri, and G. G. Borisy, eds.), pp. 233–245, Academic Press, Tokyo, New York.

Ledbetter, M. C., and Porter, K. R., 1964, The morphology of microtubules of plant cells, *Science* **144**:872–874.

Lemischka, I., and Sharp, P. A., 1982, The sequence of an expressed rat α-tubulin gene and pseudogenes with an inserted repetitive element, *Nature* **300**:330–335.

Leslie, R. J., and Pickett-Heaps, J. D., 1984, Spindle microtubule dynamics following ultraviolet-microbeam irradiations of mitotic diatoms, *Cell* **36**:717–727.

Mandelkow, E.-M., and Mandelkow, E., 1979, Junctions between microtubule walls, *J. Mol. Biol.* **129**:135–148.

Manton, I., Kowallik, K., and von Stosch, H. A., 1970, Observations of the fine structure and development of the spindle at mitosis and meiosis in a marine centric diatom (Lithodesmium undulatum). IV. The second meiotic division and conclusion, *J. Cell Sci.* **7**:407–443.

Margolis, R. L., Wilson, L., and Kiefer, B. I., 1978, Mitotic mechanism based on intrinsic microtubule behaviour, *Nature* **272**:450–452.

Mazia, D., Paweletz, N., Sluder, G., and Finze, E.-M., 1981, Cooperation of kinetochores and pole in the establishment of monopolar mitotic apparatus, *Proc. Natl. Acad. Sci. U.S.A.* **78**:377–381.

McDonald, K., and Euteneuer, U., 1983, Studies on the structure and organization of microtubule bundles in the interzone of anaphase and telophase PtK_1 cells, *J. Cell Biol.* **97**:188a.

McDonald, K., Pickett-Heaps, J. D., McIntosh, J. R., and Tippit, D. H., 1977, On the mechanism of anaphase spindle elongation in *Diatoma vulgare*, *J. Cell Biol.* **74**:377–388.

McDonald, K. L., Edwards, M. K., and McIntosh, J. R., 1979, Cross-sectional structure of the central mitotic spindle of *Diatoma vulgare*. Evidence for specific interactions between antiparallel microtubules, *J. Cell Biol.* **83**:443–461.

McGill, M., and Brinkley, B. R., 1975, Human chromosomes and centrioles as nucleating sites for the *in vitro* assembly of microtubules from bovine brain tubulin, *J. Cell Biol.* **67**:189–199.

McIntosh, J. R., and Euteneuer, U., 1984, Tubulin hooks as probes for microtubule polarity: An analysis of the method and an evaluation of data on microtubule polarity in the mitotic spindle, *J. Cell Biol.* **98**:525–533.

McIntosh, J. R., Hepler, P. K., and Van Wie, D. G., 1969, Model for mitosis, *Nature* **224**:659–663.

McIntosh, J. R., Cande, W. Z., and Snyder, J. A., 1975, Structure and physiology of the mammalian mitotic spindle, in: *Molecules and Cell Movement* (S. Inoue and R. E. Stephens, eds.), pp. 31–75, Raven Press, New York.

McIntosh, J. R., McDonald, K. L., Edwards, M. K., and Ross, B. M., 1979, Three-dimensional structure of the central mitotic spindle of *Diatoma vulgare*, *J. Cell Biol.***83**:428–442.

McKeithan, T. W., and Rosenbaum, J. L., 1984, The biochemistry of microtubules, in: *Cell and Muscle Motility* (J. W. Shay, ed.), Vol. 5, pp. 255–288, Plenum Press, New York.

McNiven, M. A., and Porter, K. R., 1984, Reversals in microtubule polarity and direction of pigment transport are both delayed by taxol in severed melanophore arms, *J. Cell Biol.* **99**:50a.

McNiven, M. A., Wang, M., and Porter, K. R., 1984, Microtubule polarity and the direction of pigment transport reverse simultaneously in surgically severed melanophore arms, *Cell* **37**:753–765.

Mitchison, T., and Kirschner, M., 1984a, Microtubule assembly nucleated by isolated centrosomes, *Nature* **312**:232–237.

Mitchison, T., and Kirschner, M., 1984b, Dynamic instability of microtubule growth, *Nature* **312**:237–242.

Mitchison, T., and Kirschner, M., 1985a, Properties of the kinetochores *in vitro*. I. Microtubule nucleation and tubulin binding, *J. Cell Biol.* **101**:755–765.

Mitchison, T., and Kirschner, M., 1985b, Properties of the kinetochore *in vitro*. II. Microtubule capture and ATP-dependent translocation, *J. Cell Biol.* **101**:766–777.

Molè-Bajer, J., 1975, The role of centrioles in the development of the astral spindle (newt), *Cytobios* **13**:117–140.

Molè-Bajer, J., Bajer, A., and Owczarzak, A., 1975, Chromosome movements in prometaphase and aster transport, *Cytobios* **13**:45–65.

Murphy, D. B., and Tilney, L. G., 1974, The role of microtubules in the movement of pigment granules in teleost melanophores, *J. Cell Biol.* **61**:757–779.

Nicklas, B. R., 1971, Mitosis, in: *Advances in Cell Biology* (D. M. Prescott, L. Goldstein, and E. McConkey, eds.), Vol. 2, pp. 225–297, Appleton-Century-Crofts, New York.

Nicklas, B. R., Brinkley, B. R., Pepper, D. A., Kubai, D. F., and Rickards, G. K., 1979, Electron microscopy of spermatocytes previously studied in life: Methods and some observations on micromanipulated chromosomes, *J. Cell Sci.* **35**:87–104.

Obika, M., Turner, W. A., Negishi, S., Menter, D. G., and Taylor, J. D., 1978, The effects of lumicolchicine, colchicine, and vinblastine on pigment migration in fish chromatophores, *J. Exp. Zool.* **205**:95–110.

Paweletz, N., 1967, Zur Funktion des "Flemming Körper" bei der Teilung tierischer Zellen, *Naturwissenschaften* **54**:533–535.

Paweletz, N., and Mazia, D., 1979, Fine structure of the mitotic cycle of unfertilized sea urchin eggs activated by ammoniacal sea water, *Eur. J. Cell Biol.* **20**:37–44.

Pepper, D. A., and Brinkley, B. R., 1977, Localization of tubulin in the mitotic apparatus of mammalian cells by immunofluorescence and immunoelectron microscopy, *Chromosoma* **60**:223–235.

Pickett-Heaps, J. D., and Tippit, D. H., 1978, The diatom spindle in perspective, *Cell* **14**:455–467.

Pickett-Heaps, J. D., Tippit, D. H., and Porter, K. R., 1982, Rethinking mitosis, *Cell* **29**:729–744.

Pickett-Heaps, J. D., Spurck, T., and Tippit, D. H., 1984, Chromosome motion and the spindle matrix, *J. Cell Biol.* **99**:137s–143s.

Rattner, J. B., and Berns, M. W., 1976, Centriole behavior in early mitosis of rat kangeroo cells (PtK_2), *Chromosoma* **54**:387–395.

Rebhun, L. I., 1972, Polarized intracellular particle transport: Saltatory movements and cytoplasmic streaming, *Int. Rev. Cytol.* **32**:93–137.

Rieder, C. L., 1982, The formation, structure and composition of the mammalian kinetochore and kinetochore fiber, *Int. Rev. Cytol.* **79**:1–58.

Rieder, C. L., and Borisy, G. G., 1981, The attachment of kinetochores to the prometaphase spindle of PtK_1 cells. Recovery from a low temperature treatment, *Chromosoma* **82**:693–716.

Rieder, C. L., and Nowogrodzki, R., 1983, Intranuclear membranes and the formation of the first meiotic spindle in *Xenos peckii (Acroschismus wheeleri)* oocytes, *J. Cell Biol.* **97**:1144–1155.

Ritter, H., Inoue, S., and Kubai, D. F., 1978, Mitosis in *Barbulanympha*. I. Spindle structure, formation, and kinetochore engagement, *J. Cell Biol.* **77**:638–654.

Roberts, K., and Hyams, J. S., 1979, *Microtubules*, Academic Press, London.

Roos, U.P., 1976, Light and electron microscopy of rat kangeroo cells in mitosis. III. Patterns of chromosome behavior during prometaphase, *Chromosoma* **54**:363–386.

Roos, U.P., and Camenzind, R., 1981, Spindle dynamics during mitosis in *Dictyostelium discoideum*, *Eur. J. Cell Biol.* **25**:248–257.

Rosenbaum, J. J., Binder, L. I., Granett, S., Dentler, W. L., Snell, W., Sloboda, R., and Haimo, L., 1975, Directionality and rate of assembly of chicken brain tubulin onto pieces of neurotubules, flagellar axonemes, and basal bodies, *Ann. NY Acad. Sci.* **253**:147–177.

Sakai, H., Mohri, H., and Borisy, G. G., 1982, *Biological Functions of Microtubules and Related Structures*, Academic Press, Tokyo, New York.

Schibler, M. J., and Pickett-Heaps, J. D., 1980, Mitosis in Oedogonium: spindle microfilaments and the origin of the kinetochore fiber, *Eur. J. Cell Biol.* **22**:687–698.

Schliwa, M., 1984, Mechanisms of intracellular organelle transport, in: *Cell and Muscle Motility* (J. W. Shay, ed.), Vol. 5, pp. 1–82, Plenum Press, New York.

Schliwa, M., and Bereiter-Hahn, J., 1973, Pigment movements in fish melanophores: morphological and physiological studies. III. The effects of colchicine and vinblastine, *Z. Zellforsch.* **147**:127–148.

Schliwa, M., Wehland, J., and Weber, K., 1979, Localization and organization of actin in a fish melanophore, and a functional test of its involvement in intracellular transport, *J. Cell Biol.* **83**:315a.

Schliwa, M., Weber, K., and Porter, K. R., 1981, Localization and organization of actin in melanophores, *J. Cell Biol.* **89**:267–275.

Schnapp, J., Vale, R. D., Sheetz, M. P., and Reese, T. S., 1985, Single microtubules from squid axoplasm support bidirectional movement of organelles, *Cell* **40**:455–462.

Sluder, G., and Rieder, C. L., 1985, Experimental separation of pronuclei in fertilized sea urchin eggs: chromosomes do not organize a spindle in the absence of centrosomes, *J. Cell Biol.* **100**:897–903.

Smith, D. S., 1971, On the significance of cross-bridges between microtubules and synaptic vesicles, *Phil. Trans. R. Soc. London Ser. B* **261**:235–272.

Stearns, M. E., and Ochs, R. L., 1982, A functional *in vitro* model for studies of intracellular motility in digitonin-permeabilized erythrophores, *J. Cell Biol.* **94**:727–739.

Stebbings, H., and Hunt, C., 1983, Microtubule polarity in the nutritive tubes of insect ovarioles, *Cell Tiss. Res.* **233**:133–141.

Stephens, R. E., 1970, Thermal fractionation of outer doublet microtubules into A- and B-tubulin, *J. Mol. Biol.* **47**:353–363.

Subirana, J. A., 1968, Role of spindle microtubules in mitosis, *J. Theoret. Biol.* **20**:117–123.

Summers, K., and Kirschner, M. W., 1979, Characteristics of the polar assembly and disassembly of microtubules observed *in vitro* by darkfield light microscopy, *J. Cell Biol.* **83**:205–217.

Telzer, B. R., and Haimo, L. T., 1981, Decoration of spindle microtubules with dynein: evidence for uniform polarity, *J. Cell Biol.* **89**:373–378.

Telzer, B. R., and Quinlan, M. S., 1984, Determination of polarity of microtubules assembled in vitro from centrosomes and kinetochores using dynein decoration observed in longitudinal sections, *J. Cell Biol.* **99**:444a.

Telzer, B. R., Moses, M. J., and Rosenbaum, J. L., 1975, Assembly of microtubules onto ki-

netochores of isolated mitotic chromosomes of HeLa cells, *Proc. Natl. Acad. Sci. U.S.A.* **72**:4023–4027.

Tilney, L. G., Bryan, J., Bush, D. J., Fujiwara, K., Mooseker, M. S., Murphy, D. B., and Snyder, D. H., 1973, Microtubules: Evidence for 13 protofilaments, *J. Cell Biol.* **59**:267–275.

Tippit, D. H., and Pickett-Heaps, J. D., 1977, Mitosis in the pennate diatom Surirella ovalis, *J. Cell Biol.* **73**:705–727.

Tippit, D. H., Schulz, D., and Pickett-Heaps, J. D., 1978, Analysis of the distribution of spindle microtubules in the diatom *Fragellaria*, *J. Cell Biol.* **79**:737–763.

Tippit, D. H., Pickett-Heaps, J. D., and Leslie, R., 1980, Cell division in two large pennate diatoms *Hantzschia* and *Nitzschia*. III. A new proposal for kinetochore function during prometaphase, *J. Cell Biol.* **86**:402–416.

Travis, J. L., Kenealy, J. F. X., and Allen, R. D., 1983, Studies on the motility of the Foraminifera. II. The dynamic microtubular cytoskeleton of the reticulopodial network of *Allogromia laticollaris*, *J. Cell Biol.* **97**:1668–1676.

Vale, R. D., Schnapp, B. J., Reese, T. S., and Sheetz, M. P., 1985, Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon, *Cell* **40**:559–569.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., and Cleveland, D. W., 1981, Nucleotide and corresponding amino acid sequences encoded by α and β tubulin mRNAs, *Nature* **289**:650–655.

Wang, E., and Goldman, R. D., 1978, Functions of cytoplasmic fibers in intracellular movements in BHK-21 cells, *J. Cell Biol.* **79**:708–726.

Witt, P. L., Ris, H., and Borisy, G. G., 1980, Origin of kinetochore microtubules in Chinese hamster ovary cells, *Chromosoma* **81**:483–505.

9

Molecular Mechanisms Controlling Tubulin Synthesis

Don W. Cleveland

1. Introduction

Microtubules, which are comprised principally of dimeric subunits of one α- and one β-tubulin polypeptide, participate in a diverse spectrum of cellular events. This repertoire of microtubule-associated functions includes formation of mitotic and meiotic spindles, establishment of some forms of intercellular and intracellular motility, establishment of programmed modifications of cell shape during morphogenesis (such as neurite outgrowth), and, in concert with actin filaments and intermediate filaments, establishment of internal cytoarchitecture.

The realization that microtubules were integral participants in this wide variety of processes led quickly to the expectation that expression of tubulin polypeptides would be a closely regulated process. Indeed, with the demonstration of the presence of multiple tubulin genes within the genomes of most eukaryotes, it initially became clear that the requisite regulatory mechanisms must control two separate aspects of tubulin gene expression. First is the selection of which among a family of similar genes are to be expressed at specific points during development and differentiation. Second is establishment of the appropriate quantitative level of expression. With regard to the first of these regulatory problems, I shall not attempt to review here the considerable work that has led to the demonstration through the efforts of a variety of investigators that the genomes of multicellular organisms contain multiple genes that are expressed in characteristic developmental and tissue-

Don W. Cleveland • Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

specific patterns. Rather, I refer the interested reader to other recent reviews (Cowan, 1983; Cleveland, 1983; Raff, 1984; Cleveland and Sullivan, 1985). In the present work, I have chosen instead to dwell at some length on reviewing the results derived from several experimental systems which have yielded provocative findings concerning the diverse mechanisms utilized for establishing the proper level of tubulin gene expression.

2. *Apparent Autoregulatory Control of Tubulin Synthesis in Animal Cells*

2.1. *Tubulin Synthesis in Animal Cells Is Sensitive to the Apparent Pool of Unpolymerized Subunits*

Even in the absence of direct evidence, it seemed obvious at the outset (at least to this observer) that animal cells must possess a sensitive mechanism(s) for regulation of their tubulin contents. Since the unpolymerized tubulin subunit concentration is generally in rapid equilibrium with the polymer (albeit through a complicated interaction of many factors), this postulated regulatory system for establishing tubulin subunit and polymer content could act by maintenance of a specified level of total tubulin content, of microtubule polymer, or of unpolymerized tubulin subunits.

But it was not until the pioneering work of Ben Ze'ev, Farmer, and Penman (1979) that some initial insight emerged as to what kind of regulatory mechanism might actually be utilized. Although many investigators had previously recorded the effects of antimicrotubule drugs on cellular microtubule arrays, it was Ben Ze'ev *et al.* who first noted that the marked alterations in the morphology of cultured animal cells following colchicine-induced microtubule depolymerization were accompanied by specific repression of new tubulin synthesis. For this demonstration, these investigators used a combination of pulse radiolabelling of newly synthesized proteins followed by two-dimensional gel electrophoresis to resolve the pattern of new protein synthesis in 3T6 cells, a mouse fibroblastic cell line. When the patterns of newly made proteins in normal cells or in colchicine-treated cells (which had lost all of their microtubule polymers) were compared, it was found that, although the overall pattern of protein synthesis was not notably affected by microtubule depolymerization, there was a dramatic repression of synthesis of new α- and new β-tubulin polypeptides. As later investigators were to show, this specific repression of tubulin synthesis in response to colchicine treatment is a general response of animal cells and has been found in all types of cultured animal cells investigated including a spectrum of different types of mouse cells (Cleveland *et al.*, 1981) and cells from organisms as diverse as humans (Fellous *et al.*, 1982; Cleveland and Havercroft, 1983), chickens (Lau *et al.*, 1985), and mosquitos (Cleveland *et al.*, 1981).

An example illustrating a two-dimensional gel analysis of protein synthetic patterns in control and colchicine-treated Chinese hamster ovary cells is displayed in Fig. 1A,B. Inspection of the patterns reveals that they are re-

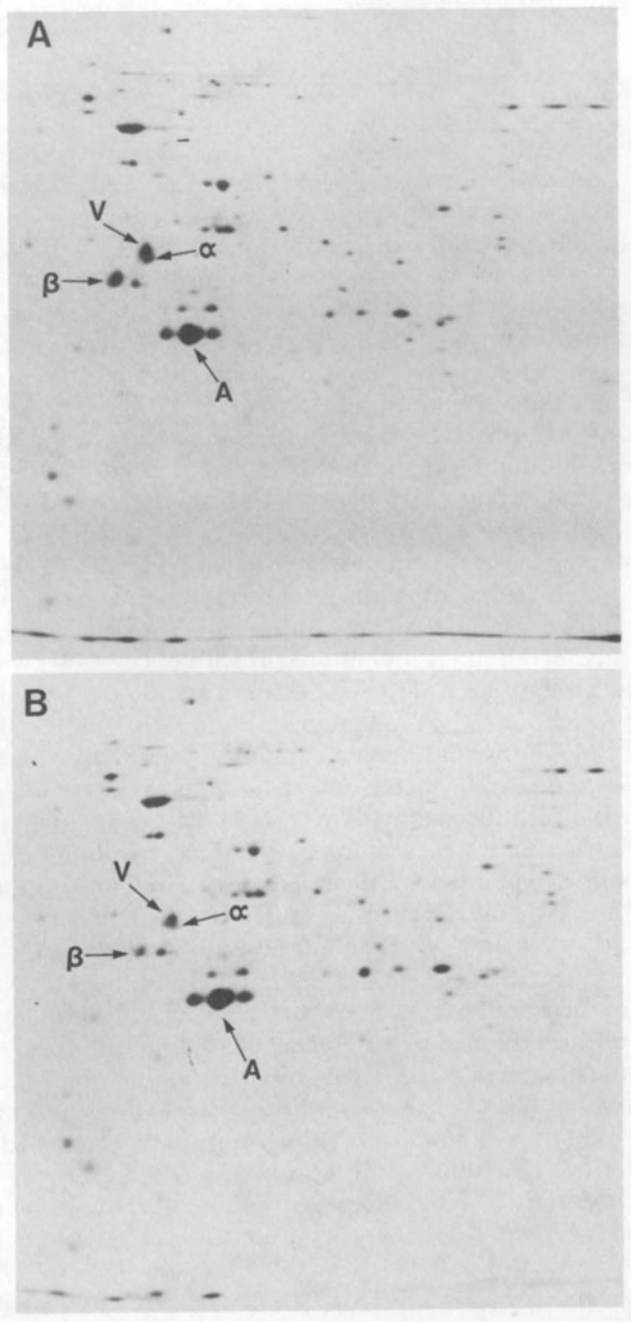


Figure 1. Specific depression in synthesis of α- and β-tubulin polypeptides following colchicine-induced microtubule depolymerization. Duplicate dishes of CHO cells were seeded in parallel. Six hours prior to harvesting, colchicine was added to one dish to a final concentration of 10 μM. Thirty minutes prior to harvesting, newly synthesized proteins in each dish were labeled by addition of [^{35}S]methionine to the media. Total cellular protein was solubilized and subjected to two-dimensional gel electrophoresis. (A) Newly synthesized proteins from control cells; (B) newly synthesized proteins from cells incubated for 6 hr in colchicine. Spots whose identities are known are labeled. α, α-tubulin; β, β-tubulin; A, actin; V, vimentin. The acidic end of the isoelectric dimension is on the left. Data from Lau *et al.* (1986). Reprinted with permission.

markably similar, except for the approximate 10-fold repression of new α- and β-tubulin synthesis (Lau *et al.*, 1986).

Moreover, in contrast to the depression of tubulin synthesis which was found when cells were incubated in colchicine, Ben Ze'ev *et al.* (1979) found that specific *elevation* of tubulin synthesis was induced both *in vivo* and *in vitro* with a second antimicrotubule drug, vinblastine. Since this drug induces not only microtubule depolymerization but also concomitant precipitation of the unpolymerized subunits into paracrystals (e.g., Bensch and Malawista, 1969; Bryan, 1972), it was proposed that the regulation of tubulin expression was achieved by a mechanism that monitors the pool of soluble unpolymerized subunits, rather than the amount of polymer.

In an effort to further characterize the events involved in this apparent autoregulation of tubulin, workers in Kirschner's laboratory used quantitative immunoprecipiation of tubulin polypeptides from pulse-labeled cells that had been treated with various antimicrotubule drugs (Cleveland *et al.*, 1981). With this assay they were able to demonstrate that the kinetics of the cellular response are rapid (half-time of repression in response to colchicine of approximately 1 hr). Moreover, consistent with the autoregulatory hypothesis, these studies demonstrated that treatment of cells with nocodazole, which, like colchicine, induces microtubule depolymerization and an increase in the pool of unpolymerized subunits (DeBrabander *et al.*, 1976), resulted in the rapid suppression of tubulin synthesis, whereas treatment with taxol, which dramatically decreases the pool of unassembled subunits (Schiff *et al.*, 1979; Schiff and Horwitz, 1980), induced a mild increase in synthesis. More recent experiments have shown that the quantitative level of this taxol-induced increase in tubulin synthesis is dependent on the cell density. Under conditions where the cells are confluent, taxol stimulates as much as a threefold specific enhancement in tubulin synthetic rates (Pachter and Cleveland, unpublished observations).

Thus, for each antimicrotubule drug investigated, all data to date are consistent with the presence of an autoregulatory control mechanism which can measure the pool of unpolymerized tubulin subunits and which can adjust the level of new synthesis in the event of suboptimal levels of subunits. These alterations in tubulin synthetic rates must represent a true synthetic control mechanism and do not simply reflect an inherent cell cycle-dependent program of tubulin synthesis which is uncovered by drug-induced cell cycle disruption (see also Section 4.2). This conclusion arises from three independent lines of evidence. First, the majority of the effect is seen within 3 hr of drug addition, a time too short to yield a substantial cell cycle blockage in an initially unsynchronized culture. Second, colchicine and nocodazole induce mitotically blocked cell populations which are essentially identical to that induced by vinblastine, even though the effects on tubulin synthetic rates are of opposite signs. Third, in contrast to the dramatic repression of tubulin synthesis induced by colchicine or nocodazole treatment, a simple calculation using the reported threefold decline in the overall rate of protein synthesis during mitosis (Fan and Penman, 1970), coupled with the known twofold relative

increase in tubulin synthesis at mitosis (Bravo and Celis, 1980), yields a predicted 6% *increase* in the relative tubulin synthetic rate following a 3-hr drug-induced mitotic arrest of cells with a doubling time of 16 hr. As we have seen (e.g., Fig. 1), in practice such treatment actually yields a 5- to 10-fold *decrease* in tubulin synthesis.

However, interpretation of these drug treatment experiments in terms of an autoregulatory control event rests entirely on the presumptive effects of the various antimicrotubule drugs. This caveat is not a trivial one, as the drugs in question obligatorily induce gross morphological alterations and the specificities of action cannot be assumed with certainty. This ambiguity was clarified, at least in part, by microinjection of purified tubulin into cells to artificially elevate the intracellular content of tubulin in the absence of drug treatments and morphological changes (Cleveland *et al.*, 1983; Lau *et al.*, 1986). All cells on a glass cover chip were serially microinjected, and after labeling with [^{35}S]methionine, newly synthesized proteins were analyzed by two-dimensional gels. An example of this approach is found in Fig. 2. Part A displays the synthetic products in control cells that were mock injected with a microtubule buffer solution only. Part B, on the other hand, shows the polypeptides synthesized by cells microinjected with a solution of purified tubulin. Comparison of these two patterns reveals that the only dramatic differences are the specific loss of newly synthesized α-tubulin and β-tubulin polypeptides in the cells injected with tubulin.

Quantitatively, it has been found (Cleveland *et al.*, 1983) that tubulin synthesis is rapidly and specifically suppressed by injection of an amount of tubulin roughly equivalent to that which would be liberated by endogenous microtubule depolymerization (25–50% of the total amount of tubulin initially present in the cell). Although in these experiments the fate of the injected tubulin subunits (i.e., unassembled or assembled) has not been directly determined, the fact that only 30% of the injected tubulin subunits could be induced to assemble under optimal *in vitro* assembly conditions in the presence of excess added microtubule-associated proteins suggests that microinjection did indeed significantly raise the unpolymerized tubulin subunit pool. Together with companion experiments in which the injected subunits were rendered polymerization incompetent *in vitro* by prebinding to colchicine (a binding event that is essentially irreversible *in vitro*), these data strongly support the hypothesis that it is the subunit form of tubulin which is monitored by a feedback control mechanism.

2.2. Possible Molecular Levels for Regulating Tubulin Synthetic Rates

Conceptually, this apparent autoregulatory control of tubulin synthesis in animal cells could be achieved through any of the following general molecular mechanisms: (1) translational sequestration or reversible inactivation of tubulin mRNAs, (2) transcriptional modulation of tubulin genes, (3) specific degradation of tubulin RNAs in the cytoplasm, or (4) inhibition of proper

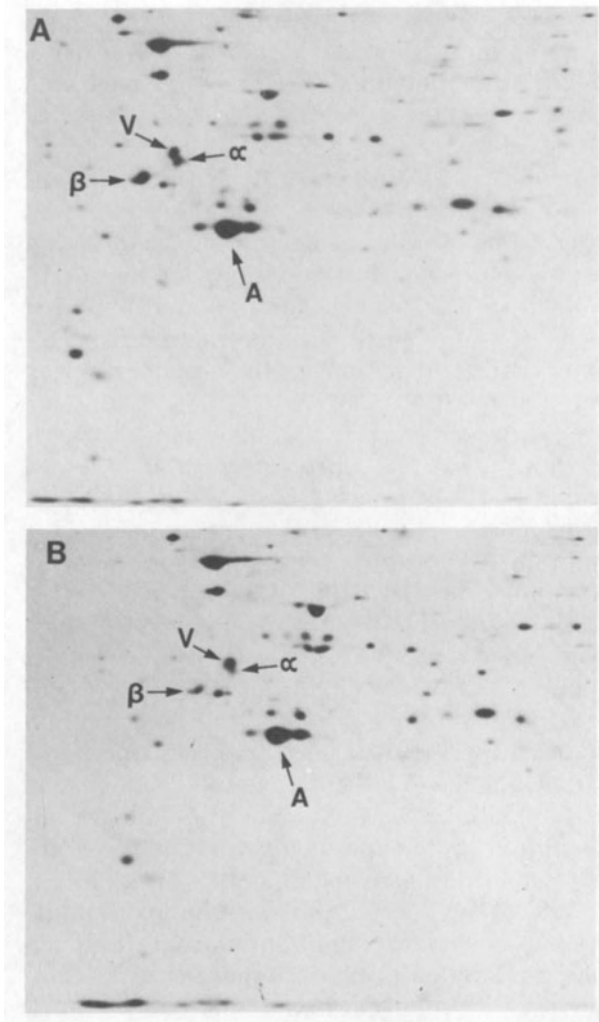


Figure 2. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. Glass cover chips were seeded with approximately 30 cells each. Each cell attached to a chip was then serially microinjected either with a standard microtubule buffer or with purified tubulin at 9.7 mg/ml in the same buffer. Following injection, both cover chips were returned to an incubator for 3 hr. Finally, newly synthesized proteins were labeled by placing the chips in media containing [^{35}S]methionine. After 1 hr of labeling, proteins were solubilized and analyzed by two-dimensional gel electrophoresis and fluorography. (A) Newly synthesized proteins in cells mock-injected with the microtubule buffer alone; (B) newly synthesized proteins in cells injected with exogenous tubulin. α, α-tubulin; β, β-tubulin; A, actin; V, vimentin. Data from Lau *et al.* (1986). Reprinted with permission.

tubulin RNA processing/transport in the nucleus. Sections 2.2.1–2.2.4 detail the progress that has been made in identifying the pathway actually utilized.

2.2.1. *Tubulin Regulation Is Not Achieved through Reversible RNA Sequestration/Modification*

The first examination of a possible mechanism that might be responsible for tubulin regulation emerged from the demonstration (Ben Ze'ev *et al.*, 1979) that the *in vivo* loss of new tubulin polypeptide synthesis was mirrored by a companion loss of ability of mRNA extracted from colchicine-treated cells to direct the synthesis of tubulin in *in vitro* translation reactions. Mechanistically, this result immediately mandated that either tubulin mRNAs were

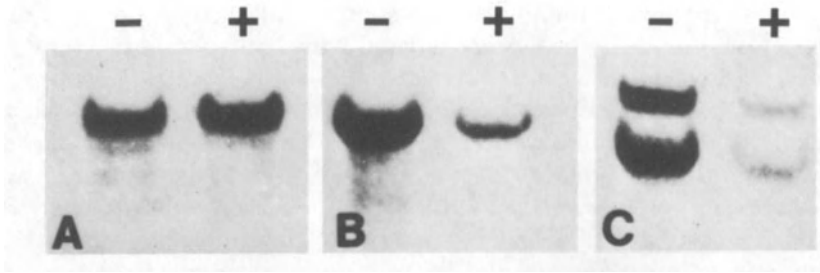


Figure 3. Demonstration that repression of newly synthesized tubulin polypeptides in response to colchicine-induced microtubule depolymerization is mediated through altered tubulin mRNA levels. Cytoplasmic RNA was prepared from control and colchicine-treated cells, and equal amounts of RNA were analyzed by RNA blotting followed by hybridization to appropriate ^{32}P-labeled probes for (A) actin, (B) α-tubulin, and (C) β-tubulin. Slots labeled − are from control cells, whereas slots labeled + are from cells treated for 6 hr with colchicine. Data are from Cleveland and Havercroft (1983). Reprinted with permission.

specifically lost from the treated cells or these mRNAs were modified in some covalent fashion that altered their translatability. Next, using cloned sequences to detect α- or β-tubulin mRNAs, it was shown by RNA blot analysis that the former of these two possibilities was in fact the case (Cleveland *et al.*, 1981; Cleveland and Havercroft, 1983). Figure 3 displays such a demonstration. Equal amounts of cytoplasmic RNA derived from control cells or from cells incubated in the presence of colchicine were analyzed for actin RNA sequences (A), α-tubulin RNA sequences (B), or β-tubulin RNA sequences (C). Clearly, α- and β-tubulin mRNAs are lost from the cells as a consequence of drug-induced microtubule depolymerization, whereas actin mRNAs are, if anything, slightly elevated in amount. In other experiments, quantitation of the levels of each RNA showed that the rate and extent of losses of α- and β-tubulin mRNAs were indistinguishable (at least within the error of the measurement) and that both were lost at a rate which quantitatively paralleled that found for the loss of new polypeptide synthesis (Cleveland *et al.*, 1981).

2.2.2. Regulation Is Not Achieved through Transcriptional Control

Since most eukaryotic genes that were previously studied in detail had been shown to be regulated principally at the level of gene transcription (e.g., globin, immunoglobulin, ovalbumin) it seemed very attractive that tubulin gene expression might also be mediated through altered rates of tubulin transcription. Indeed, based on the rough estimation of a 2-hr half-life of tubulin mRNAs in cells in which all new RNA synthesis was inhibited with actinomycin D, it was proposed that such regulation of tubulin synthesis was modulated by altered rates of formation of new tubulin mRNAs (Ben Ze'ev *et al.*, 1979), a hypothesis for which additional indirect support was forthcoming (Cleveland and Kirschner, 1982).

However, direct measurement of apparent tubulin transcription rates in later work from Cleveland and Havercroft (1983) did not detect any tran-

scriptional modulation of tubulin genes in control and colchicine-treated cells. For these measurements, nuclei were isolated from control cells or from cells pretreated with colchicine. To each set of nuclei, radiolabeled RNA precursors were added, and the nuclei were incubated under conditions in which *in vivo*-initiated RNA polymerase molecules continued to elongate their nascent RNA transcripts. The resultant labeled, heterogeneous RNAs were then analyzed by hybridization to excess cloned DNA sequences in order to determine the relative or absolute levels of new transcripts corresponding to α-tubulin, β-tubulin, and actin RNAs. In order for a transcriptional mechanism to lead to the measured 10-fold loss of cytoplasmic tubulin RNAs in the colchicine-treated cells (Fig. 3), there must be at least a corresponding 10-fold decrease in the transcription rate. However, using this nuclear runoff transcription assay with nuclei from control and colchicine-treated hamster or human cells, no diminution in the apparent rate of tubulin gene transcription could be detected. Thus, with the explicit assumption that the *in vitro* transcription system faithfully represents the *in vivo* situation (as has been shown to be the case for several previously studied genes), these data mandate that apparent autoregulation of tubulin synthesis is not achieved through a transcriptional event.

2.2.3. Regulation Is Achieved through a Cytoplasmic Event

Since apparent autoregulation of tubulin is not achieved through mRNA sequestration (Section 2.2.1) and probably not through transcriptional control (2.2.2), how can the remaining two possibilities be distinguished? Realizing that one possible mechanism (RNA processing and/or transport) represents a nuclear event, whereas the other possibility (RNA stability) is a cytoplasmic event, Pittenger and Cleveland (1985) and Caron *et al.*, (1985) prepared enucleated cells to determine whether such nuclei-free cytoplasts could respond with preferential loss of tubulin subunits. Clearly, if such cytoplasts retained the tubulin regulatory machinery, all nuclear events (either RNA process/transport or transcription) could be discarded as possible regulatory candidates.

In the actual experiments, cytoplasts were prepared from cultured cells according to the protocol originally developed by Prescott *et al.* (1972). Cells attached to glass coverslips were placed cell side down in a centrifuge tube containing media supplemented with the drug cytochalasin B to disrupt actin filament arrays. The inverted cells were then centrifuged to induce the nuclei to separate from the more adherent cytoplasm. The coverslips containing the remaining cytoplasts were then returned to an incubator to allow a period of recovery. When the pattern of new protein synthesis in control cytoplasts and in colchicine-treated cytoplasts was compared on two-dimensional gels or on one-dimensional gels following immunoprecipitation, it was found that there was a specific, 3- to 10-fold loss in the synthesis of new β-tubulin polypeptides in the drug-treated cytoplasts. These experiments unambiguously demonstrated that regulation of β-tubulin synthesis by the apparent pool of unpolymerized subunits is achieved principally through a cytoplasmic mechanism. For α-

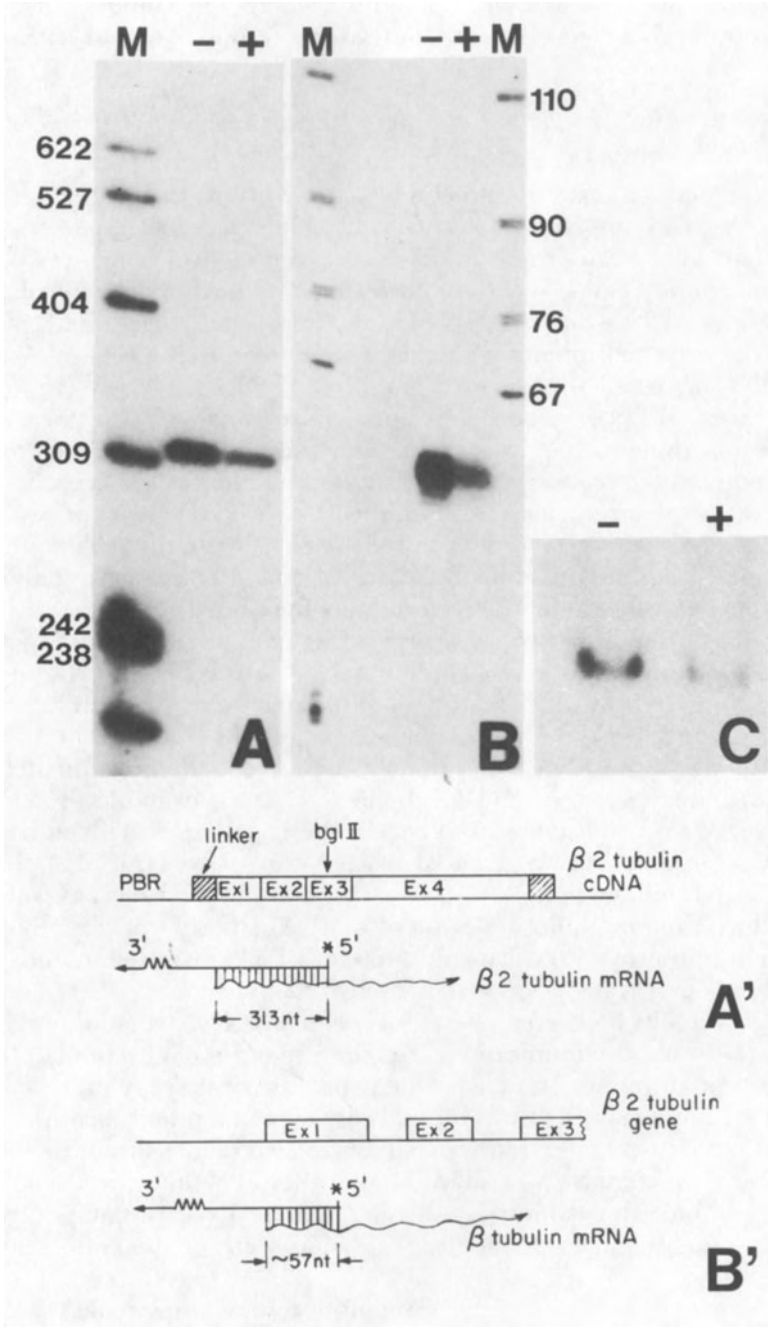
tubulin, although the response of cytoplasts was quantitatively less dramatic than that of whole cells, at least part of the regulatory machinery must also be activated through a cytoplasmic event (Pittenger and Cleveland, 1985).

2.2.4. *Reconstruction of Appropriate Regulation following Transfection of Cloned Tubulin Genes*

To further delineate the precise molecular events that underlie tubulin gene autoregulation, two groups (Lau *et al.*, 1985; N. Cowan, personal communication) have begun to define the important control sequences that are carried on a tubulin gene and/or its corresponding mRNA. The initial goal of these efforts was to determine whether appropriate regulation could be achieved on a cloned tubulin gene that was reintroduced into a cultured cell using DNA-mediated transfection. To attempt this, Lau *et al.* (1985) have utilized transient DNA transfection, a procedure that involves cellular uptake of exogenous cloned DNA under conditions where the foreign DNA remains in an unintegrated, plasmid form. Because no sequences are present on the foreign DNA to permit its replication, this DNA will only be expressed transiently (generally within 1–3 days posttransfection). With this technique, these investigators were able to document that a cloned chicken β-tubulin gene could be introduced into mouse cells and that the cloned gene was transcribed into an RNA that was properly processed to a mature transcript indistinguishable from the authentic chicken mRNA. Moreover, it was found that upon elevation of the intracellular pool of unpolymerized tubulin subunits by colchicine-induced microtubule depolymerization, the level of the transfected chicken tubulin gene RNA transcript was down-regulated coordinately with the endogenous mouse α- and β-tubulin RNAs. An example of regulated expression of a transfected chicken gene is shown in Fig. 4. With either an S1 nuclease protection assay (Fig. 4A,B) or RNA blot analysis (Fig. 4C), the level of transfected tubulin gene RNA transcripts is seen to fall substantially upon drug-induced microtubule depolymerization. Furthermore, this loss of expression in response to colchicine is found only for transfected tubulin genes, and not for other transfected genes (such as actin).

Similar results have emerged from the efforts of N. Cowan and his coworkers (personal communication). For these experiments, a human tubulin gene has been introduced onto a bovine papilloma virus vector and the hybrid plasmid introduced back into cultured cells. Exploiting the transforming potential of the virus, cloned transformed foci of cells that replicate and express the hybrid virus were then isolated. Again, when such lines were challenged with microtubule depolymerizing agents, expression of the engineered tubulin gene was found to mimic the regulation seen for the endogenous tubulin genes.

These data demonstrate that the requisite recognition signal(s) for appropriate tubulin gene regulation by the apparent pool size of depolymerized tubulin subunits must reside directly in the primary tubulin gene sequences. As a consequence, it seems very likely that by construction and analysis of hybrid tubulin genes containing different regions of tubulin sequence, the


M
-
+
M
-
+
M
622
527
404
309
242
238
110
90
76
67
A
B
-
+
C
linker
bglII
PBR
Ex1
Ex2
Ex3
Ex4
β2 tubulin cDNA
3'
*5'
β2 tubulin mRNA
313nt
A'
β2 tubulin gene
Ex1
Ex2
Ex3
3'
*5'
β tubulin mRNA
~57nt
B'

requisite regulatory sequences and pathway of regulation can be identified in the near future.

2.3. Transient Induction of Tubulin Synthesis by Interferon

In addition to apparent autoregulatory control of tubulin synthesis, Fellous *et al.* (1982) have shown that translatable tubulin mRNA levels are transiently increased by treatment of Ramos cells (a human cell line of lymphoblastoid origin) with β-interferon. Using RNA blot analysis with a cloned α-tubulin sequence, these investigators also demonstrated that treatment with either α- or β-interferon induced a transient increase in accumulated α-tubulin RNAs. In the case of β-interferon, this induction reach a maximum 4 hr after addition of interferon and yielded an accumulated level sevenfold above that in untreated cells. Remarkably, within 2 additional hours α tubulin RNA levels fell quickly back to the control level. The induction was completely blocked by cotreatment with colchicine, which, as detailed in Section 2.1, causes microtubule depolymerization and a specific down-regulation in tubulin synthesis. These investigators have interpreted this to indicate that the colchicine and interferon act through different mechanisms, although presently it seems equally plausible that the induction of tubulin RNAs could be linked to an interferon-induced, transient decrease in the tubulin subunit pool. No measurements of the subunit-to-microtubule ratio have yet been reported following interferon administration.

3. Programmed Synthesis of Tubulin during Flagellar Growth in Unicellular Organisms

Because of the ease with which mechanical shearing or alterations in growth conditions can be utilized to induce flagellar amputation or resorption (or, in the case of *Naegleria,* initial flagellar outgrowth), the stimulation of ciliary or flagellar protein synthesis during the growth of these organelles has emerged as an increasingly attractive model system with which to study the

Figure 4. Reconstruction of appropriate gene regulation on a cloned β-tubulin gene introduced into a cultured cell by DNA transfection. Cultured mouse cells were transiently transfected with a chicken β-tubulin gene. For the final 3 hr of culturing, colchicine was added to some cells to induce microtubule depolymerization. RNA was then prepared from control and drug-treated samples, and the RNAs were analyzed for the level expression of the transfected chicken gene. (A) An S1 nuclease protection experiment of RNAs from control (−) or colchicine-treated (+) cells. Lanes marked M show molecular weight markers. (B) A parallel assay using a different S1 probe to analyze RNAs from an independent transfection experiment. (A′, B′) Schematic drawings of the expected S1 nuclease-resistant probe fragments. (C) The levels of expression of the transfected chicken gene as determined by RNA blot analysis with a probe specific to the transfected chicken gene. Clearly, the level of expression of the transfected gene is sensitive to drug-induced microtubule depolymerization. Data are from Lau *et al.* (1985). Reprinted with permission.

specific induction and regulated expression of a specific set of eukaryotic genes. These investigations have centered on analysis of tubulin expression since the tubulins are among the most prominent flagellar constitutents. Not surprisingly, *in vivo* labeling in sea urchins (Stephens, 1977), *Tetrahymena* (Guttman and Gorovsky, 1979), and *Polytomella* (Brown and Rogers, 1978) demonstrated that in each of these systems large increases in synthesis of tubulin polypeptides accompany the growth of cilia or flagella. In addition, *in vitro* translations of RNA from *Tetrahymena* (Marcaud and Hayes, 1979), *Naegleria* (Lai *et al.*, 1979), and sea urchins (Merlino *et al.*, 1978) further documented that for each of these organisms, increased synthesis of flagellar proteins is due to increased levels of corresponding translatable mRNAs encoding flagellar proteins.

But without question, the organism that has received the most study and for which the flagellar induction process has been identified in greatest detail is the unicellular flagellate *Chlamydomonas*. Progress in analysis of this most tractable example is detailed in Section 3.1. and 3.2.

3.1. Regulated Synthesis of Tubulin Achieved through a Combination of Transcriptional Control and mRNA Stability in Chlamydomonas

Chlamydomonas reinhardtii is a motile unicellular eukaryote that is propelled by means of two anterior flagella. The flagella serve two primary physiological functions. They are used to move the cell into the most favorable environment for photosynthesis and are required for selective pairing of gametes during the mating process. The earliest biochemical investigations of these organelles determined that flagella lost or amputated from either gametic or vegetative cells were quickly replaced through generation of new flagella (Randall *et al.*, 1967; Rosenbaum *et al.*, 1969). As initially demonstrated by Rosenbaum *et al.* (1969), nearly full-length flagella are reassembled within 90 min of amputation. Moreover, as demonstrated by inhibition of new protein synthesis after deflagellation, sufficiently large cytoplasmic reserves of flagellar proteins are present in vegetative cells (Rosenbaum *et al.*, 1969) and in gametes (Weeks and Collis, 1976) to allow regeneration of flagella of roughly one-third to one-half normal length. However, initial experiments also determined that a specific induction of tubulin synthesis could be detected by *in vivo* labeling as early as 15 min after deflagellation. This observation was extended by *in vitro* translation of isolated polyribosomes (Weeks and Collis, 1976) and of purified RNA (Lefebvre *et al.*, 1980) to show that translatable RNAs for tubulin and other flagellar proteins accumulate rapidly after deflagellation. Indeed, increased levels of translatable tubulin RNAs were found as early as 8 min postdeflagellation (Silflow and Rosenbaum, 1981; Minami *et al.*, 1981). This accumulation peaks between 45 min and 90 min and then slowly declines to the basal level by 180 min.

Mechanistically, from the rapidity of the induction of new synthesis and the considerable size of the initial cytoplasmic pool of flagellar precursors, it was clear at the outset that the initial signal for induction could not be deple-

tion of the subunit pool (Weeks *et al.*, 1977). This point was made even more clear by the demonstration that, following deflagellation or chemically induced flagellar resorption, stimulation of tubulin synthesis was still observed even when regrowth was blocked with the drug inhibitors isobutylmethylxanthine (IBMX) or colchicine (Weeks *et al.*, 1977; Lefebvre *et al.*, 1978, 1980). However, since treatment of deflagellated gametes with IBMX led to an earlier-than-normal shutoff of new tubulin synthesis, it initially seemed possible that a feedback mechanism responsive to tubulin concentrations might be responsible for the ultimate repression of tubulin synthesis following flagellar regeneration (Weeks *et al.*, 1977). On the other hand, Lefebvre *et al.* have presented a strong argument that makes this suggestion seem unlikely. In a rather complicated experiment (Lefebvre *et al.*, 1980), these latter investigators treated cells with IBMX to induce resorption of approximately 50% of the flagellar length (and thereby to increase the pool of cytoplasmic flagellar precursors). The IBMX was then removed, but colchicine was added to block regeneration. Finally, the remaining flagella were mechanically removed by shearing. In contrast to the prediction of a feedback model, the stimulation of synthesis of flagellar proteins proceeded as normal.

The isolation of cloned sequences for the *Chlamydomonas* tubulins in Weeks' and Rosenbaum's laboratories afforded a more detailed look at the mechanism underlying the induction process. Initially, both groups used RNA blot analysis to demonstrate that increased levels of tubulin synthesis corresponded quantitatively with an increased level of α- and β-tubulin mRNAs (Silflow and Rosenbaum, 1981; Minami *et al.*, 1981). Two unlinked α- and two unlinked β-tubulin genes were shown to represent the full complement of tubulin sequences encoded within the *Chlamydomonas* genome (Silflow and Rosenbaum, 1981; Brunke *et al.*, 1982), and the mRNAs derived from each of these four tubulin genes were seen to be induced coordinately (Brunke *et al.*, 1982), although the careful quantitation experiments of Schloss *et al.* (1984) have recently argued for slightly different accumulation kinetics. An example of an RNA blot analysis that demonstrates the dramatic induction of α- and β-tubulin RNAs following deflagellation is displayed in Fig. 5.

Given the dogmatic bias endowed by most previously studied eukarytotic genes in which regulation of expression has been shown to derive principally from transcriptional mechanisms, work the from Rosenbaum laboratory produced the surprising finding that modulation in transcription rates is only part of the regulatory story for flagellar reassembly. Depending on which method of normalization was utilized, nuclei isolated from vegatative cells 20 min after deflagellation were found to transcribe between 4 and 10 times as many tubulin RNAs as nuclei isolated from cells prior to deflagellation (Keller *et al.*, 1984). Later work measuring tubulin transcription rates by *in vivo* pulse labeling with $^{32}PO_4$ (Baker *et al.*, 1984) indicated that the peak in transcriptional enhancement occurs remarkably rapidly (within 10–15 min) following deflagellation. The peak in RNA accumulation, however, does not occur until 45–60 min. The brevity of the period during which tubulin RNA synthesis is maximal (the first 20 min) is as striking as the speed of activation. However, in

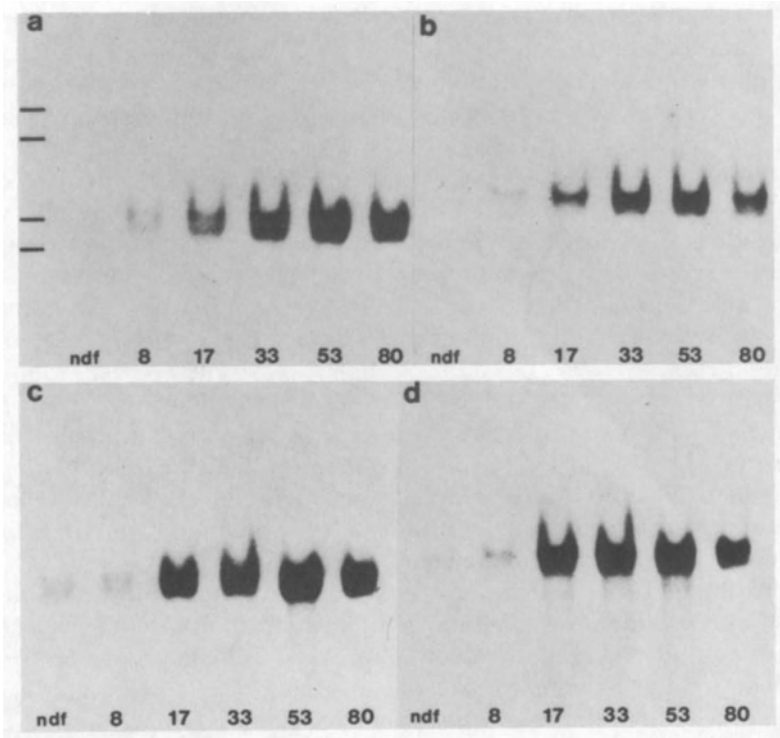


Figure 5. Demonstration by RNA blot analysis that induction of tubulin synthesis following deflagellation is the result of increased levels of tubulin mRNAs. Total cell RNA (a, b) or polyadenylated RNA prepared from total cell RNA (c, d) was prepared from vegetative cells of *Chlamydomonas* before deflagellation (ndf) or at 8, 17, 33, 53, and 80 min after mechanical deflagellation. Total cell RNA (2.4 μg) or poly(A) RNA (1 μg) was separated by size on a denaturing gel, transferred *in situ* to nitrocellulose, and tubulin RNAs identified by hybridization with ^{32}P-labeled sequences corresponding to α-tubulin (a, c) or β-tubulin (b, d). Clearly, both α- and β-tubulin RNAs increase markedly in amount within 8 min after deflagellation. Reprinted with permission from Silflow and Rosenbaum (1981).

these *in vivo* labeling experiments the peak transcription rate was estimated again to be only four- to sevenfold above the initial rate even though the accumulation of tubulin RNAs reached 10- to 14-fold above the initial levels. Careful consideration of these data mandated that in addition to an enhanced rate of tubulin gene transcription, there must be a concomitant increase of at least two- to threefold in tubulin mRNA stability.

Moreover, an additional clue as to how the induction process is achieved came from redeflagellation of cells at times before the first flagellar regeneration was completed. Such redeflagellation was found to induce another burst in tubulin RNA transcription that is identical to the first in magnitude and duration (Baker *et al.*, 1984). An example of this striking phenomenon is displayed in Fig. 6. Parts A and B represent independent experiments in which the length distribution of flagella following two successive deflagella-

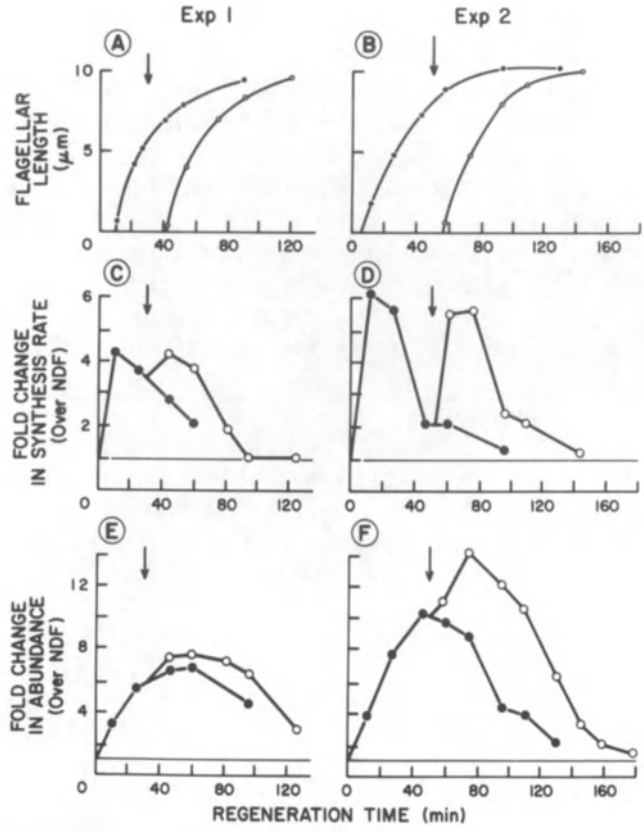


Figure 6. Redeflagellation of *Chlamydomonas* at times before the first flagellar regeneration is completed induces another burst in tubulin RNA synthesis that is identical to the first in magnitude and duration. (A, B) Flagellar regeneration curves from two different experiments in which regenerating cells were subjected to a second deflagellation induced by shearing. Arrows indicate the times at which the second deflagellation was performed. (C, D) The relative rates of synthesis of β-tubulin mRNA as determined by 5-min *in vivo* pulse labeling. ●, single deflagellation; ○, double deflagellation. (E, F) Plots of accumulated β-tubulin RNAs. NDF, nondeflagellated. Reprinted with permission from Baker *et al.* (1984).

tion events has been recorded. Parts C and D show that following the second deflagellation a new burst in the synthesis of tubulin mRNAs is induced, which in experiment 2 resulted in an accumulation of tubulin RNAs substantially greater than that produced in a single deflagellation event (Fig. 6F). These data indicate that whatever the actual induction signal, it may act simply to reprogram the tubulin genes for a transient burst of maximal synthesis.

Overall, it is now clear that the regulated expression of tubulin during flagellar regeneration results from a combined mechanism involving a transient transcriptional enhancement coupled with modulations in tubulin mRNA stability.

3.2. Identification of Candidate DNA Sequences That Mediate Tubulin Regulation in Chlamydomonas

Although no data are yet available on how mRNA stabilization is achieved, a possible identification has emerged of the DNA sequences required for the burst in transcription of tubulin gene sequences that is induced after deflagellation. Brunke *et al.* (1984a,b) have reasoned that for a family of genes that are not adjacent to each other in the genome but are coordinately induced, all gene members are likely to possess a DNA sequence feature that allows recognition by a diffusible regulatory factor. Indeed, in several previously investigated systems (e.g., heat shock genes, genes involved in histidine biosynthesis), definitive evidence had already been obtained which indicated that short regions of nucleotide homology between coordinately regulated genes were involved in modulation of inducible levels of transcription. By an analogous analysis of the sequences just 5′ to the transcribed regions of each of the four *Chlyamdomonas* tubulin genes, Brunke *et al.* have identified the putative promoter segments of each gene. As demonstrated in Fig. 7, in addition to the presence of the expected TATA promoter sequence element, they have identified a 16-base-pair consensus sequence [GCTC(G/C)AAGGC(G/T)(G/C)N-(C/A)(C/A)G] which is located multiple times within 150 base pairs of the transcriptional initiation site of each gene. Also of potential significance is a GC-rich region (denoted with +++ in Fig. 7) that flanks the TATA box sequence in each gene. Although attractive as candidates for important regulatory regions, there is at present no direct evidence for the participation of any of these sequences in inducible transcription. Modification, rearrangement, and deletion of these sequence elements, followed by reintroduction of the altered sequences back into *Chlamydomonas* by transformation, will be necessary to test definitively what role, if any, these sequence elements play in the regulation of tubulin gene expression.

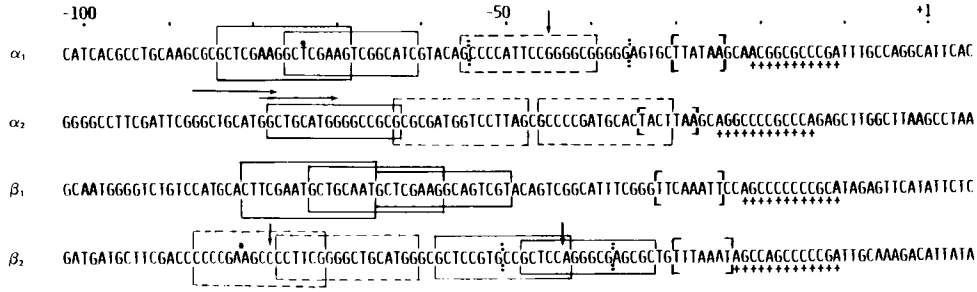


Figure 7. DNA sequences from the 5′ flanking regions of each of the four tubulin genes of *Chlamydomonas*: identification of elements of conserved sequence. Important sequence elements are indicated by the following symbols: solid brackets, TATA boxes; solid boxes, consensus-sequence elements with high homology; dashed boxes, consensus-sequence elements with weak homology; plus signs, GC-rich regions; vertical arrows, centers of dyads of symmetry; dotted vertical lines, limits of dyad of symmetry regions. Reprinted with permission from Brunke *et al.* (1984b).

3.3. The Paradigm of Tubulin Regulation in Naegleria

The unicellular eukaryote *Naegleria gruberii* can reversibly switch between two life forms, amebae and flagellates (Fulton, 1977). More specifically, when transferred from a rich growth environment to a nutrient-free aqueous environment, each ameba rapidly converts to a swimming flagellate. The flagellate form is temporary, and after a time that can very from seconds to days (depending on environmental conditions), the flagellates reconvert again to amebae. Moreover, upon placement in nutrient-free media, the differentiation of amebae into flagellates occurs remarkably rapidly (within about 60 min) and synchronously (Kowit and Fulton, 1974; Lai *et al.*, 1979). Given this rapid response, it might be anticipated that *Naegleria* would contain a substantial pool of flagellar precursors in the ameba with which to rapidly construct the flagellum. However, using an antibody prepared against *Naegleria* outer-doublet tubulin and which apparently does not recognize any other tubulin (including mitotic tubulin in extracts of *Naegleria* amebae or tubulins from other organisms), a radioimmunoassay was utilized to determine that amebae have, at most, 3% as much flagellar tubulin antigen as flagellates (Kowit and Fulton, 1974; Fulton and Simpson, 1976). Hence, flagellar assembly must be preceded by a rapid induction of flagellar tubulin synthesis. Direct evidence for this induction emerged first from demonstration in a wheat germ *in vitro* translation system that translatable flagellar tubulin mRNAs appear as early as 20 min after nutrient deprivation, reach a maximum at 60 min, and then decline in amount (Lai *et al.*, 1979). These findings suggest that flagellar tubulin synthesis during differentiation is regulated first by a programmed burst transcription of flagellar tubulin RNAs, followed by subsequent RNA degradation.

Although further support for this model arose from experiments in which actinomycin D was used to selectively inhibit both RNA synthesis and differentiation (Fulton and Walsh, 1980), it was not until the *Naegleria* α-tubulin genes themselves were cloned that definitive evidence became available. Using RNA blot techniques, Lai *et al.* (1984) directly determined the abundance of flagellar α-tubulin mRNAs during the time course of differentiation. α-Tubulin mRNA abundance was found to increase at least 80-fold in less than 50 min, followed by a decline in amount with a half-time of loss of only 8 min. Like the *Chlamydomonas* example presented in Section 3.1, the controlled synthesis of tubulin during *Naegleria* differentiation is almost certainly achieved by a dual mechanism of transcriptional control and altered mRNA stability.

3.4. Altered Tubulin Regulation in the Life Form Switch of the Parasitic Protozoa Leishmania

The life cycle of the parasitic protozoa *Leishmania* consists of two morphologically distinct forms, the first of which (the amastigote) resides inside macrophages of the mammalian host and the second of which (the promastigote) lives extracellularly and possesses a flagellum. Although both forms

contain intracellular microtubules, as might be anticipated, the flagellated form synthesizes tubulin at a higher rate (approximately threefold) than the intracellular form (Fong and Chang, 1981; Wallach *et al.*, 1982). *In vitro* translation of RNA derived from each form of *Leishmania mexicana,* however, was found to yield surprisingly similar levels of tubulin synthesis, suggesting that alterations in tubulin synthetic rates were established in this organism through reversible mRNA sequestration (Wallach *et al.*, 1982). This hypothesis was further tested by RNA blot analysis using cloned tubulin sequences from other organisms. The results were partially consistent with translational regulation. Although tubulin RNA levels were mildly higher (approximately 50%) in promastigotes than in amastigotes, this increase in tubulin RNAs was less dramatic than the difference seen with *in vivo* protein labeling (Fong *et al.*, 1984). Moreover, by RNA blot analysis a striking shift in the apparent length of β-tubulin RNAs was detected. This change in the size of the dominant β-tubulin RNAs almost certainly indicates that different tubulin gene transcription and/or RNA processing events occur in the two life forms. In view of this and in light of the failure to find evidence for a translation contribution in tubulin expression in *Leishmania enriettii* (Landfear and Wirth, 1984), the original hypothesis of translational regulation of tubulin synthesis must be considered unproven.

4. *Programmed Synthesis of Tubulin during the Cell Cycle*

4.1. *The Synchronous Cell Cycle of Physarum*

Although the regulation of tubulin synthesis throughout the cell cycle has been addressed in several organisms (see Section 4.2), a most attractive system for such a study is in the naturally synchronous cycle of the multinucleate plasmodium of *Physarum.* The tubulin polypeptides to be utilized for spindle microtubules [the only microtubules in the plasmodium (Havercroft and Gull, 1983)] have been found to be synthesized preferentially late in the G2 phase of the cell cycle, just prior to entry into mitosis (Laffler *et al.*, 1981; Schedl *et al.*, 1984a). By *in vitro* translation of RNA derived at different points in the cycle, this increased synthesis of tubulin polypeptides prior to mitosis has been found to be the result of increased levels of translatable tubulin mRNAs. Moreover, by utilization of cloned tubulin sequences as hybridization probes (Schedl *et al.*, 1984a), tubulin RNA levels in late G2 have been shown to rise exponentially to a level at least 40-fold above the level during S phase (Schedl *et al.*, 1984b). The true exponential character of this tubulin RNA accumulation is demonstrated in Fig. 8 in which the logarithm of a tubulin mRNA accumulation has been plotted versus time in the cell cycle. As is evident, this yields a linear plot. Moreover, as is also visible in Fig. 8, at the end of the 30-min mitotic event, there is a dramatic exponential loss of accumulated tubulin RNAs (half-time <20 min—about 3% of the cell cycle time) concomitant with spindle microtubule depolymerization.

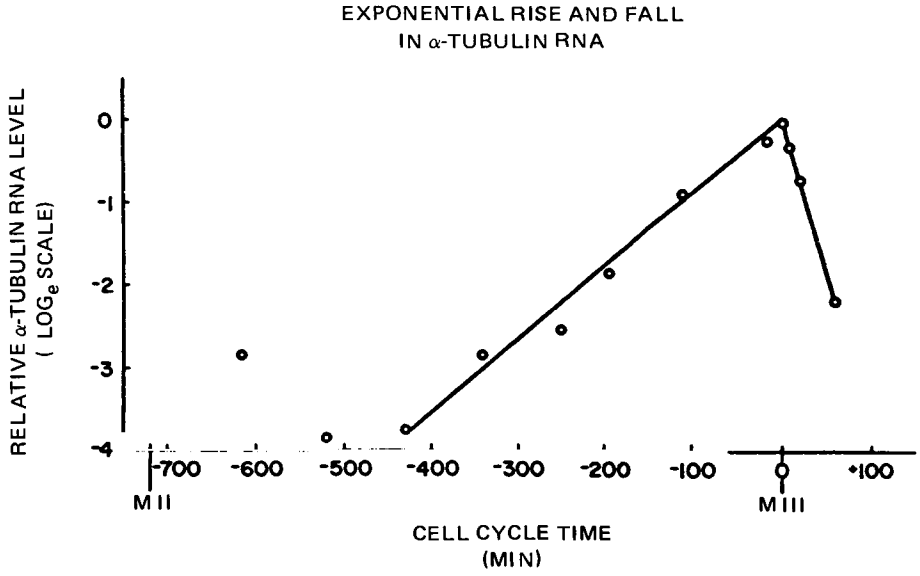


Figure 8. Relative α-tubulin RNA levels throughout the *Physarum* cell cycle. α-Tubulin RNA levels were measured by quantitative dot blot hybridization in RNA samples derived from various points in the synchronous cell cycle of *Physarum*. Data are plotted as logarithm of relative tubulin RNA level versus cell cycle time in order to demonstrate the exponential character of the accumulation and loss events. Reprinted with permission from Schedl *et al.* (1984b).

Mechanistically, since the fall in α tubulin mRNA is coincident with the depolymerization of spindle microtubules, this loss of tubulin RNAs could be achieved through a facilitated decay of tubulin RNAs as a consequence of a negative autoregulation event. The rise in tubulin RNAs in G2, however, cannot be the result of this postulated negative feedback system, since the synthesis and accumulation of tubulin polypeptides temporally precedes the assembly of spindle microtubules (Schedl *et al.*, 1984a). Nonetheless, the exponential character of the RNA rise is intriguing and suggests an autocatalytic component (Schedl *et al.*, 1984b).

4.2. Cell Cycle Regulation in Other Eukaryotes

In addition to investigating regulated synthesis of tubulin after flagellar amputation or resorption in *Chlamydomonas* (as detailed in Section 3.1), regulation of tubulin synthesis as a function of the cell cycle of this organism has also been addressed (Weeks and Colis, 1979). Most recently Howell and coworkers (Ares and Howell, 1982) have followed tubulin synthesis during the synchronous growth that occurs naturally when vegetative cells are maintained under 12-hr alternating light/dark conditions. By *in vitro* translations it was found that translatable tubulin mRNAs increase in amount by an order of magnitude just prior to the mitotic phase of the cycle. Not unexpectedly, RNA

blotting with cloned tubulin sequences demonstrated that this increase in translatable tubulin mRNA was the result of accumulation of tubulin RNA sequences. Furthermore, by measuring apparent tubulin gene transcription rates in cells made permeable to nucleotide triphosphates by treatment with toluene, an increased rate of tubulin RNA synthesis was documented to occur just prior to mitosis, thereby indicating that tubulin RNA accumulation was controlled, at least in part, by differential gene transcription (Dallman *et al.*, 1983). It should be remembered, however, that the cell cycle fluctuations investigated here reflect the superposition of true cell cycle requirements on top of the natural induction of flagellar proteins that occurs after mitosis.

Finally, cell cycle regulation of tubulin synthesis has also been investigated in cultured animal cells. For this analysis, after synchronization by mitotic shakeoff, Bravo and Celis (1980) pulse-labeled cells at various times and then analyzed the newly synthesized proteins by two-dimensional gels. Quantitations of many resultant spots were performed by excision of individual spots and liquid scintillation counting. A remarkably constant pattern of overall protein synthesis was found, although the tubulins were among the few polypeptides seen to vary by a factor of 2 or so during the cell cycle of HeLa cells. This relative increase in new tubulin synthesis was seen to peak during mitosis [although the overall rate of new protein synthesis falls by about a factor of 3 in mitotic cells (Fan and Penman, 1970)].

5. Summary

The examination of the molecular processes that underlie the regulated expression of tubulin both during normal cell cycle events and during specialized events of differentiation has been initiated in a variety of promising experimental systems. Already substantial data have been accumulated that indicate the presence in cultured animal cells of an autoregulatory apparatus that monitors the level of unpolymerized tubulin subunits. In response to suboptimal levels of unpolymerized subunits, this system is able to alter the rate of tubulin polypeptide synthesis through a cytoplasmic control event that specifically alters cytoplasmic tubulin mRNA stability. Similarly, detailed investigation of induction of flagellar growth in unicellular flagellates and in *Physarum* has shown that the proper regulation is achieved by coupling transient transcriptional enhancement of tubulin gene expression with altered mRNA stabilities.

This substantial progress notwithstanding, the determinations of the detailed pathways of tubulin gene regulation remain in their infancy. However, with the application of ever more powerful approaches (such as reintroduction of cloned, engineered genes using DNA transfection), we anticipate for the future an explosive period of growth in our understanding of how proper levels of tubulin subunits are established and how such regulatory mechanisms contribute to overall microtubule function.

References

Ares, M., and Howell, S. H., 1982, Cell cycle stage-specific accumulation of mRNAs encoding tubulin and other polypeptides in *Chlamydomonas, Proc. Natl. Acad. Sci. USA* **79:**5577.

Baker, E. J., Schloss, J. A., and Rosenbaum, J. L., 1984, Rapid changes in tubulin RNA synthesis and stability induced by deflagellation in *Chlamydomonas, J. Cell Biol.* **99:**2074.

Ben Ze'ev, A., Farmer, S. R., and Penman, S., 1979, Mechanisms of regulating tubulin synthesis in cultured mammalian cells, *Cell* **17:**319.

Bensch, K. G., and Malawista, S. E., 1969, Microtubular crystals in mammalian cells, *J. Cell Biol.* **40:**95.

Bravo, R., and Celis, J. E., 1980, A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells, *J. Cell Biol.* **84:**795.

Brown, D. L., and Rogers, K. A., 1978, Hydrostatic pressure-induced internalization of flagellar axonemes, disassembly, and reutilization during flagellar regeneration in *Polytomella, Exp. Cell Res.* **117:**313.

Bryan, J., 1972, Vinblastine and microtubules. I. Induction and isolation of crystals from sea urchin oocytes, *Exp. Cell Res.* **66:**129.

Brunke, K. J., Young, E. E., Buchbinder, B. U., and Weeks, D. P., 1982, Coordinate regulation of the four tubulin genes of *Chlamydomonas reinhardtii,* Mechanisms regulating expression of tubulin, *Nucleic Acids Res.* **10:**1295.

Brunke, K., Anthony, J., Kalish, F., Sternberg, E., and Weeks, D. P., 1984a, Coordinate expression of the four tubulin genes in *Chlamydomonas,* in: *Molecular Biology of the Cytoskeleton* (G. Borisy, D. Cleveland, and D. Murphy, eds.), pp. 367–379, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Brunke, K. J., Anthony, J. G., Sternberg, E. J., and Weeks, D. P., 1984b, Repeated consensus sequence and pseudopromoters in the four coordinately regulated tubulin genes of *Chlamydomonas reinhardtii, Mol. Cell. Biol.* **4:**1115.

Caron, J., Jones, A. L., Rall, L. B., and Kirschner, M.W., 1985, Autoregulation of tubulin synthesis in enucleated cells, *Nature* **317:**648.

Cleveland, D. W., 1983, The tubulins: From DNA to RNA to protein and back again, *Cell* **34:**330.

Cleveland, D. W., and Havercroft, J. C., 1983, Is apparent autoregulatory control of tubulin synthesis non-transcriptionally regulated? *J. Cell Biol.* **97:**919.

Cleveland, D. W., and Kirschner, M. W., 1982, Autoregulatory control of the expression of α and β tubulins: Implications for microtubule assembly, *Cold Spring Harbor Symp. Quant. Biol.* **46:**171.

Cleveland, D. W., and Sullivan, K. F., 1985, Molecular biology and genetics of tubulin, *Ann. Rev. Biochem.* **54:**331.

Cleveland, D. W., Lopata, M. A., Sherline, P., and Kirschner, M. W., 1981, Unpolymerized tubulin modulates the level of tubulin mRNAs, *Cell* **25:**537.

Cleveland, D. W., Pittenger, M. F., and Feramisco, J. R., 1983, Elevation of tubulin levels by microinjection suppresses new tubulin synthesis, *Nature* **305:**738.

Cowan, N. J., 1983, Tubulin isotypes and the multigene tubulin families, *Int. Rev. Cytol.* **85:**147.

Dallman, T., Ares, M., and Howell, S. H., 1983, Analysis of transcription during the cell cycle in toluenized *Chlamydomonas reinhardtii* cells, *Mol. Cell. Biol.* **3:**1537.

DeBrabander, M. J., Van De Veire, R. M. L., Aerts, F. E. M., Borgers, M., and Janssen, P. A. J., 1976, The effects of methyl [5-(2-thienylcarbonyl)-1H-benzimicazol-2-yl carbamate (R 17 934: NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured *in vitro, Cancer Res.* **36:**905.

Fan, H., and Penman, S., 1970, Regulation of protein synthesis in mammalian cells, *J. Mol. Biol.* **50:**655.

Fellous, A., Ginzburg, I., and Littauer, U. Z., 1982, Modulation of tubulin mRNA levels by interferon in human lymphoblastoid cells, *EMBO J.* **1:**835.

Fong, D., and Chang, K. P., 1981, Tubulin biosynthesis in the developmental cycle of a parasitic

protozoan, *Leishmania mexicana:* Changes during differentiation of motile and nonmotile stages, *Proc. Natl. Acad. Sci. USA* **78:**7624.

Fong, D., Wallach, M., Keithly, J., Melera, P. W., and Chang, K. P., 1984, Differential expression of mRNAs for α and β tubulin during differentiation of the parasitic protozoan *Leshmania mexicana, Proc. Natl. Acad. Sci. USA* **81:**5782.

Fulton, C., 1977, Cell differentiation in *Naegleria gruberii, Ann. Rev. Microbiol.* **31:**597.

Fulton, C., and Simpson, P., 1976, Selective synthesis and utilization of flagellar tubulin: the multi-tubulin hypothesis, in: *Cell Motility* (R. Goldman, T. Pollard, and J. Rosenbaum, eds.), pp. 987–1006, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Fulton, C., and Walsh, C., 1980, Cell differentiation and flagellar elongation in *Naegleria gruberii, J. Cell Biol.* **85:**346.

Guttman, S. D., and Gorovsky, M. A., 1979, Cilia regeneration in starved *Tetrahymena:* An inducible system for studying gene expression and organelle biosynthesis, *Cell* **17:**307.

Havercroft, J. C., and Gull, K., 1983, Demonstration of different patterns of microtubule organization in *Physarum polycephalum* myxamoebae and plasmodia using immunofluorescence microscopy, *Eur. J. Cell Biol.* **32:**67.

Keller, L. R., Schloss, J. A., Silflow, C. D., and Rosenbaum, J. R., 1984, Transcription of α and β tubulin genes *in vitro* in isolated *Chlamydomonas reinhardtii* nuclei, *J. Cell Biol.* **98:**1138.

Kowit, J. D., and Fulton, C., 1974, Programmed synthesis of tubulin for the flagella that develop during cell differentiation in *Naegleria gruberii, Proc. Natl. Acad. Sci. USA* **71:**2877.

Laffler, T. G., Chang, M. T., and Dove, W. F., 1981, Periodic synthesis of microtubule proteins in the cell cycle of *Physarum, Proc. Natl. Acad. Sci. USA* **78:**5000.

Lai, E. Y., Walsh, C., Wardell, D., and Fulton, C., 1979, Programmed appearance of translatable flagellar tubulin mRNA during cell differentiation in *Naegleria, Cell* **17:**867.

Lai, E. Y., Remillard, S. P., and Fulton, C., 1984, Tubulin and actin: Yin–yang gene expression during *Naegleria* differentiation, in: *Molecular Biology of the Cytoskeleton* (G. Borisy, D. Cleveland, and D. Murphy, eds.), pp. 257–266, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Landfear, S. M., and Wirth, D. F., 1984, Control of tubulin gene expression in the parasitic protozoan *Leishmania enriettii, Nature* 309:716.

Lau, J. T. Y. L., Pittenger, M. F., and Cleveland, D. W., 1985, Reconstruction of appropriate tubulin and actin gene regulation following transient transfection of cloned β tubulin and β actin genes, *Mol. Cell. Biol.* **5:**1611.

Lau, J. T. Y., Pittenger, M. F., Havercroft, J. C., and Cleveland, D.W., 1986, Reconstruction of tubulin gene regulation in cultured mammalian cells, *Ann. N. Y. Acad. Sci.* (in press).

Lefebvre, P. A., Nordstrom, S. A., Moulder, J. E., and Rosenbaum, J. L., 1978, Flagellar elongation and shortening in *Chlamydomonas, J. Cell Biol.* **78:**8.

Lefebvre, P. A., Silflow, C. D., Wieben, E. D., and Rosenbaum, J. L., 1980, Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella, *Cell* **20:**469.

Marcaud, L., and Hayes, D., 1979, RNA synthesis in starved deciliated *Tetrahymena pyriformis, Eur. J. Biochem.* **98:**267.

Merlino, G. T., Chamberlin, J. P., and Kleinsmith, L. J., 1978, Effects of deciliation on tubulin messenger RNA activity in sea urchin embryos, *J. Biol. Chem.* **253:**7078.

Minami, S. A., Collis, P. S., Young, E. E., and Weeks, D. P., 1981, Tubulin incubation in *C. reinhardtii:* Requirement for tubulin mRNA synthesis, *Cell* **24:**89.

Pittenger, M. F., and Cleveland, D. W., 1985, Retention of autoregulatory control of tubulin synthesis in cytoplasts: Demonstration of a cytoplasmic mechanism that regulates the level of tubulin expression, *J. Cell Biol.* **101:**1941.

Prescott, D. M., Myerson, D., and Wallace, J., 1972, Enucleation of mammalian cells with cytochalasin B, *Exp. Cell Res.* **71:**480.

Raff, E. C., 1984, Genetics of microtubule systems, *J. Cell Biol.* **99:**1.

Randall, J., Cavalier-Smith, T., McVittie, A., Warr, J. R., and Hopkins, J. M., 1967, Developmental and control processes in the basal bodies and flagella of *Chlamydomonas reinhardtii, Dev. Biol. Suppl.* **1:**43.

Rosenbaum, J. L., Moulder, J. E., and Ringo, D. L., 1969, Flagellar elongation and shortening in *Chlamydomonas*, *J. Cell Biol.* **41**:600.

Schedl, T., Burland, T. G., Gull, K., and Dove, W. F., 1984a, Cell cycle regulation of tubulin RNA level, tubulin protein synthesis, and assembly of microtubules in *Physarum*, *J. Cell Biol.* **99**:155.

Schedl, T., Burland, T. G., Dove, W. F., Roobol, A., Paul, E. C. A., Foster, K. E., and Gull, K., 1984b, Tubulin expression and the cell cycle of the *Physarum plasmodium*, in: *Molecular Biology of the Cytoskeleton* (G. Borisy, D. Cleveland, and D. Murphy, eds.), pp. 235–244, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schiff, P. B., and Horwitz, S. B., 1980, Taxol stabilizes microtubules in mouse fibroblast cells, *Proc. Natl. Acad. Sci. USA* **77**:1561.

Schiff, P. B., Fant, J., and Horwitz, S. B., 1979, Promotion of microtubule assembly *in vitro* by taxol, *Nature* **277**:665.

Schloss, J. S., Silflow, C. D., and Rosenbaum, J. L., 1984, mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*, *Mol. Cell. Biol.* **4**:424.

Stephens, R. E., 1977, Differential protein synthesis and utilization during cilia formation in sea urchin embryos, *Dev. Biol.* **61**:311.

Silflow, C. D., and Rosenbaum, J. L., 1981, Multiple α and β tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation, *Cell* **24**:81.

Wallach, M., Fong, D., and Chang, K. P., 1982, Post-transcriptional control of tubulin biosynthesis during leishmanial differentiation, *Nature* **299**:650.

Weeks, D. P., and Collis, P. S., 1976, Induction of microtubule protein synthesis in *Chlamydomonas reinhardtii* during flagellar regeneration, *Cell* **9**:15.

Weeks, D. P., and Collis, P. S., 1979, Induction and synthesis of tubulin during the cell cycle and life cycle of *Chlamydomonas reinhardtii*, *Dev. Biol.* **69**:400.

Weeks, D. P., Collis, P. S., and Gealt, M., 1977, Control of induction of tubulin synthesis in *Chlamydomonas reinhardtii*, *Nature* **268**:667.

10

Regulation of Tubulin Expression in Brain

Marcelle R. Morrison and W. Sue T. Griffin

1. Introduction

The α- and β-tubulins are the major polypeptide components of microtubules. In the immature nervous system, microtubules are involved in cell division, in the determination of cell shape, and in neuronal growth and migration. In the mature nervous system, microtubules are also involved in synaptogenesis and in axonal transport (Olmsted and Borisy, 1973; Dustin, 1978).

Brain α- and β-tubulins are more heterogeneous than are their counterparts in other organs, and many of the tubulin isoforms are developmentally regulated. There are multiple copies of the α- and β-tubulin genes, providing the transcriptional substrates for some of these isoforms. Posttranslational modification also contributes to isoform heterogeneity. The biological significance of the different tubulin isoforms and the factors regulating their expression *in vivo* are unknown.

This chapter reviews tubulin expression in cultured neural cells and in avian and mammalian brain. Several recent reviews can be consulted for additional information (Cowan, 1983; Raff, 1984; Cleveland and Sullivan, 1985; Littauer and Ginzburg, 1985). The control of tubulin gene expression is reviewed by Cleveland in Chapter 9 of this volume.

Marcelle R. Morrison • Department of Neurology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235. ***W. Sue T. Griffin*** • Department of Cell Biology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

2. *Tubulin Proteins*

2.1. *Different Isoforms of* α- *and* β-*Tubulins*

Mammalian brain microtubules are a complex of α- and β-tubulin heterodimers (Bryan, 1974; Bryan and Wilson, 1971; Feit *et al.*, 1971, 1977b; Olmsted *et al.*, 1971; Amos, 1979). The α- and β-tubulins have similar molecular weights (Lee *et al.*, 1973) but different amino acid sequences (Luduena and Woodward, 1973; Nelles and Bamburg, 1979).

Microheterogeneity in the individual β-tubulin subunits was shown by hydroxylapatite chromatography (Little, 1979). On two-dimensional gels, the α-subunit is resolved into two molecular weight species—α_1(58 kD) and α_2(57 kD) (Morrison *et al.*, 1981). The isoelectric points of the two α-tubulin subunits are similar to each other but are more basic than are those of the β-subunits. The two β-subunit isoforms have molecular weights of approximately 54 kD. They differ in isoelectric points, β_2 being more acidic than β_1 (Marotta *et al.*, 1978; Morrison *et al.*, 1981). High-resolution, one-dimensional isoelectric focusing separates between 9 and 21 brain α- and β-tubulin isoforms (Gozes and Littauer, 1978; Gozes *et al.*, 1979; Forgue and Dahl, 1979; George *et al.*, 1981; Stromska, 1982; Wolff *et al.*, 1982; Sullivan and Wilson, 1984; Field *et al.*, 1984). These isoforms are highly conserved (Field *et al.*, 1984).

There is now evidence that at least some of this isoform heterogeneity is due to the transcription of multiple tubulin genes. For example, tryptic peptide maps of three brain α-tubulin isoforms showed that each had at least seven unique peptides; three β-tubulin isoforms each had at least two unique peptides (George *et al.*, 1981). Four unique isoforms of the α-subunit and two of the β-subunit have been identified by amino acid analysis of porcine brain tubulin (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981). By sequencing individual recombinant DNA clones, it was determined that most of these isoforms, whether subspecies of α- or β-subunits, differ from each other by no more than a few amino acid substitutions (reviewed in Littauer and Ginzburg, 1985). There are two positions in the α-chain and two in the β-chain that contain most of the amino acid substitutions. These variable regions are at positions 265–273 and 340 in the α-chain (Ponstingl *et al.*, 1981; Ginzburg *et al.*, 1981; Lemischka *et al.*, 1981; Littauer and Ginzburg, 1985) and 48–57 in the β-chain as well as at the carboxy terminus in the β chain (Krauhs *et al.*, 1981; Littauer and Ginzburg, 1985). The relationship between the tubulin isoforms with different isoelectric points and those with unique amino acid and nucleic acid sequences is as yet unclear. It is clear, however, that the number of different isoforms is greater than the number of different primary sequences. Posttranslational modifications such as glycosylation, deamidation, tyrosylation, and acetylation of several or all of the primary translation products (Feit and Shelanski, 1975; Nelles and Bamburg, 1979; Nath and Flavin, 1978; L'Hernault and Rosenbaum, 1985) must account for the additional isoelectric heterogeneity (see Section 2.3).

The heterogeneity of tubulin isoforms in brain (Gozes and Littauer,

1978; Gozes *et al.*, 1979) could simply be a consequence of the heterogeneity of brain cell types. Alternatively, it may reflect the variety of functions subserved by brain microtubules. Analysis of isoform levels in discrete brain regions does show differential isoform distributions (Gozes *et al.*, 1980). That isoform heterogeneity is not solely a consequence of cell type diversity was clearly established when Gozes and Sweadner (1981) showed that multiple isoforms are present in a single neuron. In their experiments, radiolabeled tubulins were isolated from one sympathetic neuron grown in culture; seven distinct α- and β-tubulin isoforms were separated by isoelectric focusing and four α- and four β-isoforms were identified by two-dimensional gel electrophoresis. Failure to detect the 20 or more previously identified brain isoforms could be due to (1) tissue culture conditions that alter tubulin expression; (2) rapid turnover of some isoforms (the labeling period was 18 hr); (3) absence of cell-specific isoforms that are not synthesized in the particular sympathetic neurons analyzed; or (4) the relatively low resolution of their isoelectric focusing gels.

Recent studies of Little and Ludueña (1985) suggest that different tubulin isoforms have distinct functions. They show that the major bovine β-tubulin isoform, β_1, contains two cysteines that may be important in colchicine binding and microtubule assembly. One of these is absent in the minor isoform, β_2 (Little and Ludueña, 1985). This structural difference may underlie functional differences between the two isoforms.

2.2. *Differential Localization of Brain α- and β-Tubulin Isoforms*

2.2.1. *Subcellular Distribution of Different Isoforms*

One approach to determining the function of the different tubulin isoforms is to generate monoclonal antibodies against isoform-specific epitopes and localize them intracellularly. Gozes and Barnstable (1982) showed that monoclonal antibodies (TUB 2.1 and TUB 2.5) immunoprecipitate all β-tubulin isoforms although different gel bands were labeled to different intensities with the two antibodies. Using monoclonal antibody TUB 2.1 (Gozes and Barnstable, 1982), Cumming *et al.* (1982) showed that the parallel fibers of the molecular layer and the axonal processes of the white matter of adult rat cerebellum were immunoreactive, as were, to a lesser extent, Purkinje cell dendrites and Bergmann glial fibers.

Using their own monoclonal antibodies against β-tubulin (Tu3B, Tu9B, and Tu12B), Carceres *et al.* (1984) showed identical subcellular localization to that using a polyclonal tubulin antibody in rat cerebellum and hippocampus.

The epitope recognized by a monoclonal antibody against α-tubulin (YL1/2; Kilmartin *et al.*, 1982) has been characterized as a linear sequence that includes the tyrosylated carboxy terminal residues (Wehland *et al.*, 1983, 1984). YL1/2 immunoreactivity in adult rat cerebellum was localized in Purkinje dendrites and Bergmann glial fibers. Little immunoreactivity was localized either in the somas of these cells or in the parallel fiber axons. In

contrast, YOL/34, a monoclonal antibody against the more basic α-tubulin isoforms (Kilmartin *et al.*, 1982), has the same cerebellar distribution as the TUB 2.1 and 2.5 β-tubulin antibodies; these antibodies are localized to parallel fiber axons (Cumming *et al.*, 1983a). Using another monoclonal antibody against α-tubulin (TU 01; Viklicky *et al.*, 1982), Hajos and Rostomian (1984) showed immunoreactivity exclusively in Bergmann glial fibers.

These immunocytochemical localization studies indicate that, although both α- and β-tubulins are present in all brain cell types, unique isoforms of each may be preferentially localized in different regions within a cell and/or within discrete cell types. Such findings suggest that tubulin heterogeneity may be related to functional specificity.

An axon-specific, unique tubulin isoform has now been identified in guinea pig (Tashiro and Komiya, 1983). Although the isoelectric point and the peptide map of this isoform (T_{AX}) are similar to those of the α-tubulin subunits isolated from whole brain or from axons, its molecular weight is more similar to that of the β-subunits (Tashiro and Komiya, 1983). Such a unique, highly localized isoform as T_{AX} is an ideal candidate for the study of the relationship between the presence of a specific isoform and specialized regional functions in a cell.

2.2.2. *Myelin-Bound Isoforms*

Colchicine binding studies show that tubulin is associated with rat brain myelin. Myelin-associated tubulin comigrates with purified rat brain tubulin, and its peptide map is identical (Gozes and Richter-Landsberg, 1978). Whether or not tubulin is bound to myelin was questioned by Waehneldt and Malotka (1980) after they demonstrated that tubulin was readily solubilized from the myelin fraction. More recently, de Néchaud *et al.* (1983) showed that three isoforms of α-tubulin and nine of β-tubulin do colocalize with myelin.

2.2.3. *Cytoplasmic versus Membrane-Bound Isoforms*

Tubulins are present in both cytoplasm and membranes. A fraction of the cytoplasmic α-tubulin is tyrosylated *in vivo* whereas no membrane-bound tyrosylated α-tubulin has been identified (Nath and Flavin, 1978).

Both α- and β-tubulin immunoreactivity is associated with plasma membranes in neurites of cultured rat superior cervical ganglion cells. In contrast to the soluble tubulin pool, the particulate tubulin is fucosylated. At least part of the tubulin is exposed on the exterior of the cell surface (Estridge, 1977). Both α- and β-tubulins are also present in the postsynaptic density (Feit *et al.*, 1977a).

Some studies show that the α-tubulins are preferentially associated with presynaptic membranes and synaptosomes (Gozes and Littauer, 1979; Zisapel *et al.*, 1980; Cumming *et al.*, 1983b). For example, by isoelectric focusing, molecular weight determination, peptide mapping, and vinblastine binding, Gozes and Littauer (1979) showed that isolated synaptosomes contain signifi-

cant levels of α-tubulin. Other studies indicate that α- and β-subunits are present in equal amounts in synaptosomes and that a novel α-tubulinlike protein is also present (Marotta *et al.*, 1979a).

The mechanisms by which neuronal tubulins are prevented from assembly into microtubules and are instead permitted to migrate within the cell to distant insertion site(s) are unknown. In the case of cortical neurons projecting to the spinal cord, the presynaptic membrane may be a meter or more from the soma. Some specific tubulin isoforms, because of either unique peptide sequences or posttranslational modification, could be prevented from interaction with the isoforms in microtubules. Soifer and Czosnek (1980) propose that only nascent tubulin peptides can interact with membranes; once tubulins assemble into microtubules they cannot be inserted into membranes.

2.3. Evidence for Posttranslational Modification

The number of unique genes that code for tubulins of different primary structure is less than the number of tubulin isoforms and therefore cannot account for the isoform heterogeneity detected by isoelectric focusing (see Sections 3 to 5). One source of microheterogeneity is differential tyrosylation of the α-tubulin isoforms. Tyrosine residues are encoded by two different α-tubulin mRNAs (Ginzburg *et al.*, 1981; Valenzuela *et al.*, 1981). Tyrosine can also be added posttranslationally to the carboxy terminus (Raybin and Flavin, 1975; Barra *et al.*, 1974). This reaction is reversible (Arce and Barra, 1985), does not utilize tyrosyl-tRNA, and is energy dependent (Barra *et al.*, 1974). Tyrosylation may be functionally important, as tyrosylated α-tubulins are differentially distributed to the cytoplasm rather than to the membrane *in vivo* (Nath and Flavin, 1978). α-Tubulin cannot be detyrosylated unless it is in microtubules, perhaps because the detyrosylating enzyme is associated with microtubules (Arce and Barra, 1985). In chick, tyrosylation of tubulin is developmentally regulated (Rodriguez and Borisy, 1978).

Another source of microheterogeneity could be phosphorylation of the α- and β-tubulin subunits (Eipper, 1972; Reddington *et al.*, 1976; Forgue and Dahl, 1979). The *in vivo* phosphate content per mole of brain microtubule protein dimer is high in the β-tubulins, whereas the carbohydrate and amino sugar levels are negligible (Eipper, 1972). In *in vitro* studies where [^{32}P]ATP was the substrate, five different peptide fragments were phosphorylated (Eipper, 1974a) whereas only one peptide is phosphorylated *in vivo* (Eipper, 1974b). However, sequence analysis of pig brain tubulins shows no evidence for posttranslational modifications (Krauhs *et al.*, 1981; Ponstingl *et al.*, 1981).

Early attempts to phosphorylate neuroblastoma tubulin *in vivo* were unsuccessful (Littauer *et al.*, 1976; Solomon *et al.*, 1976; Schmitt, 1976; Eddé *et al.*, 1982), but, recently, Gard and Kirschner (1985) showed that a minor isoform of β-tubulin is phosphorylated in neuroblastoma cells. Phosphorylation increases with neuroblastoma differentiation. Phosphorylation is also coupled to the levels of intracellular microtubules, suggesting that tubulin

phosphorylation is regulated by the levels of microtubules during differentiation (Gard and Kirschner, 1985).

Both rat brain and glioma tubulin can act as a substrate for ribosylation reactions *in vitro* (Amir-Zaltsman *et al.*, 1982; Hawkins and Browning, 1982). *In vivo* ribosylation has not yet been demonstrated.

Posttranslational modification must occur during axoplasmic transport in mouse brain since there is a progressive alteration in the pattern of α-tubulin isoforms when tubulin is transported down the optic nerve (Brown *et al.*, 1982). Electrophoretic separation of the α- and β-tubulins from [^{35}S]methionine-labeled rat sympathetic cells in culture also shows that, relative to pulse time, there are late-arising, more acidic, tubulin α-isoforms (Black and Kurdyla, 1983) that resemble the posttranslationally modified isoforms described by Brown *et al.* (1982). A more rapidly moving α-tubulin subunit, Tax, is found in axons of the guinea pig dorsal motor nucleus (Tashiro and Komiya, 1983). A functional difference between axonally transported tubulin and total brain tubulin has recently been described by Brady *et al.* (1984). In contrast to the cold lability of total brain tubulin, approximately 50% of the α- and β-axonally-transported tubulins are cold-stable. The α-tubulins in this fraction are so basic that they do not focus on equilibrium isoelectric focusing gels (Brady *et al.*, 1984). Nothing is known either about the nature of this modification or its functional significance. From these studies, one may conclude that posttranslational modifications can occur at relatively long times after translation and at sites distant from the cell body. Therefore, particular tubulin isoforms may not only perform different intracellular functions, but one isoform may perform more than one function depending on its posttranslational modification.

2.4. Developmental Regulation of Tubulin Isoforms

Fellous *et al.* (1975) showed that immature rat brain tubulin assembles into microtubules less readily than that of the adult. This could be related to the low levels of the assembly-initiator proteins, tau (Witman *et al.*, 1976), in immature brain. Mareck *et al.* (1980) have shown that the tau proteins increase in activity and heterogeneity with brain development. This heterogeneity is controlled at the mRNA level (Ginzburg *et al.*, 1982). For a further review of the roles of tau and MAP proteins in tubulin function, see Littauer and Ginzburg (1985).

Total tubulin levels are highest in the immature brain and decrease with age (Littauer *et al.*, 1976; Schmitt *et al.*, 1977; Morrison *et al.*, 1981). The levels of soluble tubulin increase in the rat and kitten visual cortex (but not rat motor cortex) during the two week interval after eye opening (Cronly-Dillon and Perry, 1976, 1978). This increase does not occur in dark-reared rats, and only takes place if rats are exposed to light during the critical period in visual development (Perry and Cronly-Dillon, 1978).

Superimposed on the developmental decrease in total brain tubulin, there is a regulated change in the steady-state levels of different α- and β-tubulin isoforms. Using isoelectric focusing, Dahl and Weibel (1979) showed

an age-related increase in rat brain tubulin isoform heterogeneity. In addition, they showed that the levels of the different isoforms were developmentally regulated; one β-tubulin decreased to adult levels after postnatal day 8, and two more acidic β-tubulin forms—not present in the day 1 rat brain—appeared after day 5 and reached adult levels between days 14 and 17. One tubulin isoform that was present in the neonates, but not in the adult, could not be categorized as either an α- or β-isoform. Such changes in microheterogeneity may reflect changes in assembly properties of microtubule proteins, changes in the distribution of mitotic spindles, and growth of axons and dendrites.

Developmental changes in tubulin isoform heterogeneity have also been studied in the mouse. There are five to six α-isoforms and 10 or more β-isoforms in mouse brain (von Hungen *et al.*, 1981). Two of the isoforms (α6 and β2) decreased to undetectable levels during development, while two others (β6 and β10), only detectable postnatally, were at their highest levels in the adult. Denoulet *et al.* (1982) showed that the number of total isoforms increases from six (four α, two β) in embryonic mouse brain to 11 (six α, five β) in the adult. In addition, the α- and β-tubulin isoforms were shown to be independently regulated; the changes in the α-isoforms occur prenatally whereas the increase in β-isoform heterogeneity is largely postnatal.

The isoelectric variants of tubulin in the chick brain include seven α-isoforms and 10 β-isoforms; most are present in the 13-day embryo, but their relative levels are developmentally modulated (Sullivan and Wilson, 1984). As noted in the mouse and rat, changes in the levels of a number of the chick tubulin isoforms can be temporally correlated with developmental events occurring in the embryo and in the chick, after hatching (Sullivan and Wilson, 1984).

Using the α- and β-tubulin antibodies mentioned in Section 2.2.1. (α; YL1/2 and YOL/34, Kilmartin *et al.*, 1982; and β; TUB 2.1, Gozes and Barnstable, 1982), Cumming *et al.*, (1984a) have shown that α-tubulin undergoes modification during cerebellar development. This modification occurs in granule cell axons (parallel fibers) concomitant with synapse formation, but not in other cell bodies or their processes. Cerebellar granule-cell-enriched cultures did not show similar modifications in α-tubulin during cell synapse formation (Cumming *et al.*, 1984b).

The increase in tubulin heterogeneity with brain development can be correlated with periods of intense proliferation of neurons in the cerebellum as well as migration, synapse formation, and growth of axons and dendrites in both cerebellum and cerebral cortex. Thus, increased heterogeneity and changes in the levels of specific isoforms may reflect (1) the utilization of a specific isoform(s) for a specific function(s) in all cell types or (2) the requirement of a particular cell type for a specific isoform.

Hormones are one of the possible regulators of tubulin synthesis and heterogeneity. Chaudhury and Sarkar (1983) have shown that thyroid hormone (T_3) increase tubulin synthesis by 60–80% in organ cultures of the perinatal rat brain. This induction is dose dependent and tissue specific;

physiological doses are most effective, and the sensitivity is greatest when the T_3 receptors on brain cell nuclei are at a maximum. Similarly, Gonzales and Geel (1978) showed that the concentration of cellular tubulin was less in 25-day-old hypothyroid rats.

Assembly of microtubules is also dependent on adequate levels of thyroid hormone in the neonatal rat brain (Fellous *et al.*, 1979). The rate of microtubule assembly can be increased by adding either sonicated microtubule fragments or MAPs, suggesting that thyroid hormone may regulate functional MAP levels. In contrast, the assembly of adult brain microtubules is not thyroid hormone sensitive. Hypothyroid neonatal guinea pig, with its prenatally developed brain, is similarly thyroid hormone insensitive (Fellous *et al.*, 1979). Thus, the accelerated rate of microtubule assembly necessary for the growth of neural cell processes is thyroid hormone dependent during development.

3. Tubulin Genes in Higher Eukaryotes

3.1. α-Tubulin Genes

The electrophoretic heterogeneity of the α- and β-tubulin *in vitro* translation products was the first evidence that these proteins might be products of a multigene family (Gozes and Littauer, 1978; Marotta *et al.*, 1978). Direct peptide sequencing of porcine brain tubulins shows that at least four different α-tubulins are expressed in the adult of this species (Ponstingl *et al.*, 1981). Southern analysis using the chick α-tubulin cDNA probe showed that chick genomic DNA contains approximately four homologous sequences (Cleveland *et al.*, 1980), whereas human, rat, and mouse genomic DNA each contain between 10 and 20 (Cleveland *et al.*, 1980; Littauer and Ginzburg, 1985). The four chick α-tubulin genes are not physically clustered in the genome (Cleveland *et al.*, 1981); at least one is on chromosome 1 and another is on chromosome 8.

Nine genomic clones that hybridized to the chick α-tubulin probe were isolated from two independently constructed human genomic libraries (Wilde *et al.*, 1982a). Restriction mapping of the cloned fragments showed that these clones contained a minimum of two and a maximum of four different α-tubulin genes (Wilde *et al.*, 1982a,b). The number of functional human α-tubulin sequences and nonexpressed pseudogenes remains to be determined. In rat, one α-tubulin gene and two α-tubulin pseudogenes have been described (Lemischka and Sharp, 1982; Littauer and Ginzburg, 1985).

3.2. β-Tubulin Genes

The first β-tubulin cDNA clone was also isolated from the embryonic chick brain cDNA library of Cleveland *et al.* (1980). This β-tubulin clone also exhibits strong cross-species homology; in Southern analysis, it hybridized to

as many as four DNA fragments in chick and between 15 and 20 fragments in human genomic DNA (Cleveland *et al.*, 1980). Four unique clones, β1–4, were isolated from a chick genomic library (Lopata *et al.*, 1983). Each of the four genes is transcribed into a specific mRNA (see Section 5.2.1). A recent report from this group indicates that there may be an additional three to five β-tubulin genes in the chick genome (Sullivan and Cleveland, 1984). The chick β-tubulin genes are located on at least two different chromosomes, one of which is chromosome 2 (Cleveland *et al.*, 1981).

Unlike the chick, most of the members of the human β-tubulin multigene family are probably nonexpressed pseudogenes. Of the 10 human β-tubulin sequences analyzed by Cowan's group, five are intronless pseudogenes, two are pseudogenes with intervening sequences, and only three are transcribed into functional mRNAs (Cowan *et al.*, 1981; Wilde *et al.*, 1982c; Hall *et al.*, 1983; Gwo-Shu Lee *et al.*, 1983). Although three different rat β-tubulin cDNA clones have been isolated (Bond *et al.*, 1984; see Section 5.2.2.), no sequence analysis of the rat β-tubulin gene family has been published to date.

4. *Expression of α-Tubulin mRNAs*

4.1. *In Vitro Translation Products of α-Tubulin mRNAs*

The decrease in the steady-state levels of total brain tubulins during brain development is accompanied by a decrease in the levels of translationally active tubulin mRNAs (Littauer *et al.*, 1976; Schmitt *et al.*, 1977; Sato *et al.*, 1978; Morrison *et al.*, 1981). Although multiple α-tubulin isoforms are resolved when total brain tubulin is separated on high-resolution isoelectric focusing gels (Sullivan and Wilson, 1984; Field *et al.*, 1984), only two α-tubulin *in vitro* translation products have been resolved (Gozes *et al.*, 1980). Separation of *in vitro* translation products of mRNAs isolated from mouse neuroblastoma cells and chick, rat, and human brain by two-dimensional gel electrophoresis resolves two α-tubulin isoforms with the same isoelectric points but different molecular weights (Morrison *et al.*, 1979, 1981, 1983; Bryan *et al.*, 1978; Marotta *et al.*, 1979b; Strocchi *et al.*, 1981; Morrison and Griffin, 1981; Gilbert *et al.*, 1981). The levels of the mRNAs encoding these α-tubulin isoforms decrease during neuroblastoma differentiation (Morrison *et al.*, 1980). Their levels also decrease concomitant with neuronal differentiation in rat and human cerebellum and cortex (Morrison *et al.*, 1981, 1983; Morrison and Griffin, 1985a,b). There is no evidence from *in vitro* translation data that different α-tubulin isoforms are developmentally regulated in the brain.

4.2. *Hybridization Analysis of Different α-Tubulin mRNAs*

Sequencing and hybridization analyses of the clones isolated from chick and mammalian cDNA libraries provide evidence for the regulated transcription of several α-tubulin genes. Cowan *et al.* (1983) isolated an α-tubulin clone

(kα1) from a human keratinocyte cDNA library and one (bα1) from a human fetal brain cDNA library. Sequence analysis showed that the coding regions of the mRNAs have 97% homology, yet the 3′ untranslated regions are completely dissimilar (Cowan *et al.*, 1983). Hybridization to a 3′ region subclone of the bα1 cDNA showed that its mRNA is expressed only in fetal brain (adult brain was, however, not tested).

Sequence comparisons between two rat brain cDNAs indicate that at least two different α-tubulin mRNAs are expressed in the adult rat (Ginzburg *et al.*, 1980, 1981; Lemischka *et al.*, 1981). The two mRNAs have strong sequence homology in their coding regions and show sequence divergence in the 3′ noncoding regions. Three different α-tubulin cDNA clones have been isolated from a Chinese hamster ovary cDNA library (Elliott *et al.*, 1985). Again, there is little homology in the 3′ noncoding sequences of these different mRNAs.

The 3′ untranslated region of the mRNA corresponding to the human bα1 clone (Cowan *et al.*, 1983) was compared to the mRNA corresponding to the rat brain pILαT1 clone (Lemischka *et al.*, 1981), and to that of the Chinese hamster ovary, gene 2 α-tubulin mRNA (Table 1). Surprisingly, greater than 80% homology was found between the 3′ untranslated regions of all three mRNAs, showing strong cross-species conservation of this region (Cowan *et al.*, 1983; Elliott *et al.*, 1985). There is 42% homology between the 3′ noncoding region of the chick α-tubulin clone, pT1, and that of the human bα1 clone (Elliott *et al.*, 1985). Similarly, there is 64% homology between the 3′ noncoding regions of the kα1 human clone and another Chinese hamster ovary clone, Gene 1, and 53% homology between Kα1 and the pT25 rat clone (Ginzburg *et al.*, 1981; Cowan *et al.*, 1983; Elliott *et al.*, 1985). The α-tubulins encoded by the kα1, Gene 1, and pT25 mRNAs have identical carboxy termini (Elliott *et al.*, 1985). A third expressed α-tubulin gene, Gene 3, has been found only in Chinese hamster ovary cells (Elliott *et al.*, 1985). In addition, the mRNA transcribed from this gene encodes a protein that differs from the other Chinese hamster ovary transcripts by 4 out of 25 amino acids at the carboxy terminus, and it is two amino acids shorter. Elliott *et al.* (1985) determined, by hybridization to oligonucleotide probes specific for each mRNA, that 71% of the α-tubulin mRNAs expressed in Chinese hamster ovary cells

Table 1. Relationship between α-Tubulin mRNAs from Different Species[a]

Species	Chinese Hamster Ovary	Human	Rat	Chick
Clones	Gene I	Kα1	pT25.	?
	Gene II	bα1	pIIαT1	pT1
	Gene III	?	?	?

[a]Modified from Elliot *et al.*, 1985.

are encoded by Gene 1, 5% by Gene 2, and 24% by Gene 3. No hybridization analysis of the different brain α-tubulin mRNAs has been published.

5. *Expression of* β-*Tubulin mRNAs*

5.1. *In Vitro Translation Products of* β-*Tubulin mRNAs*

5.1.1. *Mouse Neuroblastoma Cells*

Two β-tubulin isoforms, β_1 and β_2, are resolved when unlabeled mouse neuroblastoma proteins are electrophoresed on two-dimensional gels (Morrison *et al.*, 1979 and Fig. 1). Our results on cytoplasmic compartmentalization, translational efficiency, and degree of adenylation in neuroblastoma show that a minimum of two separate and independently regulated mRNAs must encode these isoforms (Morrison and Griffin, 1985b). One way that these mRNAs can be differentiated is by their cytoplasmic compartmentation. Approximately 30% of neuroblastoma mRNAs are not polysomal but rather are found in cytoplasmic mRNA ribosome protein particles (mRNPs; Croall and Morrison, 1980). Comparison of *in vitro* translation products of the polysomal mRNAs and mRNPs showed that the mRNA encoding the more acidic β_1-subunit was present on polysomes but was virtually absent from the mRNP fraction. The mRNA encoding the β_2-subunit was present in both fractions.

To test the possibility that—as suggested by our results—the β_1-tubulin mRNA is intrinsically a better translational initiator than is the β_2-mRNA, we compared the translational efficiencies of the β_1- and β_2-mRNAs under conditions where ribosomes and initiating factors were rate limiting. Contrary to our prediction, the β_1-mRNA is not a better initiator than β_2; it is less well translated than the β_2-mRNA (Croall and Morrison, 1980). Therefore, factors other than the intrinsic translational efficiency of the β_1- and β_2-mRNAs must determine the distribution of these mRNAs between polysomes and mRNP particles *in vivo*.

In addition to their differential cytoplasmic distribution, the neu-

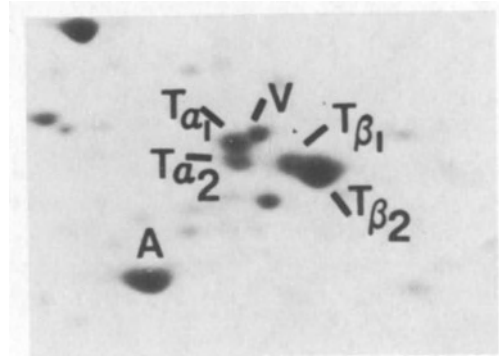


Figure 1. Two-dimensional gel electrophoresis of unlabeled proteins from neuroblastoma S-20 cells.

Only the tubulin area of the gel is shown in this and subsequent electrophoretograms. The isoelectric focusing dimension is from left to right. The molecular weight dimension is from top to bottom; A, actin; V, vimentin; $T\alpha_1$, $T\alpha_2$, α_1- and α_2-tubulins; $T\beta_1$, $T\beta_2$, β_1- and β_2-tubulins.

roblastoma β_1- and β_2-tubulin mRNAs have different degrees of adenylation. Comparison of the *in vitro* translation products of adenylated and nonadenylated neuroblastoma mRNAs showed a relative enrichment of the β_2-mRNA in the nonadenylated mRNA fraction, whereas almost 100% of the β_1-mRNA is adenylated (Morrison *et al.*, 1979).

Some aspects of neuronal differentiation can be mimicked in neuroblastoma cells by increasing intracellular cAMP levels (Prashad and Rosenberg, 1978). cAMP-treated neuroblastoma and glioma cells have transiently increased steady-state total tubulin mRNA levels (Ginzburg *et al.*, 1983a). Three days after cAMP treatment, tubulin mRNA levels are less than control values (Morrison *et al.*, 1980; Ginzburg *et al.*, 1983a). It is possible that hybridization with cDNA subclones specific for individual mRNAs may demonstrate cAMP-induced changes in the relative steady-state levels of different tubulin mRNAs. Neurite outgrowth in neuroblastoma is accompanied by a posttranslational modification of one of the β-tubulin subunits (Eddé *et al.*, 1981).

5.1.2. Rat Cerebellum and Cortex

Developmental changes in the different β-tubulin isoforms have been most extensively documented in rat brain. *In vitro* translation products of fetal head and 30-day postnatal rat brain are resolved into five isoforms, each differing in isoelectric point (Gozes *et al.*, 1980). Synthesis of β-tubulin isoforms 5 and 6 decrease developmentally whereas synthesis of isoform 7 is relatively increased (Gozes *et al.*, 1980). These authors showed that the mRNAs encoding the different isoforms can be partially separated on sucrose gradients.

To facilitate correlation of changes in mRNA levels with specific developmental events, we evaluated the *in vitro* synthesis of the different β-tubulin isoforms during the development of the rat cerebellum and cortex. After short-term *in vivo* labeling, two radiolabeled cerebellar β-tubulins are resolved on two-dimensional gels (Morrison *et al.*, 1981). These correspond in molecular weights and isoelectric points to the β_1- and β_2-tubulins previously identified in neuroblastoma (see Section 5.1.1). There is relatively more of the β_1-isoform synthesized in neonatal cerebellum than in adult although the synthesis of both isoforms was lower in the adult. These results show that the developmental decreases in β-tubulins *in vivo* are, at least in part, the result of altered levels of synthesis.

In order to directly quantitate tubulin mRNA levels, total RNA was isolated from rat cerebellum at 2, 6, 14, 30, and 90 days postnatal, and *in vitro* translation products were displayed on two-dimensional gels (Morrison *et al.*, 1981). Two β-tubulins are clearly resolved (Saborio *et al.*, 1978; Marotta *et al.*, 1979b; Strocchi *et al.*, 1981). The minor component, β_1, is present at approximately 25% of the major β_2-isoform level at early developmental stages when the granule cells in the external granule layer were proliferating and then migrating into the internal granule layer (Addison, 1911; Altman, 1972;

Zagon and McLaughlin, 1979). The level of the β_1-mRNA decreases by greater than 75% between days 14 and 30, a time during which granule cell proliferation finally ceases and the rate of synaptogenesis and myelination are high (Altman, 1972; Clark *et al.*, 1978; Campagnoni *et al.*, 1978). The levels of the major cerebellar tubulin mRNA, β_2, follow approximately the same time course although its levels remain relatively high in the adult (Morrison *et al.*, 1981).

Similar results were obtained when the translation products of poly(A)$^+$ mRNAs isolated from 1-month and adult human cerebellum were compared. The β_1-tubulin mRNA, present at 1 month, is barely detectable in the adult. As in the rat, the amount of β_2-mRNA is only slightly decreased in adult cerebellum (Fig. 2 and Morrison and Griffin, 1985b).

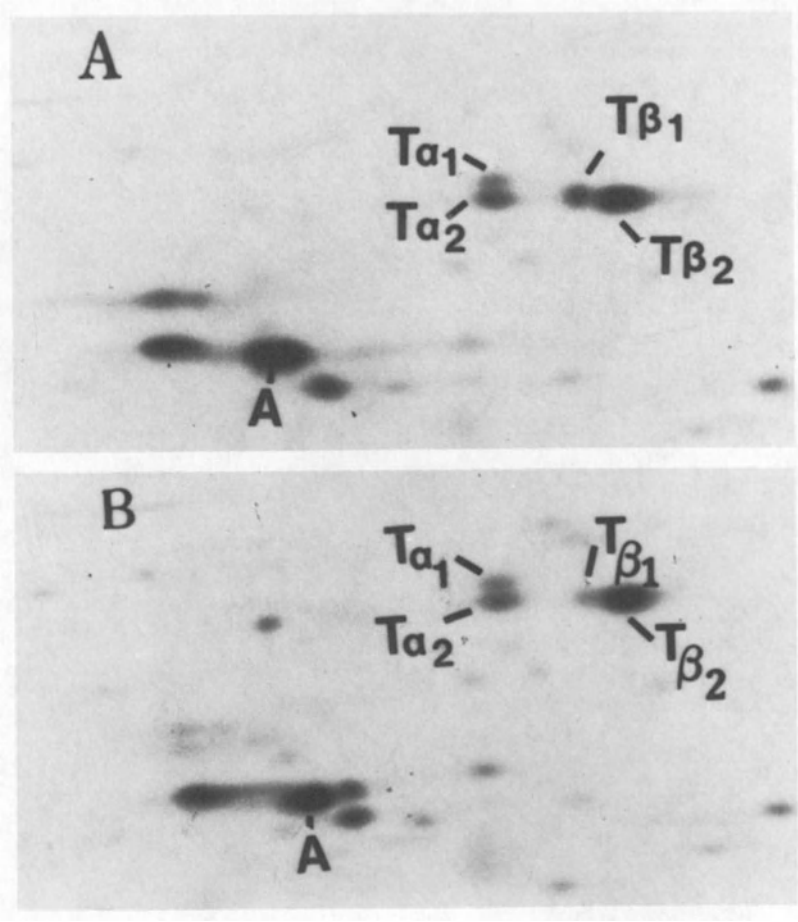


Figure 2. Two-dimensional gel electrophoresis of wheat germ [^{35}S]-labeled *in vitro* translation products of poly(A)$^+$ RNAs isolated from 1-month (A) and adult (B) human cerebella.

In order to further clarify the roles of these differentially regulated tubulins, we analyzed the levels of their mRNAs in developing and adult cerebral cortex, where the time frame of development is earlier than in cerebellum. Division of virtually all cortical cells takes place prenatally (Berry *et al.*, 1964; Hicks and D'Amato, 1968). Migration and axonal elaboration primarily occur during the first week of postnatal life (Eayrs and Goodhead, 1959). Most dendritic arborization and synaptogenesis occurs between days 12 and 20 postnatal (Eayrs and Goodhead, 1959).

In fetal rat cortex, the levels of both the β_1- and β_2-mRNAs are higher than in adult cortex. The level of mRNA encoding the β_1-tubulin isoform is equivalent to that encoding the β_2-isoform, whereas, even at the earliest stage of cerebellar development, the β_1-mRNA is much less abundant (Morrison and Griffin, 1985,a,b). The levels of cortical β_1-tubulin mRNA are equivalent to cerebellar levels by 10 days and, as in cerebellum, are barely detectable in the adult. β_2-mRNA levels also remain relatively high in adult cortex. The developmental profile of β_1- and β_2-mRNAs in fetal and adult human cortex mirrors that of rat cortex (Fig. 3 and Morrison *et al.*, 1983).

As in neuroblastoma, the levels of β_1-tubulin mRNA are higher than the levels of β_2 in the fetal cortex poly(A)$^+$ fraction, while the β_2-mRNA is more abundant than β_1 in the poly(A)$^-$ fraction (Morrison and Griffin, 1985b). These results show that the relative rate of deadenylation and/or the stability of the nonadenylated RNAs is similar in neuroblastoma and fetal cortex. Therefore, the rate of metabolism of the poly(A) tails may be an intrinsic property of these mRNAs.

Our results suggest that the β_1-tubulin may be more abundant in cell types characteristic of the developing cortex. Interestingly, the cortex does have a higher proportion of large cells than the cerebellum (Jacobson, 1978) whereas the small granule interneurons are the most abundant cerebellar cell type at all developmental stages (Addison, 1911). Another possible interpretation of our results would be that the β_1-tubulin is expressed at higher levels because, relative to cerebellum, during early stages of cortex development, a higher proportion of cells are synthesizing structures for which this tubulin subunit is essential.

5.2. Hybridization Analysis of β-Tubulin mRNAs

5.2.1. Chick and Human β-Tubulin mRNAs

Subclones (β1–β4) have been isolated that are specific for four chick β-tubulin mRNAs (Havercroft and Cleveland, 1984). Table 2 shows the molecular weights of the chick mRNAs, identified by hybridization to subclones specific for each transcript, and their relative steady-state levels in brain (Havercroft and Cleveland, 1984).

In all tissues examined, β4 is a major and β1 a minor component. β2 is present at relatively high levels in brain and lung and at lower levels in other tissues. β3 is the predominant β-tubulin mRNA in testis. It is also present in

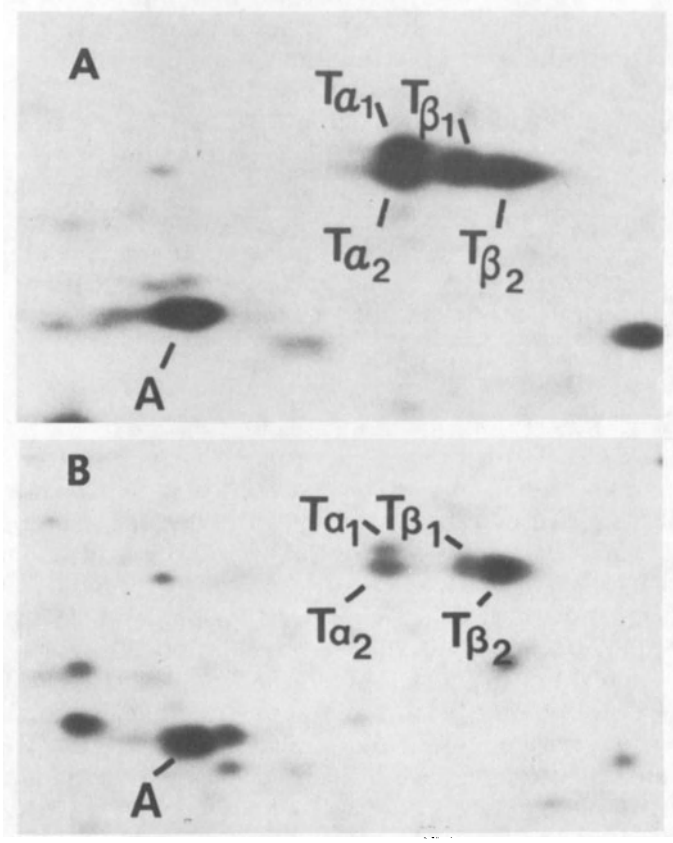


Figure 3. Two-dimensional gel electrophoresis of wheat germ [^{35}S]-labeled *in vitro* translation products of poly(A)$^+$ RNAs isolated from fetal (A) and adult (B) human cortex.

Table 2. The Size and Abundance of β-Tubulin mRNAs in Embryonic Chick Brain

Clone	mRNA size (kb)	Expression in chick brain
β1	4000 1800	Peaks in 12-day embryonic brain and decreases 50% by hatching
β2	1800	The major β-tubulin mRNA in embryonic brain; peaks at gestational day 12, decreasing to low levels at hatching
β3	1800	Fourfold increase during brain development
β4	3500	High levels in early fetal brain, decreasing to low levels at hatching

several other tissues. Usually, several of the β-tubulin mRNAs are present in one cell type. There is no evidence that any of the β-tubulin mRNAs are brain-specific, although the β2 mRNA is the predominant species in embryonic chick brain. In the adult brain, all four β-tubulin mRNAs are present at similarly low levels (Havercroft and Cleveland, 1984).

Two human β-tubulin cDNA clones, Dβ-1 and 5β, have been isolated (Hall *et al.*, 1983). These differ in the sequences at the carboxy termini and in the 3′ noncoding regions. In HeLa cells, a 3′ Dβ-1 subclone hybridizes to two mRNAs, 1.8 and 2.6 kb in length, suggesting that these mRNAs are derived from the same gene by differential processing (Hall *et al.*, 1983). A separate 2.6-kb mRNA is transcribed from the 5β gene (Hall *et al.*, 1983). These authors report that mRNAs transcribed from both genes are also found in human brain.

5.2.2. Rat β-Tubulin mRNAs

β-Tubulin mRNAs of different molecular weight and degree of tissue expression are also found in rat (Bond *et al.*, 1984). The results from this group are summarized in Table 3. In contrast to the lack of brain specificity observed with the chick gene-specific probes (Havercroft and Cleveland, 1984), the RβT.1 and RβT.2 mRNAs appear to be present only in brain (Bond *et al.*, 1984). However, the radiolabeled rat probes were hybrid to total RNAs isolated from other tissues whereas the chick probes were hybridized to isolated chick poly(A)$^+$ mRNAs. Therefore, it may be that the RβT.1 and RβT.2 mRNAs are present at low levels in tissues other than brain.

In order to correlate our *in vitro* translation data with the expression of particular tubulin genes, we have hybridized the chick β2-tubulin probe of Cleveland *et al.* (1980) to total RNAs isolated from different stages of cortical and cerebellar development. At early stages of cerebellar development, Northern analysis shows that the β2-probe only hybridizes to a 1.8-kb mRNA. At later developmental stages, hybridization to the 1.8-kb mRNA is decreased 10-fold, and hybridization to a 2.5-kb mRNA becomes detectable (Morrison and Griffin, 1985b). This pattern of hybridization is similar to that seen when the RβT.1 clone of Bond *et al.* (1984) was hybridized to rat cerebellar RNAs (Bond and Farmer, 1983; Bond *et al.*, 1984). The pattern of hybridization differs from that observed with the RβT.3 clone, encoding the predominant β-tubulin mRNA sequence in adult rat brain (Bond *et al.*, 1984). We conclude

Table 3. The Size and Abundance of Different β-Tubulin mRNAs in Rat Brain

Clone	mRNA size (kb)	Expression in rat brain
RβT.1	1.8	High levels early in development, decreasing to negligible levels in the adult
RβT.2	2.5	Only in adult
RβT.3	1.8	High levels at all stages of development but severalfold lower in adult
	2.9	High levels early in development

that the chick tubulin clone hybridizes predominantly to the RβT.1 mRNA at early developmental stages and to the 2.5-kb mRNA transcribed from the RβT.2 gene in the adult cerebellum. The faint hybridization of the chick probe to the 1.8-kb mRNA in the adult probably indicates that this clone does cross-hybridize to a lesser extent with the RβT.3 mRNA. This would be expected from the high-sequence homology between the tubulin coding sequences.

The results from our slot-blot analysis of β-tubulin mRNA levels in developing rat cerebellum and cerebral cortex are also very similar to those described by Bond and Farmer (1983) for their RβT.1 clone. Immediately postnatal, the β-tubulin mRNA levels are severalfold higher in cortex than they are in cerebellum (Fig. 4). At postnatal day 8, the levels in both brain areas are similar and gradually decrease with age. These hybridization results correlate with the levels of the β_1-tubulin isoform translated *in vitro* (see Section 5.1), suggesting that the β_1 subunit is encoded by the RβT.1 mRNA. The RβT.3 mRNA of Bond *et al.* (1984) probably encodes the major *in vitro* translation product, β_2. The minor β-tubulin mRNAs identified by hybridization analyses must encode proteins that comigrate with β_1 and β_2 and are not resolved on our two-dimensional gel system.

The pattern of hybridization of the chick β2 probe (RβT.1?) in cerebral cortex allows us to correlate particular developmental events with the requirement for its corresponding mRNA. The levels of this mRNA in cerebral cortex are higher several days after cell division has ceased (4 days postnatal) than they are perinatally (although they have not yet been quantitated earlier than fetal day 18). Taken together with the results in cerebellum, it appears that the levels of this tubulin mRNA correlate best with neuronal migration and process formation. This correlation would also explain the lower levels of this mRNA in cerebellum since the predominant neuronal cell type (granule) in this brain area has less dendritic arborization than does the predominant neuronal cell type (stellate/pyramidal) in cortex. The RβT.3 mRNA may code for tubulin isoform(s) that maintain cell shape in neurons at all developmental stages; the tubulin isoform encoded by the RβT.2 mRNA may be important in maintenance of cell/cell communication or in axonal transport in the mature

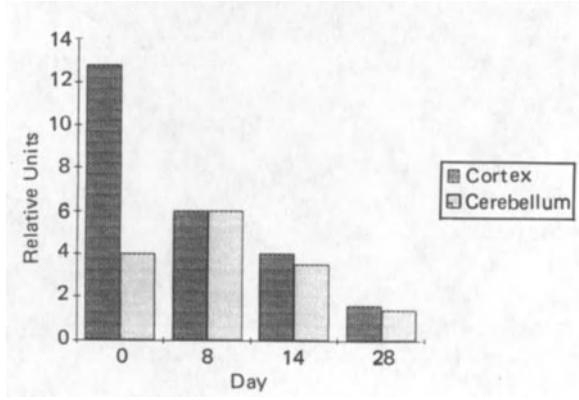


Figure 4. Relative levels of β-tubulin mRNAs during development of rat cortex and cerebellum. Total RNAs were isolated from rat cortex and cerebellum at different postnatal ages (Ilaria *et al.*, 1985); 1-μg and 2-μg total RNA inputs were slot-blotted and hybridized to the [^{32}P]-nick-translated chick β2-tubulin probe of Cleveland *et al.* (1980). Signals were quantitated by densitometric scanning.

nervous system. In addition, one or all of these mRNAs may be more abundant in glia (see Section 5.3).

Differential tissue expression and sequence homologies suggest relationships between the β-tubulin mRNAs expressed in chick, rat, and man. The original β-tubulin clone, chick β2, probably corresponds to the rat RβT.1 clone of Bond *et al.* (1984) as they both hybridize to the most abundant β-tubulin mRNAs (mol. wt. = 1.8 kb) in developing brain and their levels decrease similarly during development. Sequence comparisons indicate that the β-tubulin encoded by the human 5β gene has the greatest sequence homology to these mRNAs, although the mRNA transcribed by this gene is 2.6 kb in length (Hall *et al.*, 1983). Based solely on the relative hybridization of cDNA probes to fetal and adult brain, the chick β4 clone may be equivalent to the rat RβT.3 clone of Bond *et al.* (1984). Possible relationships between the other clones cannot be predicted at the present time.

5.3. In Situ Hybridization of Chick β2-Tubulin cDNA in Rat Cerebellum

We used the technique of *in situ* hybridization to determine (1) whether β-tubulin mRNAs, hybridizing to the chick β2-tubulin cDNA, are differentially distributed among cerebellar cell types and (2) the developmental stage at which the levels of this mRNA are modulated in a specific cell type. We assessed total poly(A)$^+$ mRNA content in different cell types to correct for possible variation in (1) access of probe to its complementary mRNA or (2) mRNA leakage from cells during tissue preparation. Total poly(A)$^+$ mRNA was quantitated by hybridizing tissue sections to [^{3}H]poly(U). Adjacent sections were hybridized with the ^{3}H chick β2 DNA probe. After autoradiography, the amount of the specific mRNA in each cell type relative to the total poly(A)$^+$ mRNA was determined by quantitating the autoradiographic grain counts over individual cells in the adjacent sections (Griffin *et al.*, 1983, 1985; Griffin and Morrison, 1985). *In situ* hybridization of cerebellar sections from 14-day-old rats showed that autoradiographic grains were distributed over the cytoplasm of individual cells. Areas of the sections corresponding to axon terminals where no mRNAs should be present were devoid of autoradiographic grains (Figs. 5 and 6).

Control experiments showed that [^{3}H]poly(U) did not hybridize to sections pretreated with ribonuclease (Fig. 5D) and that there was negligible hybridization to sections hybridized to the [^{3}H]pBR322 vector alone (Fig. 6D). The grain counts over individual sections were highly reproducible (Table 4).

In contrast to the relatively uniform distribution of poly(A)$^+$ mRNA (Fig. 5), there was a differential distribution of the β-tubulin mRNAs in different cell types (Fig. 6 and Table 4). The relative amount of β-tubulin mRNA in the immature granule cells of the EGL was approximately twofold greater than that in the mature granule cells of the IGL (Table 4). It is clear from this result that the EGL cells require more of the β-tubulin mRNA, perhaps to synthesize enough of this tubulin for axon formation during migration through the molecular layer of the cerebellum into the IGL, than do IGL cells.

The relative amount of β-tubulin mRNA in the Purkinje and stellate cells of the 14-day cerebellum is approximately threefold less than in the granule cells of the IGL (Table 4). The Purkinje cells are formed prenatally whereas the stellate cells are formed postnatally (days 8–10; Addison, 1911; Altman, 1972). Both cell types are elaborating dendrites at 14 days, a process that may require less of this β-tubulin mRNA than does the maintenance of the axons of the granule cells of the IGL. A similar analysis of the β-tubulin mRNA distribution at earlier stages of cerebellar development will show whether the Purkinje and stellate cells, at more immature stages, contain greater amounts of this mRNA. In addition, such a developmental analysis will show whether perinatal EGL cells have the same amount of a particular β-tubulin mRNA as do late postnatal EGL cells.

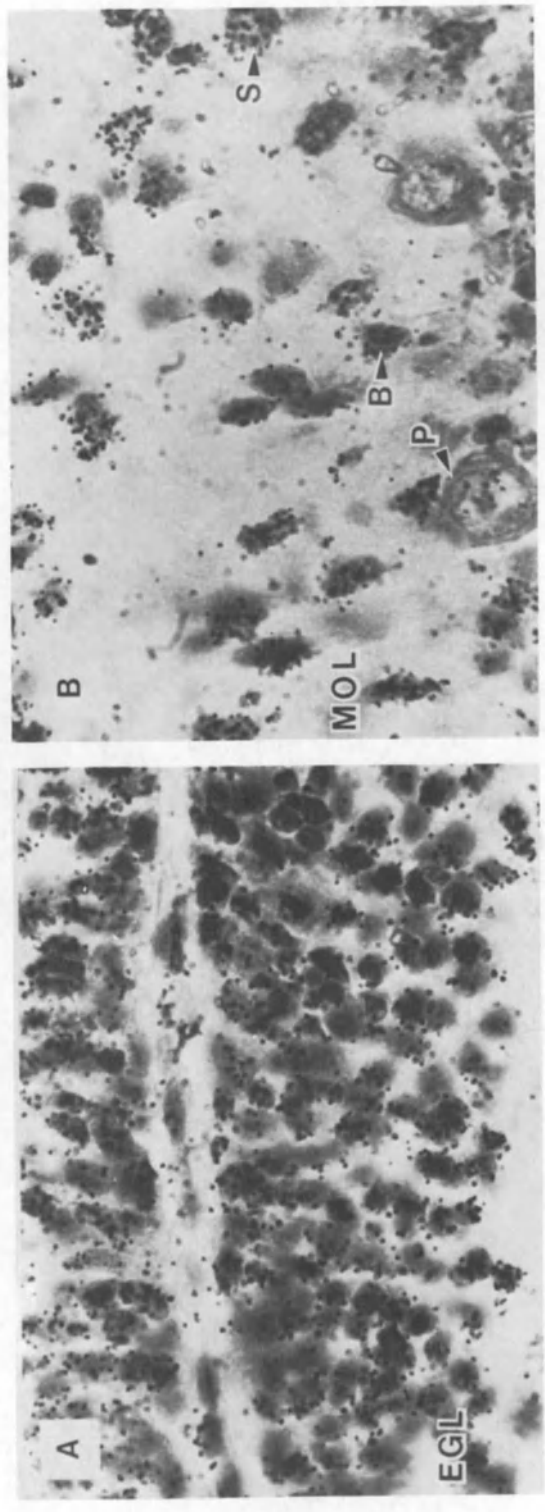
The β-tubulin mRNA hybridized in our experiments is not neuron-specific as it is also found in glial cells of the cerebellar white matter (Figure 7). Therefore, the differential distribution of this β-tubulin mRNA is not solely a function of neuronal process elaboration.

6. *Conclusions*

Several α- and β-tubulin genes are transcribed in different vertebrate species. Most regions of the encoded proteins are very similar, possibly because of the number of evolutionarily conserved domains necessary for tubulin function. Since the number of tubulin isoforms resolved on isoelectric focusing gels is greater than the number of genes expressed, some primary translation products must be posttranslationally modified *in vivo*. The levels of different steady-state tubulin isoforms and the levels of the mRNAs encoded by the different genes may be tissue specific and vary with developmental stage.

The functional significance of tubulin heterogeneity is still unclear. The different tubulin genes may have evolved so that cells could modulate tubulin synthesis in response to different stimuli. If this were true, the only physiologically significant difference between the tubulin genes would be in the regions of DNA controlling their transcription; evolutionary variation in their encoded proteins would not be functionally significant. It is more likely that the differences in the coding regions signify unique cellular functions for the tubulin isoforms.

Now that many of the different tubulin genes have been isolated and sequenced, definitive experiments can be performed to determine the relationship between the structure of the different tubulin isoforms and their function. For example, antibodies raised against *in vitro* synthesized peptides specific for the carboxy termini of the different tubulin isoforms could be used to determine whether these epitopes are differentially distributed within a cell or are present in different cell types. *In situ* hybridization with specific cDNA subclones would conclusively demonstrate the extent to which the mRNAs for specific isoforms are differentially distributed in different cell


A
EGL
B
MOL
S
B
P

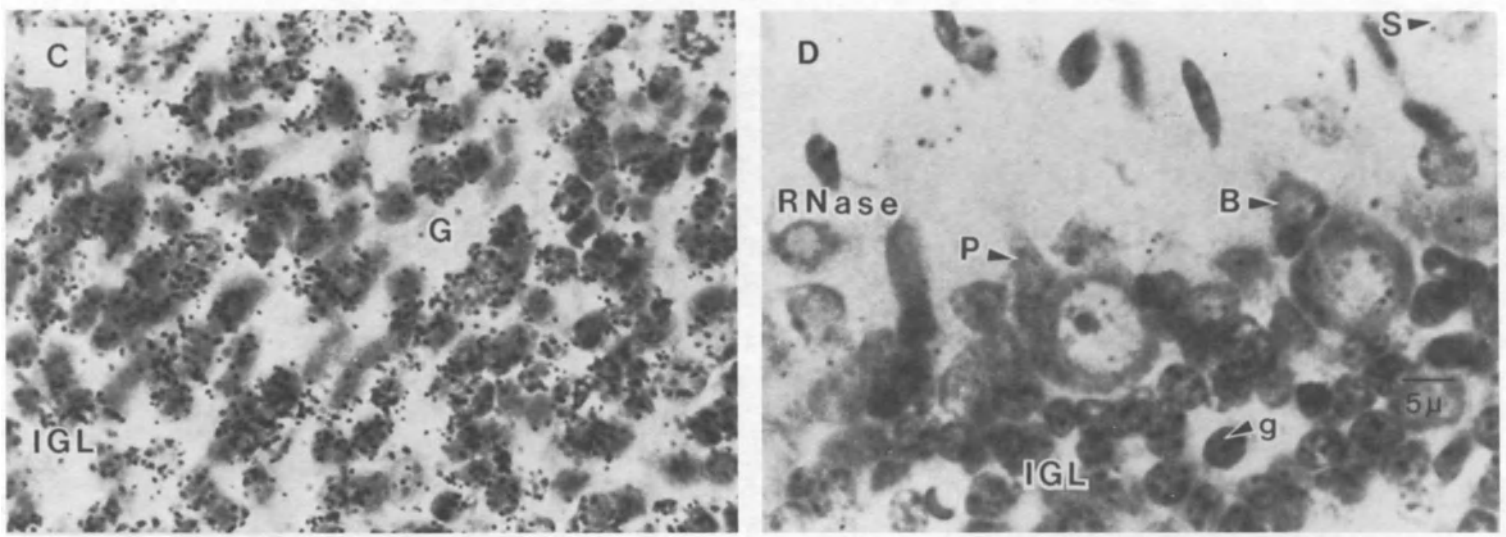
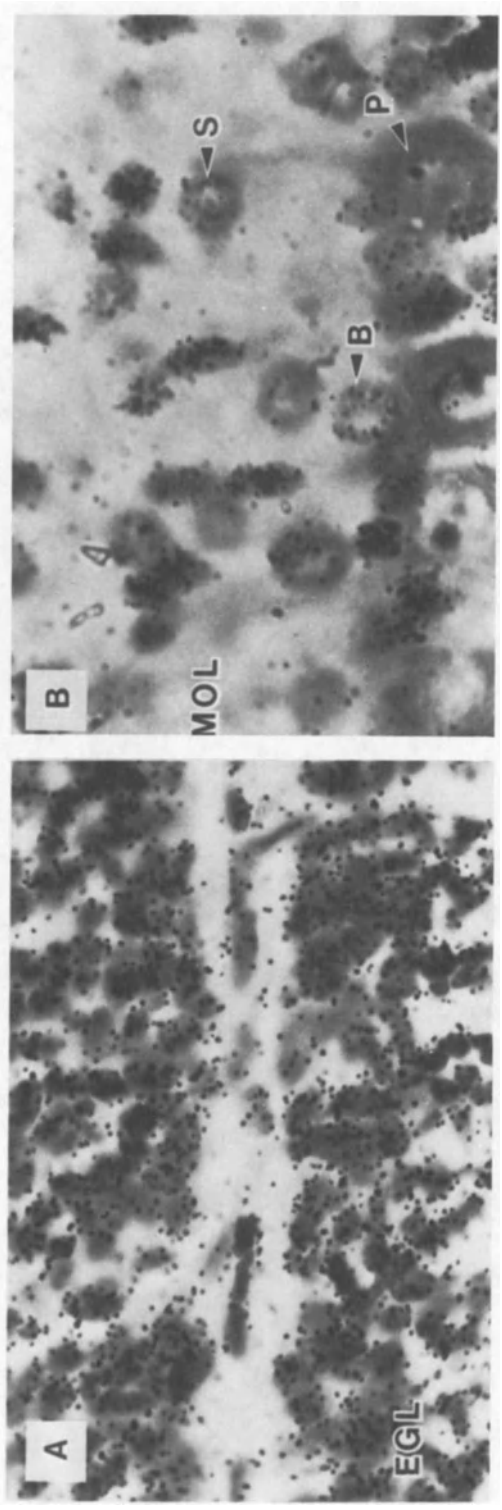


Figure 5. Autoradiographs of *in situ* hybridization of [^{3}H]poly(U). [^{3}H]poly(U) (3 Ci/mole; 39,500 cpm/section) was hybridized to cerebellar sections from 14-day-old rats. (A) Grain density over cells in the EGL. (B) Grain density in Purkinje cells (P) as well as in stellate (S) and basket (B) cells in the molecular layer. (C) Grain density in granule cells of the IGL. Note the paucity of grains over the mossy fiber axon terminals in the glomerulus (G). (D) Grain density in Purkinje cells and granule (g) cells of the IGL. The low density of grains over cells in D is due to pretreatment with RNAse A before hybridizations.


A
EGL
B
MOL
S
P
B

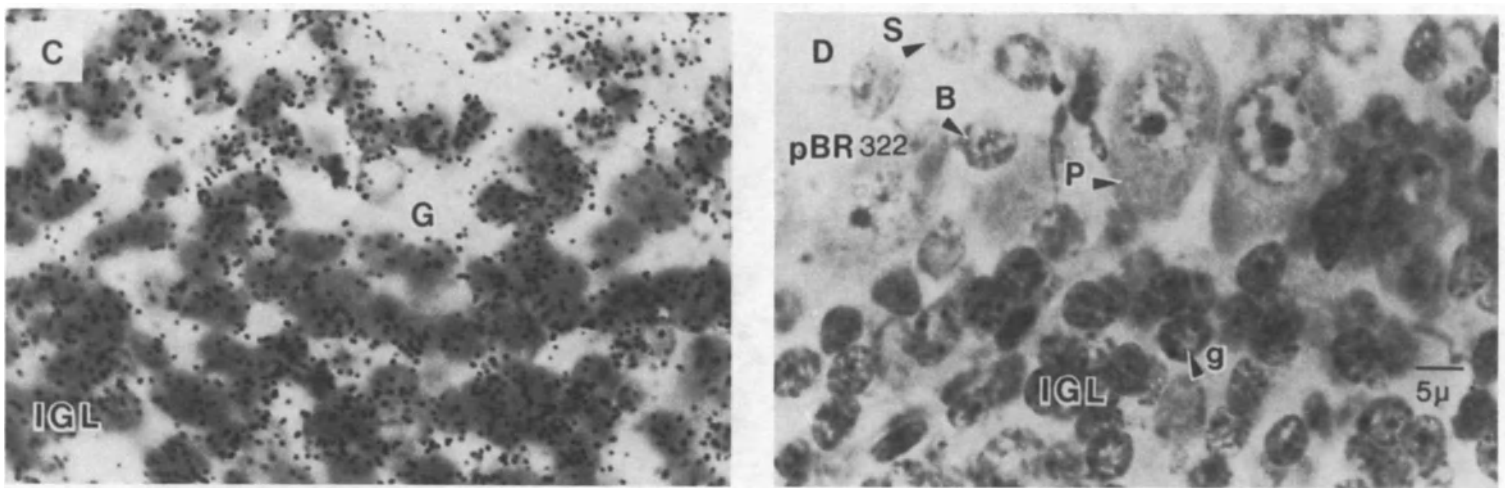


Figure 6. Autoradiographs of *in situ* hybridization of [^{3}H]-β2-tubulin probe and [^{3}H]-native pBR322. [^{3}H]-β2-tubulin probe (60,000 cpm/section (A–C) and native pBR322 (60,000 cpm/section) (D) were hybridized to cerebellar sections from 14-day-old rats. (A) Grain density over cells in the EGL. (B) Grain density in Purkinje cells (P) as well as in the stellate (S) and basket (B) cells in the molecular layer. (C) Grain density in granule cells (g) of the IGL. The low density of grains over cells in D shows the lack of hybridization of radiolabeled native pBR322 to cerebellar cells.

Table 4. The Distribution of Relative Levels of β-Tubulin mRNAs and Total Poly(A) mRNAs over Purkinje and Stellate Cells and the Granule Cells in the EGL and IGL[a]

	Grains per cell	Relative levels of β-tubulin mRNA
Purkinje cells		
β-tubulin	14.7 ± 0.3	0.48
poly (U)	30.1 ± 3.4	
Stellate cells		
β-tubulin	24.7 ± 1.8	0.35
poly (U)	70.6 ± 2.6	
EGL granule cells		
β-tubulin	53.0 ± 2.0	2.30
poly (U)	23.0 ± 2.4	
IGL granule cells		
β-tubulin	33.0 ± 2.4	1.36
poly (U)	24.0 ± 3.0	

[a]Grains were counted over cells such as those shown in Figs. 5 and 6. The 5-μm cerebellar sections, examined at 1000 diameters magnification, yielded "grains/cell" (mean ± SD of at least 100 cells of each cell type from three different experiments). Grains were counted by focusing at every level of the emulsion-coated slide. The relative level of β-tubulin mRNA in each cell type is the ratio of grains/cell type in sections hybridized to the [^{3}H]-β2-tubulin probe to the grains/cell type in sections hybridized to [^{3}H]poly (U).

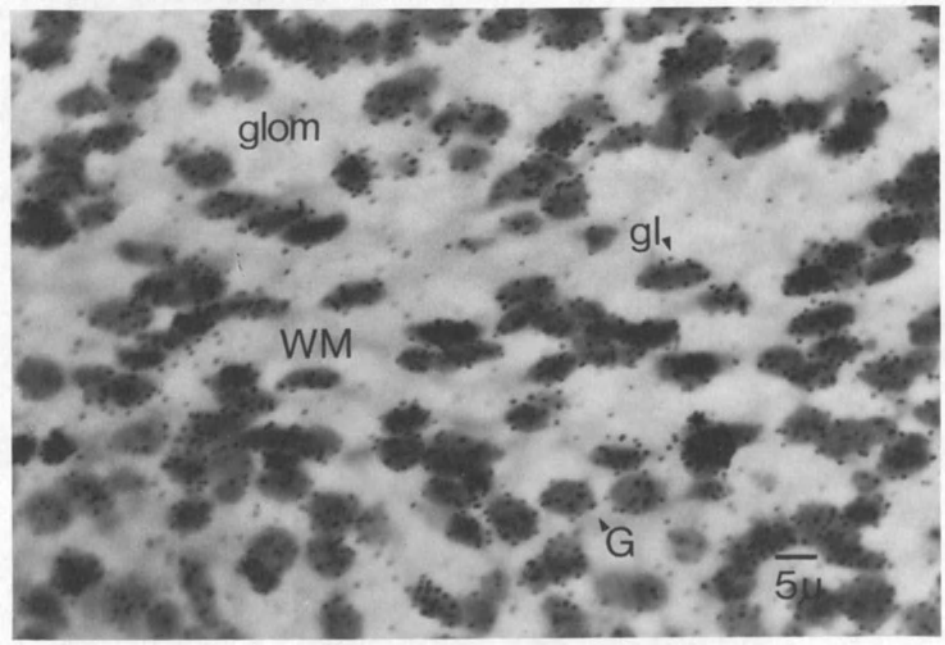


Figure 7. Autoradiographs of *in situ* hybridization of [^{3}H]-β2-tubulin probe to the arbor vitae of the cerebellum. [^{3}H]-β2-tubulin probe (60,000 cpm/section) was hybridized to cerebellar sections from 14-day-old rats. Grain density over glial (gl) cells in the arbor vitae (white matter, WM) and over granule cells (G) surrounding the glomerulus (glom).

types. Whether or not specific mRNAs are localized and translated in different regions of the cell could also be assessed by *in situ* hybridization.

Using a combination of specific antibodies and cDNA probes, the relationship between the different tubulin genes, their primary translation products, and any posttranslationally modified species could be clarified. For example, hybrid-selected translation using the specific tubulin subclones could be used to identify the primary translation product of each gene. Western blotting could then be used in combination with high-resolution isoelectric focusing to determine which tubulin isoform *in vivo* contains a particular epitope.

The cloned genes are being used to determine how the concentration of tubulin subunits modulates steady-state tubulin mRNA levels (see Chapter 9 by Cleveland in this volume). They will also be instrumental in determining the environmental influences regulating transcription of the different genes *in vivo*. The cloned genes could also be used in expression systems to determine the extent to which structurally different tubulin isoforms are utilized in different subsets of microtubules.

ACKNOWLEDGMENTS. This work was supported in part by NIH grants HD 14886, AI 14666, and AG 05537 and by the Leland Fikes Foundation. We acknowledge the excellent technical assistance of Sibile Pardue, Michael Alejos, and Riki Ison. We thank E. Katherine Miller and Lanya Lonsberry for editing and Annette Brown and Chris Claeson for typing the manuscript.

References

Addison, W. H. F., 1911, The development of the Purkinje cells and of the cortical layers in the cerebellum of the albino rat, *J. Comp. Neurol.* **21**:459.

Altman, J., 1972, Postnatal development of the cerebellar cortex in the rat, *J. Comp. Neurol.* **145**:353.

Amir-Zaltsman, Y., Ezra, E., Scherson, T., Zutra, A., Littauer, U. Z., and Salomon, Y., 1982, ADP-ribosylation of microtubule proteins as catalyzed by cholera toxin, *EMBO J.* **1**:181.

Amos, L. A., 1979, Structure of microtubules, in: *Microtubules* (K. Roberts and J. S. Hyams, eds.), pp. 1–64, Academic Press, New York.

Arce, C. A., and Barra, H. S., 1985, Release of C-terminal tyrosine from tubulin and microtubules at steady state, *Biochem. J.* **226**:311.

Barra, H. S., Arce, C. A., Rodriguez, J. A., and Caputto, R., 1974, Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins, *Biochem. Biophys. Res. Commun.* **60**:1384.

Berry, M., Rogers, A. W., and Eayrs, J. T., 1964, Pattern of cell migration during cortical histogenesis, *Nature* **203**:591.

Black, M. M., and Kurdyla, J. T., 1983, Post-translational modification of tubulin in cultured neurons, *Brain Res.* **289**:355.

Bond, J. F., and Farmer, S. R., 1983, Regulation of tubulin and actin mRNA production in rat brain: Expression of a new β-tubulin mRNA with development, *Mol. Cell Biol.* **3**:1333.

Bond, J. F., Robinson, G. S., and Farmer, S. R., 1984, Differential expression of two neural cell-specific β-tubulin mRNAs during rat brain development, *Mol. Cell. Biol.* **4**:1313.

Brady, S. T., Tytell, M., and Lasek, R. J., 1984, Axonal tubulin and axonal microtubules: Biochemical evidence for cold stability, *J. Cell Biol.* **99**:1716.

Brown, B. A., Nixon, R. A., and Marotta, C. A., 1982, Posttranslational processing of α-tubulin during axoplasmic transport in CNS axons, *J. Cell Biol.* **94**:159.

Bryan, J., 1974, Biochemical properties of microtubules, *Fed. Proc.* **33**:152.

Bryan, J., and Wilson, L., 1971, Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. USA* **68**:1762.

Bryan, R. N., Cutter, G. A., and Hayashi, M., 1978, Separate mRNAs code for tubulin subunits, *Nature* **272**:81.

Caceres, A., Binder, L. I., Payne, M. R., Bender, P., Rebhun, L., and Steward, O., 1984, Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies, *J. Neurosci.* **4**:394.

Campagnoni, C. W., Carey, G. D., and Campagnoni, A. T., 1978, Synthesis of myelin basic proteins in the developing mouse brain, *Arch. Biochem. Biophys.* **190**:118.

Chaudhury, S., and Sarkar, P. K., 1983, Stimulation of tubulin synthesis by thyroid hormone in the developing rat brain, *Biochim. Biophys. Acta* **763**:93.

Clark, B. R., Weichsel, M. E., Jr., and Poland, R. E., 1978, Correlation of DNA accumulation rate with thymidylate synthetase activity in developing rat cerebellum: Effect of hypothyroidism, *Biol. Neonate* **34**:209.

Cleveland, D. W., and Sullivan, K. F., 1985, Molecular biology and genetics of tubulin, *Ann. Rev. Biochem.* **54**:331.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirschner, M. W., 1980, Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes, *Cell* **20**:95.

Cleveland, D. W., Hughes, S. H., Stubblefield, E., Kirschner, M. W., and Varmus, H. E., 1981, Multiple α and β tubulin genes represent unlinked and dispersed gene families, *J. Biol. Chem.* **256**:3130.

Cowan, N. J., 1983, Tubulin isotypes and the multigene tubulin families, *Int. Rev. Cytol.* **85**:147.

Cowan, N. J., Wilde, C. D., Chow, L. T., and Wefald, F. C., 1981, Structural variation among human β-tubulin genes. *Proc. Natl. Acad. Sci. USA* **78**:4877.

Cowan, N. J., Dobner, P. R., Fuchs, E. V., and Cleveland, D. W., 1983, Expression of human α-tubulin genes: Interspecies conservation of 3′ untranslated regions, *Mol. Cell. Biol.* **3**:1738.

Croall, D. E., and Morrison, M. R., 1980, Polysomal and non-polysomal messenge rRNA in neuroblastoma cells: Lack of correlation between polyadenylation or initiation efficiency and messenger RNA location, *J. Mol. Biol.* **140**:549.

Cronly-Dillon, J. R., and Perry, G. W., 1976, Tubulin synthesis in developing rat visual cortex, *Nature* **261**:581.

Cronly-Dillon, J. R., and Perry, G. W., 1978, Tubulin synthesis in developing cerebral cortex of rat and kitten, *J. Physiol.* **287**:26.

Cumming, R., Burgoyne, R. D., and Lytton, N. A., 1982, Differential immunocytochemical localisation of α-tubulin and β-tubulin in cerebellum using monoclonal antibodies, *Cell Biol. Int. Rep.* **6**:1047.

Cumming R., Burgoyne, R. D., and Lytton, N. A., 1983a, Axonal sub-populations in the central nervous system demonstrated using monoclonal antibodies against α-tubulin, *Eur. J. Cell Biol.* **31**:241.

Cumming, R., Burgoyne, R. D., Lytton, N. A., and Gray, E. G., 1983b, Immunocytochemical evidence for tubulin in the presynaptic terminal of synaptosomes, *Neurosci. Lett.* **37**:215.

Cumming, R., Burgoyne, R. D., and Lytton, N. A., 1984a, Immunocytochemical demonstration of α-tubulin modification during axonal maturation in the cerebellar cortex, *J. Cell Biol.* **98**:347.

Cumming, R., Burgoyne, R. D., and Lytton, N. A., 1984b, Immunofluorescence distribution of α tubulin, β tubulin and microtubule-associated protein 2 during *in vitro* maturation of cerebellar granule cell neurones, *Neuroscience* **12**:775.

Dahl, J. L., and Weibel, V. J., 1979, Changes in tubulin heterogeneity during postnatal development of rat brain, *Biochem. Biophys. Res. Commun.* **86**:822.

de Néchaud, B., Wolff, A., Jeantet, C., and Bourre, J-M., 1983, Characterization of tubulin in mouse brain myelin, *J. Neurochem.* **41:**1538.

Denoulet, P., Eddé, B., Jeantet, C., and Gros, F., 1982, Evolution of tubulin heterogeneity during mouse brain development, *Biochimie* **64:**165.

Dustin, P., 1978, *Microtubules*, Springer-Verlag, New York.

Eayrs, J. T., and Goodhead, B., 1959, Postnatal development of the cerebral cortex in the rat, *J. Anat.* **93:**385.

Eddé, B., Jeantet, C., and Gros, F., 1981, One β-tubulin subunit accumulates during neurite outgrowth in mouse neuroblastoma cells, *Biochem. Biophys. Res. Comm.* **103:**1035.

Eddé, B., Portier, M.-M., Sahuquillo, C., Jeantet, C., and Gros, F., 1982, Changes in some cytoskeletal proteins during neuroblastoma cell differentiation, *Biochimie* **64:**141.

Eipper, B. A., 1972, Rat brain microtubule protein: purification and determination of covalently bound phosphate and carbohydrate, *Proc. Natl. Acad. Sci. USA* **69:**2283.

Eipper, B. A., 1974a, Rat brain tubulin and protein kinase activity, *J. Biol. Chem.* **249:**1398.

Eipper, B. A., 1974b, Properties of rat brain tubulin, *J. Biol. Chem.* **249:**1407.

Elliott, E. M., Okayama, H., Sarangi, F., Henderson, G., and Ling, V., 1985, Differential expression of three α-tubulin genes in Chinese hamster ovary cells, *Mol. Cell Biol.* **5:**236.

Estridge, M., 1977, Polypeptides similar to the α and β subunits of tubulin are exposed on the neuronal surface, *Nature* **268:**60.

Feit, H., and Shelanski, M. L., 1975, Is tubulin a glycoprotein? *Biochem. Biophys. Res. Commun.* **66:**920.

Feit, H., Slusarek, L., and Shelanski, M. L., 1971, Heterogeneity of tubulin subunits, *Proc. Natl. Acad. Sci. USA* **68:**2028.

Feit, H., Kelly, P., and Cotman, C. W., 1977a, Identification of a protein related to tubulin in the postsynaptic density, *Proc. Natl. Acad. Sci. USA* **74:**1047.

Feit, H., Neudeck, U., and Baskin, F., 1977b, Comparison of the isoelectric and molecular weight properties of tubulin subunits, *J. Neurochem.* **28:**697.

Fellous, A., Francon, J., Virion, A., and Nunez, J., 1975, Microtubules and brain development, *FEBS Lett.* **57:**5.

Fellous, A., Lennon, A. M., Francon, J., and Nunez, J., 1979, Thyroid hormones and neurotubule assembly *in vitro* during brain development, *Eur. J. Biochem.* **101:**365.

Field, D. J., Collins, R. A., and Lee, J. C., 1984, Heterogeneity of vertebrate brain tubulins, *Proc. Natl. Acad. Sci. USA* **81:**4041.

Forgue, S. T., and Dahl, J. L., 1979, Rat brain tubulin: subunit heterogeneity and phosphorylation, *J. Neurochem.* **32:**1015.

Gard, D. L., and Kirschner, M. W., 1985, A polymer-dependent increase in phosphorylation of β-tubulin accompanies differentiation of a mouse neuroblastoma cell line, *J. Cell Biol.* **100:**764.

George, H. J., Misra, L., Field, D. J., and Lee, J. C., 1981, Polymorphism of brain tubulin, *Biochemistry* **20:**2402.

Gilbert, J. M., Strocchi, P., Brown, B. A., and Marotta, C. A., 1981, Tubulin synthesis in rat forebrain: studies with free and membrane-bound polysomes, *J. Neurochem.* **36:**839.

Ginzburg, I., de Baetselier, A., Walker, M. D., Behar, L., Lehrach, H., Frischauf, A. M., and Littauer, U. Z., 1980, Brain tubulin and actin cDNA sequences: Isolation of recombinant plasmids, *Nucl. Acids Res.* **8:**3553.

Ginzburg, I., Behar, L., Givol, D., and Littauer, U. Z., 1981, The nucleotide sequence of rat α-tubulin: 3′-end characteristics, and evolutionary conservation, *Nucl. Acids Res.* **9:**2691.

Ginzburg, I., Scherson, T., Giveon, D., Behar, L., and Littauer, U. Z., 1982, Modulation of mRNAs for microtubule-associated proteins during brain development, *Proc. Natl. Acad. Sci. USA* **79:**4892.

Ginzburg, I., Rybak, S., Kimhi, Y., and Littauer, U. Z., 1983a, Biphasic regulation by dibutyryl cyclic AMP of tubulin and actin mRNA levels in neuroblastoma cells, *Proc. Natl. Acad. Sci. USA* **80:**4243.

Gonzales, L. W., and Geel, S. E., 1978, Quantitation and characterization of brain tubulin (Colchicine-binding activity) in developing hypothyroid rats, *J. Neurochem.* **30:**237.

Gozes, I., and Barnstable, C. J., 1982, Monoclonal antibodies that recognize discrete forms of tubulin, *Proc. Natl. Acad. Sci. USA* **79**:2579.

Gozes, I., and Littauer, U. Z., 1978, Tubulin microheterogeneity increases with rat brain maturation, *Nature* **276**:411.

Gozes, I., and Littauer, U. Z., 1979, The α-subunit of tubulin is preferentially associated with brain presynaptic membrane, *FEBS Lett.* **99**:86.

Gozes, I., and Richter-Landsberg, C., 1978, Identification of tubulin associated with rat brain myelin, *FEBS Lett.* **95**:169.

Gozes, I., and Sweadner, K. J., 1981, Multiple tubulin forms are expressed by a single neurone, *Nature* **294**:477.

Gozes, I., Saya, D., and Littauer, U. Z., 1979, Tubulin microheterogeneity in neuroblastoma and glioma cell lines differs from that of the brain, *Brain Res.* **171**:171.

Gozes, I., de Baetselier, A., and Littauer, U. Z., 1980, Translation *in vitro* of rat brain mRNA coding for a variety of tubulin forms, *Eur. J. Biochem.* **103**:13.

Griffin, W. S. T., and Morrison, M. R., 1985, *In situ* hybridization–visualization and quantitation of genetic expression in brain, *Peptides* **6 (Suppl. 2)**:89.

Griffin, W. S. T., Alejos, M., Nilaver, G., and Morrison, M. R., 1983, Brain protein and messenger RNA identification in the same cell, *Brain Res. Bull.* **10**:597.

Griffin, W. S. T., Alejos, M., Cox, E. J., and Morrison, M. R., 1985, The differential distribution of beta tubulin mRNAs in individual mammalian brain cells, *J. Cell. Biochem.* **27**:205.

Gwo-Shu Lee, M., Lewis, S. A., Wilde, C. D., and Cowan, N. J., 1983, Evolutionary history of a multigene family: An expressed human β-tubulin gene and three processed pseudogenes, *Cell* **33**:477.

Hajos, F., and Rostomian, M. A., 1984, Localization of α-tubulin immunoreactivity to cerebellar Bergmann glia with the TU 01 antibody, *Histochemistry* **81**:297.

Hall, J. L., Dudley, L., Dobner, P. R., Lewis, S. A., and Cowan, N. J., 1983, Identification of two human β-tubulin isotypes, *Mol. Cell. Biol.* **3**:854.

Havercroft, J. C., and Cleveland, D. W., 1984, Programmed expression of β-tubulin genes during development and differentiation of the chicken, *J. Cell Biol.* **99**:1927.

Hawkins, D. J., and Browning, E. T., 1982, Tubulin adenosine diphosphate ribosylation is catalyzed by cholera toxin, *Biochemistry* **21**:4474.

Hicks, S. P., and D'Amato, C. J., 1968, Cell migration to the isocortex in the rat, *Anat. Rec.* **160**:619.

Ilaria, R., Wines, D., Pardue, S., Jamison, S., Ojeda, S., Snider, J., and Morrison, M. R., 1985, A rapid microprocedure for isolating RNA from multiple samples of human and rat brain, *J. Neurosci. Meth.* **15**:165.

Jacobson, M., 1978, *Developmental Neurobiology*, Plenum Press, New York.

Kilmartin, J. V., Wright, B., and Milstein, C., 1982, Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line, *J. Cell Biol.* **93**:576.

Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, W., and Ponstingl, H., 1981, Complete amino acid sequence of β-tubulin from porcine brain, *Proc. Natl. Acad. Sci. USA* **78**:4156.

Lee, J. C., Frigon, R. P., and Timasheff, S. N., 1973, The chemical characterization of calf brain microtubule protein subunits, *J. Biol. Chem.* **248**:7253.

Lemischka, I., and Sharp, P. A., 1982, The sequences of an expressed rat α-tubulin gene and a pseudogene with an inserted repetitive element, *Nature* **300**:330.

Lemischka, I. R., Farmer, S., Racaniello, V. R., and Sharp, P. A., 1981, Nucleotide sequence and evolution of a mammalian α-tubulin messenger RNA, *J. Mol. Biol.* **151**:101.

L'Hernault, S. W., and Rosenbaum, J. L., 1985, Chlamydomonas alpha-tubulin is posttranslationally modified by acetylation on the epsilon amino group of a lysine, *Biochemistry* **24**:473.

Littauer, U. Z., and Ginzburg, I., 1985, Expression of microtubule proteins in brain, in: *Gene Expression in Brain* (C. Zomzely-Neurath and W. A. Walker, eds.), pp. 125–156, Wiley, New York.

Littauer, U. Z., Schmitt, H., and Gozes, I., 1976, Properties and synthesis of tubulin in neuroblastoma cells, *J. Natl. Cancer Inst.* **57**:647.

Little, M., 1979, Identification of a second β chain in pig brain tubulin, *FEBS. Lett.* **108:**283.

Little, M., and Ludueña, R. F., 1985, Structural differences between brain β1- and β2-tubulins: Implications for microtubule assembly and colchicine binding, *EMBO J.* **4:**51.

Lopata, M. A., Havercroft, J. C., Chow, L. T., and Cleveland, D. W., 1983, Four unique genes required for β tubulin expression in vertebrates, *Cell* **32:**713.

Luduena, R. F., and Woodward, D. O., 1973, Isolation and partial characterization of α- and β-tubulin from outer doublets of sea-urchin sperm and microtubules of chick embryo brain, *Proc. Natl. Acad. Sci. USA* **70:**3594.

Mareck, A., Fellous, A., Francon, J., and Nunez, J., 1980, Changes in the composition and activity of microtubule-associated proteins during brain development, *Nature* **284:**353.

Marotta, C. A., Harris, J. L., and Gilbert, J. M., 1978, Characterization of multiple forms of brain tubulin subunits, *J. Neurochem.* **30:**1431.

Marotta, C. A., Strocchi, P., and Gilbert, J. M., 1979a, Subunit structure of synaptosomal tubulin, *Brain Res.* **167:**93.

Marotta, C. A., Strocchi, P., and Gilbert, J. M., 1979b, Biosynthesis of heterogeneous forms of mammalian brain tubulin subunits by multiple messenger RNAs, *J. Neurochem.* **33:**231.

Morrison, M. R., and Griffin, W. S. T., 1981, The isolation and *in vitro* translation of undegraded messenger RNAs from human postmortem brain, *Anal. Biochem.* **113:**318.

Morrison, M. R., and Griffin, W. S. T., 1985a, Molecular biology of the mammalian brain, in: *Gene Expression in Brain* (C. Zomzely-Neurath and W. A. Walker, eds.), pp. 57–98, Wiley, New York.

Morrison, M. R., and Griffin, W. S. T., 1985b, Quantitation and in situ localization of tubulin mRNA in the mammalian nervous system, *Ann. NY Acad. Sci.* (in press).

Morrison, M. R., Brodeur, R., Pardue, S., Baskin, F., Hall, C. L., and Rosenberg, R. N., 1979, Differences in the distribution of poly(A) size classes in individual messenger RNAs from neuroblastoma cells, *J. Biol. Chem.* **254:**7675.

Morrison, M. R., Pardue, S., Prashad, N., and Brodeur, R., 1980, Relative increase in polysomal mRNA for R1 cAMP binding protein in neuroblastoma cells treated with 1,N^6-dibutyryl-adenosine 3′,-5′-phosphate, *Eur. J. Biochem.* **106:**463.

Morrison, M. R., Pardue, S., and Griffin, W. S. T., 1981, Developmental alterations in the levels of translationally active messenger RNAs in the postnatal rat cerebellum, *J. Biol. Chem.* **256:**3350.

Morrison, M. R., Pardue, S., and Griffin, W. S. T., 1983, Altered expression of different tubulin electrophoretic variants during human cortex development, *J. Neurogenet.* **1:**105.

Nath, J., and Flavin, M., 1978, A structural difference between cytoplasmic and membrane-bound tubulin of brain, *FEBS Lett.* **95:**335.

Nelles, L. P., and Bamburg, J. R., 1979, Comparative peptide mapping and isoelectric focusing of isolated subunits from chick embryo brain tubulin, *J. Neurochem.* **32:**477.

Olmsted, J. B., and Borisy, G. G., 1973, Microtubules, *Ann. Rev. Biochem.* **42:**507.

Olmsted, J., Witman, G., Carlson, K., and Rosenbaum, J. L., 1971, Comparison of the microtubule proteins of neuroblastoma cells, brain, and chlamydomonas flagella, *Proc. Natl. Acad. Sci. USA* **68:**2273.

Perry, G. W., and Cronly-Dillon, J. R., 1978, Tubulin synthesis during a critical period in visual cortex development, *Brain Res.* **142:**374.

Ponstingl, H., Krauhs, E., Little, M., and Kempf, T., 1981, Complete amino acid sequence of α-tubulin from porcine brain, *Proc. Natl. Acad. Sci. USA* **78:**2757.

Prashad, N., and Rosenberg, R. N., 1978, Induction of cyclic AMP binding proteins by dibutyryl cyclic AMP in mouse neuroblastoma cells, *Biochim. Biophys. Acta* **539:**459.

Raff, E. C., 1984, Genetics of microtubule systems, *J. Cell Biol.* **99:**1.

Raybin, D., and Flavin, M., 1975, An enzyme tyrosylating α-tubulin and its role in microtubule assembly, *Biochem. Biophys. Res. Commun.* **65:**1088.

Reddington, M., Tan, L. P., and Lagnado, J. R., 1976, The phosphorylation of brain microtubular proteins *in situ* and *in vitro*, *J. Neurochem.* **27:**1229.

Rodriguez, J. A., and Borisy, G. G., 1978, Modification of the C-terminus of brain tubulin during development, *Biochem. Biophys. Res. Commun.* **83:**579.

Saborio, J. L., Palmer, E., and Meza, I., 1978, *In vivo* and *in vitro* synthesis of rat brain α- and β-tubulins, *Exp. Cell Res.* **114**:365.

Sato, M., Yoshida, Y., and Takahashi, Y., 1978, *In vitro* biosynthesis of tubulin on total, free and membrane-bound polysomes from the developing rat brain, *J. Neurochem.* **31**:1361.

Schmitt, H., 1976, Control of tubulin and actin synthesis and assembly during differentiation of neuroblastoma cells, *Brain Res.* **115**:165.

Schmitt, H., Gozes, I., and Littauer, U. Z., 1977, Decrease in levels and rates of synthesis of tubulin and actin in developing rat brain, *Brain Res.* **121**:327.

Soifer, D., and Czosnek, H., 1980, The possible origin of neuronal plasma membrane tubulin, in: *Microtubules and Microtubule Inhibitors* (M. De Brabander and J. De Mey, eds.) pp. 429–447, Elsevier/North Holland, Amsterdam.

Solomon, F., Gysin, R., Rentsch, M., and Monard, D., 1976, Purification of tubulin from neuroblastoma cells: Absence of covalently bound phosphate in tubulin from normal and morphologically differentiated cells, *FEBS Lett.* **63**:316.

Strocchi, P., Marotta, C. A., Bonventre, J., and Gilbert, J. M., 1981, The subunit composition of cerebellar tubulin: Evidence for multiple β-tubulin messenger mRNAs, *Brain Res.* **211**:206.

Stromska, D. P., 1982, Isoelectric focusing of tubulin in a new type of charge-balanced agarose, *Anal. Biochem.* **119**:387.

Sullivan, K. F., and Cleveland, D. W., 1984, Sequence of a highly divergent β-tubulin gene reveals regional heterogeneity in the β-tubulin polypeptide, *J. Cell Biol.* **99**:1754.

Sullivan, K. F., and Wilson, L., 1984, Developmental and biochemical analysis of chick brain tubulin heterogeneity, *J. Neurochem.* **42**:1363.

Tashiro, T., and Komiya, Y., 1983, Subunit composition specific to axonally transported tubulin, *Neuroscience* **9**:943.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., and Cleveland, D. W., 1981, Nucleotide and corresponding amino acid sequences encoded by α and β tubulin mRNAs, *Nature* **289**:650.

Viklický, V., Dráber, P., Hásek, J., and Bártek, J., 1982, Production and characterization of a monoclonal antitubulin antibody, *Cell Biol. Int. Rep.* **6**:725.

von Hungen, K., Chin, R. C., and Baxter, C. F., 1981, Brain tubulin microheterogeneity in the mouse during development and aging, *J. Neurochem.* **37**:511.

Waeheldt, T. V., and Malokta, J., 1980, Comparative electrophoretic study of the wolfgram proteins in myelin from several mammalia, *Brain Res.* **189**:582.

Wehland, J., Willingham, M. C., and Sandoval, I. V., 1983, A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. 1. Biochemical characterization, effects on microtubule polymerization *in vitro,* and microtubule polymerization and organization *in vivo, J. Cell Biol.* **97**:1467.

Wehland, J., Schröder, H. C., and Weber, K., 1984, Amino acid sequence requirements in the epitope recognized by the α-tubulin-specific rat monoclonal antibody YL1/2, *EMBO J.* **3**:1295.

Wilde, C. D., Chow, L. T., Wefald, F. C., and Cowan, N. J., 1982a, Structure of two human α-tubulin genes, *Proc. Natl. Acad. Sci. USA* **79**:96.

Wilde, C. D., Crowther, C. E., and Cowan, N. J., 1982b, Isolation of a multigene family containing human α-tubulin sequences, *J. Mol. Biol.* **155**:533.

Wilde, C. D., Crowther, C. E., Cripe, T. P., Lee, M. G.-S., and Cowan, N. J., 1982c, Evidence that a human β-tubulin pseudogene is derived from its corresponding mRNA, *Nature* **297**:83.

Witman, G. B., Cleveland, D. W., Weingarten, M. D., and Kirschner, M. W., 1976, Tubulin requires tau for growth onto microtubule initiating sites, *Proc. Natl. Acad. Sci. USA* **73**:4070.

Wolff, A., Denoulet, P., and Jeantet, C., 1982, High level of tubulin microheterogeneity in the mouse brain, *Neurosci. Lett.* **31**:323.

Zagon, I. S., and McLaughlin, P. J., 1979, Morphological identification and biochemical characterization of isolated brain cell nuclei from the developing rat cerebellum, *Brain Res.* **170**:443.

Zisapel, N., Levi, M., and Gozes, I., 1980, Tubulin: An integral protein of mammalian synaptic vesicle membranes, *J. Neurochem.* **34**:26.

11

Tubulins from Plants, Fungi, and Protists

A REVIEW

Louis C. Morejohn and Donald E. Fosket

1. Introduction: Scope and Objectives

Because brain tissue is a readily available source of abundant and easily purified microtubule protein, much of our knowledge of the biochemistry of microtubules and tubulin came first from studies on animal tubulin. Subsequently tubulin was isolated and characterized from extracts of numerous types of vertebrate and invertebrate animal tissues. The aggregate of this work has given us a general model for the biochemistry of tubulin. In recent years investigators have begun to purify and characterize the tubulins from plants, fungi, and protists, and we have summarized the methodologies and results herein with some historical perspective. Apparently the tubulins from these nonanimal sources exhibit the general structural characteristics observed with animal tubulins. However, recent immunological, pharmacological, electrophoretic, peptide mapping, and sequencing data indicate that nonanimal tubulins possess several interesting structural properties that are not fully shared with tubulins from animal sources. These results suggest more divergence of tubulins than was previously thought to exist. Our purpose in this review is to catalogue the recent body of work that has come from studies on tubulins from plants, fungi, and protists and to provide some

Louis C. Morejohn • Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108. ***Donald E. Fosket*** • Department of Developmental and Cell Biology, University of California, Irvine, California 92717.

perspective on the differences and similarities between these nonanimal tubulins and their animal counterparts.

We have included in each section of this chapter selected aspects of animal tubulin biochemistry to provide a background for comparison with results obtained on nonanimal tubulins. For a comprehensive background on related aspects of tubulin and microtubules both *in vivo* and *in vitro*, we refer the reader to recent reviews by Dustin (1984), Ludueña (1979), Burton (1981), Correia and Williams (1983), Hill and Kirschner (1983), and Cowan and Dudley (1983). Some reviews that have concentrated on nonanimal systems include those by Fedtke (1982), Sabnis and Hart (1982), Gunning and Hardham (1982), and Burland and Gull (1984).

2. *Isolation and Identification of Tubulin*

Microtubules are filamentous subcellular structures found in all eukaryotic cells and are the major structural components of several organelles including mitotic spindles, centrioles, basal bodies, cilia, flagella, axopodia, and cytoskeletons. As components of these organelles, microtubules participate in a diverse spectrum of cellular functions including cell motility, chromosome movement during mitosis, organelle transport, cytokinesis, cell plate formation, orientation of cellulose microfibril deposition in developing plant cell walls, and maintenance of cell shape.

The major component of microtubules is the heterodimeric protein tubulin, which is composed of equal amounts of $\simeq$50,000 dalton α- and β-subunit polypeptides. Tubulin dimers are dumbbell-shaped and arranged end to end in linear protofilaments, and usually 13 of these are aligned laterally to form the 24-nm-diameter microtubule (Amos, 1979). The conditions under which these microtubule arrays form and their sensitivities to environmental factors and to drugs vary considerably, despite the fact that individual microtubules are ultrastructurally similar. There have been reports of microtubules and tubulinlike proteins in prokaryotes, and the reader is referred to the recent review by Dustin (1984) for more information on this topic.

Because of the conserved structure of tubulin and microtubules, a previously developed method for tubulin purification from a given organism can often be applied successfully and without modification to widely disparate biological systems. Nevertheless, it has become clear to many investigators who have attempted to isolate and purify tubulins from plants, fungi, and protists that several standardized methods designed for animal systems are not always applicable without considerable modification. In this section we have described and compared the efficacy of the most commonly employed methods that have been used to identify, quantitate, or purify tubulin from animals, plants, fungi, and protists.

2.1. Assays for Tubulin

The isolation and identification of a cellular protein requires a reliable and specific assay for the detection of that protein both in extracts prepared from cells and in subsequent fractions prepared from the extracts. Several types of enzymatic activity have been found to copurify with microtubule proteins. These include a cyclic AMP-dependent protein kinase and ATPase and GTPase activities (Sandoval and Cuatrecasas, 1976; Soifer *et al.*, 1975; Maccioni and Seeds, 1977; David-Pfuety *et al.*, 1977). However, both the ATPase and protein kinase activities can be separated from tubulin and therefore are not intrinsic properties of this protein, while the GTPase activity is intrinsic to the dimer but is exhibited primarily during tubulin assembly into microtubules. For these reasons, the observed enzymatic activities associated with tubulin or other microtubule proteins have not been used as assays for the isolation and identification of tubulin.

The most widely employed method for the detection of tubulin in animal extracts has been the colchicine-binding assay. Taylor and co-workers (Taylor, 1965; Borisy and Taylor, 1967a,b; Shelanski and Taylor, 1967) and Wilson and Friedkin (1967) demonstrated that this plant alkaloid bound to a specific cellular protein, and they presented strong circumstantial evidence that its target was a microtubule protein. Weisenberg *et al.* (1968) isolated and partially characterized the colchicine-binding protein from brain tissue for which Mohri (1968) suggested the name "tubulin." Because colchicine binds to animal tubulins nearly irreversibly and with high affinity and specificity, colchicine binding has been used in a large number of studies both to detect and to quantitate tubulin in cellular extracts (for example, Bamburg *et al.*, 1973; Pipeleers *et al.*, 1977)

Although colchicine binding is not without problems as a tubulin assay in crude extracts of animal tissues (for example, see Sherline *et al.*, 1979), it has been generally unsatisfactory as either a qualitative or quantitative assay for tubulins from plants, fungi, and protists at any level of tubulin purity. In early studies colchicine-binding activity was reported to be undetectable in extracts of the alga *Chlamydomonas*, the protozoan *Tetrahymena*, the yeast *Schizosaccharomyces*, and higher plants (Borisy and Taylor, 1967a; Burns, 1973). It was not clear from these early studies whether the lack of colchicine binding was due to low tubulin concentrations, a low affinity of the tubulins for colchicine, or the inactivation of the colchicine-binding sites during preparation of the extracts. Subsequent studies have demonstrated that purified tubulins and tubulinlike proteins from nonanimal sources do bind colchicine (Rubin and Cousins, 1976; Davidse and Flach, 1977; Maekawa, 1978; Roobol *et al.*, 1980a; Mizuno *et al.*, 1981; Okamura, 1983; Morejohn *et al.*, 1984, 1986a). However, with the possible exception of carrot tubulin (Okamura, 1983; Morejohn *et al.*, 1984), these tubulins have shown very low levels of binding activity, indicating that they lack a high-affinity colchicine-binding site. The use of the colchicine-binding assay as a method for the identification, detection, or quantitation of

tubulins from nonanimal sources is of questionable reliability not only because of the low binding affinity, but also because there is evidence that these organisms contain colchicine-binding proteins whose relationship with tubulin is unclear (Hart and Sabnis, 1973, 1976a,b; Olson, 1973; Heath, 1975a).

The specific binding of antibodies to antigens has led to the development of immunological assays for tubulins. Antibodies or antisera have been raised against the tubulins from diverse tissues and species. The specific characteristics of some of these antitubulin preparations and their uses will be discussed in Section 3.4. For our purposes here it is sufficient to state that while many of these antibody preparations have been shown to bind specifically to tubulin, they do not always cross-react identically with the tubulins from a wide spectrum of organisms. As a result, antitubulin antibody binding has been used with different degrees of success in numerous types of immunological assays for the identification and quantitation of tubulin in preparations from heterologous species.

In lieu of a reliable assay for nonanimal tubulins, the unambiguous criterion for the identification of tubulin is the demonstration of its self-assembly into microtubules *in vitro*. Microtubules have been polymerized from cytoplasmic tubulins isolated from protozoans, yeasts, slime molds, algae, and higher plants (Maekawa and Sakai, 1978; Roobol *et al.*, 1980a,b; Stearns and Brown, 1979; McKeithan and Rosenbaum, 1981; Kilmartin, 1981; Morejohn and Fosket, 1982; Morejohn *et al.*, 1984; Mizuno, 1985). However, microtubule assembly may be different or impossible to achieve in crude or partially purified extracts of some organisms. As a result, there have been numerous reports of the isolation of presumptive tubulins from nonanimal sources in which the identification criteria have included characteristics of polyanionic charge, electrophoretic composition and mobility, peptide mapping pattern, drug-binding activity, cross-reaction with antitubulin antibody, and/or copolymerization with added heterologous tubulin (Hart and Sabnis, 1973; Rubin and Cousins, 1976; Davidse and Flach, 1977; Baum *et al.*, 1978; Clayton *et al.*, 1979; Slabas *et al.*, 1980; Mizuno *et al.*, 1981; Fong and Chang, 1981; Yadav and Filner, 1983; White *et al.*, 1983; Okamura, 1983).

2.2. Ion-Exchange Chromatography

The first successful isolation of tubulin was from an extract of mammalian brain and utilized anion-exchange chromatography to separate the colchicine-binding protein from most other cellular proteins (Weisenberg *et al.*, 1968). Brain tubulin isolated by this procedure with minor modifications is 95–98% pure and has been shown to undergo self-assembly *in vitro* to form microtubules (Lee and Timasheff, 1977). This chromatography method, with certain modifications, has been employed for the isolation of tubulins or putative tubulins from a number of plants, fungi, and protists. In some cases, anion-exchange chromotographic fractionation of cell extracts has yielded tubulinlike proteins, which were shown either to bind antimicrotubule drugs or to have mobilities similar to bona fide brain tubulin on SDS–polyacryla-

mide gels (Haber *et al.*, 1972; Hart and Sabnis, 1973, 1976a,b; Heath, 1975a; Rubin and Cousins, 1976; Davidse and Flach, 1977; Okamura, 1980, 1983; Mizuno *et al.*, 1981).

Tubulins capable of self-assembly have been isolated by ion-exchange chromatography from a yeast, a protist, an alga, and higher plant cells (Maekawa and Sakai, 1978; Stearns and Brown, 1979; Kilmartin, 1981; Morejohn and Fosket, 1982; Morejohn *et al.*, 1984; Mizuno, 1985). The DEAE–Sephadex fractionation of soluble proteins from cultured cells of *Rosa, Daucus,* and *Hibiscus* is shown in Fig. 1. Tubulinlike polypeptides eluted from columns in the 0.4–0.8 M KCl step and were 85–90% pure according to quantitative densitometry of stained gels. Verification of their identities as tubulins was accomplished by glycerol- or taxol-induced microtubule polymerization (Morejohn and Fosket, 1982; Morejohn *et al.*, 1984). Kilmartin (1981) was able to subject yeast DEAE-tubulin to successive rounds of microtubule assembly and disassembly, while Maekawa and Sakai (1978) used

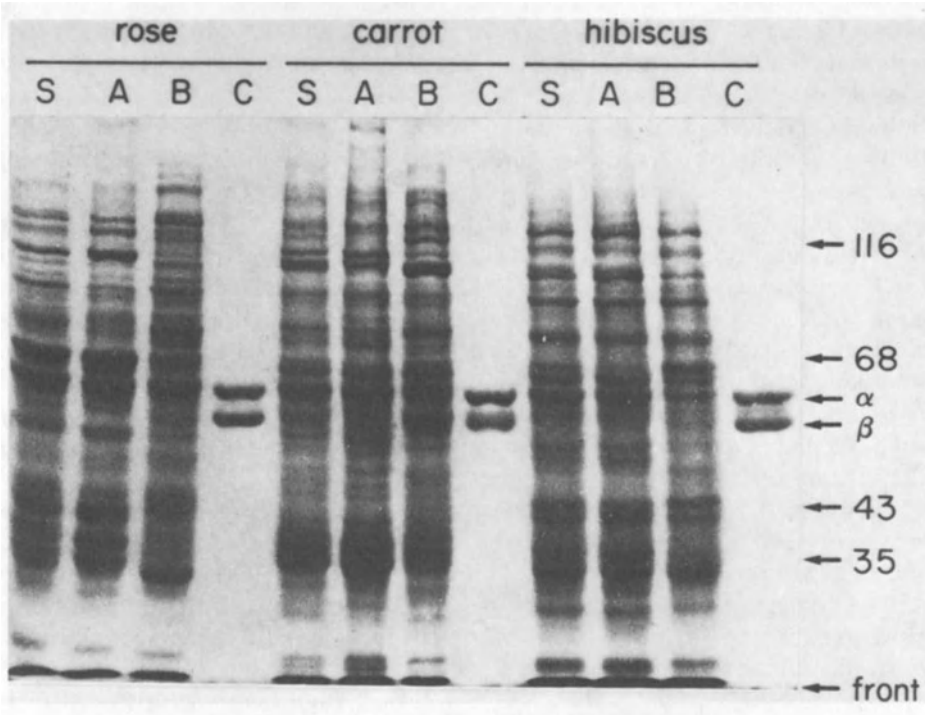


Figure 1. Isolation of tubulin from cultured rose, carrot, and hibiscus cells. Extracts of cultured cells of three higher plants (rose, carrot, and hibiscus) were prepared, and supernatants were fractionated by DEAE–Sephadex A50 chromatography into three protein-containing fractions (A, B, and C) and run on a SDS/7.5% polyacrylamide gel. S, unfractionated supernatants; A, unbound proteins of fraction A; B, weakly bound proteins (0.4 M KCl step) of fraction B; C, tightly bound proteins (0.4–0.8 M KCl step) of fraction C (tubulin). Apparent molecular weights $\times 10^{-3}$ and α and β subunit positions are indicated on the right. Reproduced from Morejohn *et al.* (1984).

Tetrahymena ciliary outer doublet fibers as seeds to initiate polymerization of soluble *Tetrahymena* DEAE-tubulin. Stearns and Brown (1979) used *Polytomella* microtubule organizing centers to initiate phosphocellulose-isolated tubulin assembly. The broad success of ion-exchange chromatography in tubulin isolations indicates that the strong polyanionic charge of the tubulin dimer has been conserved over evolution and can be exploited for the isolation of tubulins from extracts of diverse species.

2.3. Drug Affinity Methods

Animal tubulins have been isolated by drug-affinity column chromatography by taking advantage of their highly specific binding to colchicine (Hinman *et al.*, 1973; Morgan and Seeds, 1975; Sandoval and Cuatrecasas, 1976; Pipeleers *et al.*, 1977; Launay *et al.*, 1983). Colchicine affinity columns have been constructed, but because colchicine lacks the functional groups necessary for its attachment to sepharose, the drug must first be converted to a mixture of deacetylcolchicine and isodeacetylcolchicine before linkage to the resin (Hinman *et al.*, 1973; Morgan and Seeds, 1975). Farrell and Burns (1975) used a colchicine column to isolate a presumptive tubulin from *Chlamydomonas* cytoplasm, but while the protein so isolated was similar to brain tubulin in its polyanionic charge and electrophoretic mobility, they were unable to assemble it into microtubules under conditions that would promote the *in vitro* assembly by brain tubulin. Despite these findings, it remains to be demonstrated that the affinity of nonanimal tubulins for colchicine is sufficiently high to make colchicine affinity chromatography an effective tubulin isolation method for these groups of organisms. In addition, there is even some doubt about the basis for the separation obtained with animal extracts using colchicine affinity columns. Morgan and Seeds (1975) noted that they were able to isolate brain tubulin on a colchicine affinity column even when the tubulin had been saturated with colchicine prior to the chromatographic step. This suggests that the column may have bound tubulin for some reason other than the affinity of tubulin for colchicine.

Despite these uncertainties, some types of affinity chromatography may ultimately prove to be useful in the isolation of tubulins from nonanimal sources. Numerous herbicides and fungicides have been shown to produce antimitotic effects in plants, fungi, or protists (see Section 4). Some of these bind to tubulin and, thus, could be useful in affinity chromatography. For example, Mizuno *et al.* (1981) used a combination of ethyl-*N*-phenylcarbamate (EPC) affinity chromatography and anion exchange and gel filtration chromatographies to purify a putative tubulin from azuki bean epicotyls. Their data indicate that the putative tubulin bound to the EPC affinity column, although a pure preparation of the protein was not obtained by the affinity method alone. This procedure has been used to isolate plant tubulins from a variety of species (Mizuno *et al.*, 1985; Mizuno, 1985).

Isolation of tubulin from cell supernatant solutions has been accomplished by vinblastine precipitation. This alkaloid binds to tubulin and in-

duces the formation of rings, spirals, or crystals that can be sedimented by centrifugation (Bensch *et al.*, 1969; Marantz *et al.*, 1969; Bryan, 1971, 1972a). Williams *et al.* (1979) isolated cytoplasmic tubulin from *Tetrahymena* by vinblastine-induced precipitation. While the tubulin was not pure, the precipitate contained ~60% tubulin. Rather similar results were obtained with *Physarum* precipitates (Roobol and Gull, 1980). Because vinblastine will also precipitate a number of other proteins including actin, pure tubulin precipitates should not be expected when obtained from whole cell supernatants (Wilson *et al.*, 1970). It remains to be shown that vinblastine precipitation can be used for the isolation of tubulin from extracts of plants and fungi.

2.4. Microtubule Polymerization and Depolymerization

After Weisenberg (1972) and Borisy and Olmsted (1972) demonstrated that the soluble tubulin in brain extracts would self-assemble into microtubules after warming in the presence of GTP, magnesium, and the calcium chelator EGTA, several tubulin isolation procedures were devised on the basis of microtubule assembly *in vitro* (for example, Shelanski *et al.*, 1973; Sloboda *et al.*, 1975; Borisy *et al.*, 1975). These procedures rely on cyclic temperature-dependent assembly and disassembly of tubulin dimers and microtubules, respectively. The microtubule protein concentration must be maintained above a certain critical concentration, or microtubules will not spontaneously assemble. The critical concentration can vary by three orders of magnitude (0.015–4.0 mg/ml) depending on the origin and composition of microtubule protein, the type of assembly-promoting constituents used in the polymerization buffer, and the temperature used for polymerization (for example, Gaskin *et al.*, 1974; Sloboda *et al.*, 1975; Schiff *et al.*, 1979; Suprenant and Rebhun, 1983; Morejohn and Fosket, 1984b). When this method of microtubule purification is applied to crude high-speed supernatants of cellular extracts, usually three or more successive rounds of microtubule polymerization and depolymerization are required for the separation of microtubule proteins from most other cellular proteins.

Several renditions of the cyclic polymerization–depolymerization isolation method have been developed, with the major difference between them being the particular type of polymerization-enhancing components in the solvent. The methods of Shelanski *et al.* (1973) and Sloboda *et al.* (1975), which incorporate 4 M glycerol into the assembly buffer, have been shown to reduce by an order of magnitude the critical tubulin dimer concentration required for microtubule polymerization (Lee and Timasheff, 1977). Glycerol stabilizes the native state of tubulin as shown by its ability to prevent the loss of colchicine-binding activity (Solomon *et al.*, 1973; Keates, 1981). Sucrose apparently has similar stabilizing effects (Frigon and Lee, 1972; Shelanski *et al.*, 1973). The procedure of Borisy *et al.* (1975) does not use glycerol in the assembly buffer, although the procedure is otherwise similar to that of Shelanski *et al.* (1973). Asnes and Wilson (1979) modified the procedure of Borisy and co-workers by employing a sodium glutamate buffer and charac-

terized the optimum conditions for the cyclic assembly–disassembly isolation of brain microtubule proteins without glycerol.

The cyclic polymerization–depolymerization procedures do not yield pure tubulin from either bird or mammalian brain tissues. Rather, these procedures yield tubulin as a major component and stoichiometric amounts of a few high-molecular-weight, microtubule-associated proteins (MAPs) as minor components. In electron micrographs the MAPs are seen as projections attached to microtubule walls (Dentler *et al.*, 1975; Murphy and Borisy, 1975). These copolymerized MAPs can be separated from tubulin by ion-exchange chromatography on either DEAE–Sephadex or phosphocellulose (Weingarten *et al.*, 1975; Murphy and Borisy, 1975; Sloboda *et al.*, 1975). Three rounds of microtubule assembly and disassembly yield a protein preparation that is approximately 75% tubulin (Murphy and Borisy, 1975; Sloboda *et al.*, 1975). However. Langford (1981) used the procedure of Shelanski *et al.* (1973) to isolate tubulin from the brain of the dogfish, *Mustelus* and showed that it lacked MAPs as analyzed by SDS–gel electrophoresis and electron microscopy.

While the precise functions of MAPs *in vivo* are not known, MAPs have been shown to be associated with microtubules in brain and cultured mammalian cells by indirect immunofluorescence studies (Sherline and Schiavone, 1977; Sheterline, 1978). Although tubulins will assemble into microtubules in the absence of MAPs (Lee and Timasheff, 1975; Himes *et al.*, 1976), MAPs stabilize the *in vitro* assembled microtubules and reduce the critical tubulin concentration required for the initiation of microtubule polymerization (Weingarten *et al.*, 1975; Murphy *et al.*, 1977).

Cytoplasmic tubulins have been purified from a wide spectrum of animal tissues and species using cycles of microtubule assembly and disassembly or a combination of this method and ion-exchange chromatography. There have been few reports of the successful isolation of cytoplasmic microtubule protein directly from extracts of nonanimal species using the cyclic assembly–disassembly procedure. Roobol *et al.* (1980a,b) obtained microtubule proteins from extracts of myxamebae of the slime mold *Physarum* by an assembly–disassembly procedure that used glycerol. Microtubule protein subjected to three rounds of assembly and disassembly was 98% pure tubulin. Subsequent phosphocellulose chromatography removed minor copolymerizing protein components. These minor proteins facilitated the *in vitro* assembly of the tubulin and presumably represented *Physarum* MAPs. Okamura (1984) observed polymorphic structures formed from cultured carrot cell supernatants following two rounds of temperature-dependent depolymerization and polymerization (Shelanski *et al.*, 1973). Electron micrographs of tannic acid-stained polymers revealed microtubulelike structures, yet the protein composition of the polymers was not examined. Kantharaj *et al.* (1985) reported that tubulin could be purified from *Phaseolus* by cyclic assembly–disassembly and phosphocellulose chromatography, and that indolebutyric acid promoted polymerization in the presence of microsomal fractions. However, no evi-

dence was provided that the structures that contributed to turbidity increase were microtubules.

Maekawa and Sakai (1978), Kilmartin (1981), Morejohn and Fosket (1982), and Russell *et al.* (1984) used DEAE-chromatography to separate tubulin from most other cellular proteins before the microtubule assembly steps. Figure 2 shows electron micrographs of yeast microtubules polymerized twice from DEAE-isolated tubulin. Most microtubules (~90%) contained 13 protofilaments while ~10% were composed of 12 protofilaments (Kilmartin, 1981). Some of the possible reasons why an exclusive use of the microtubule assembly–disassembly technique has not been more successful for the isolation of tubulin and other microtubule proteins from nonanimal species are discussed in Section 2.7.

2.5. Copolymerization with Heterologous Tubulins

Putative tubulins from numerous species have been isolated by copolymerization with purified carrier tubulin. A tentative identification of the copolymerized radiolabeled tubulin is accomplished by separating the microtubule polypeptides on one- or two-dimensional electrophoretic gels and comparing the composition and relative mobilities of polypeptides observed on fluorographic exposures with those of authentic carrier tubulin polypeptides. This technique has proven successful for the isolation of putative tubulins from the algae *Polytomella* and *Chlamydomonas,* the yeast *Saccharomyces,* the fungus *Aspergillus,* the slime molds *Physarum* and *Dictyostelium,* and the higher plants *Daucus, Rosa,* and *Nicotiana* (Davidse, 1975; Sheir-Neiss *et al.,* 1976, 1978; Water and Kleinsmith, 1976; Weeks and Collis, 1976; Baum *et al.,* 1978; Cappuccinelli *et al.,* 1978; Lai *et al.,* 1979; Clayton *et al.,* 1979; Roobol *et al.,* 1980a,b; Slabas *et al.,* 1980; Fosket *et al.,* 1981; Kilmartin, 1981; McKeithan and Rosenbaum, 1981; Yadav and Filner, 1983; White *et al.,* 1983). Because of the possibility that nontubulin polypeptides with electrophoretic properties similar to those of tubulin could adventitiously interact with and copurify with carrier tubulin, this method alone cannot be used to conclusively identify tubulin. However, comparative peptide mapping of the presumptive tubulin polypeptides and authentic tubulins can considerably strengthen the prudent investigator's claim that the copolymerized polypeptides are tubulin subunits. The apparent widespread success of the copolymerization technique indicates that the dimer–dimer binding sites in microtubules have been highly conserved among extremely diverse species.

2.6. Tubulin-Rich Organelles

Eukaryotic cilia and flagella contain a cylindrical axoneme composed of nine outer doublet microtubules arranged around a central pair of microtubules. Since cilia and flagella contain many fewer proteins than the whole cytoplasm, and tubulin may make up 20–25% of the total flagellar protein

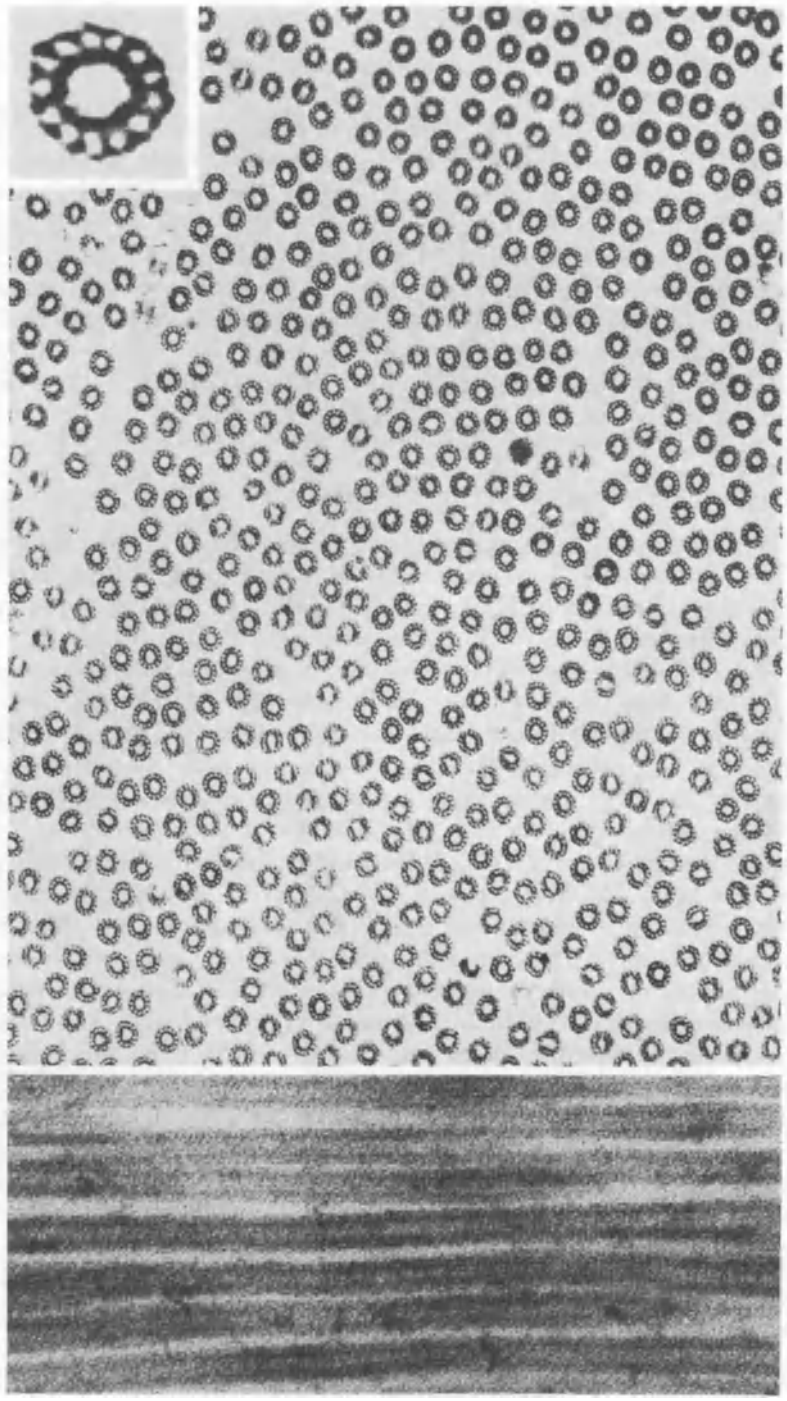


Figure 2. Electron micrographs of purified yeast microtubules. Upper panel shows cross-sections of microtubules, and the inset displays a high-resolution image of a single microtubule containing 13 protofilaments. The lower panel shows microtubules in longitudinal section. Micrographs were supplied by Dr. John Kilmartin.

(Piperno *et al.*, 1977; Lefebvre *et al.*, 1980), the isolation of these organelles represents a substantial initial enrichment step in the purification of tubulin. Flagella or cilia can be removed from the cell bodies by a variety of procedures, including pH shock (Rosenbaum and Child, 1967), treatment with high levels of calcium (Gibbons, 1965; Witman *et al.*, 1972), sonication (Binder and Rosenbaum, 1978), or mechanical shearing (Kowit and Fulton, 1974; Lefebvre *et al.*, 1978; Ludueña *et al.*, 1980; McKeithan and Rosenbaum, 1981). The flagella are collected by differential centrifugation and, in some cases, separated from cell debris by banding in a Percoll gradient (McKeithan and Rosenbaum, 1981). The isolated cilia or flagella may be demembranated with a detergent (Stephens, 1970). The central pair microtubules are readily solubilized by dialysis against an EDTA-containing buffer at basic pH (Stephens, 1970), but solubilization of the outer doublet microtubules is more difficult. Sonication has been shown to solubilize the outer doublet microtubules of axonemes from a number of plant and animal species (Kuriyama, 1976; Binder and Rosenbaum, 1978; Farrell and Wilson, 1978; Ludueña *et al.*, 1980).

With regard to the organisms under consideration in this review, flagellar doublet tubulin has been assembled *in vitro* only from the protozoans *Tetrahymena* (Kuriyama, 1976) and *Giardia* (Russell *et al.*, 1984) although flagellar tubulin has been isolated and some of its properties studied from *Chlamydomonas*, *Polytomella*, the protozoan *Naegleria*, and the ferns *Marselia* and *Pteridium* (Witman *et al.*, 1972; Kowit and Fulton, 1974; Fulton and Kowit, 1975; Piperno and Luck, 1976; Weeks and Collis, 1976; Ludueña *et al.*, 1980; McKeithan and Rosenbaum, 1981; Little *et al.*, 1981a).

An analogous approach to the cilia or flagella enrichment method is the isolation of cytoskeletal structures. The cytoskeletons of plants, fungi, and protists vary considerably in structure and composition, but because microtubules represent a major component of cytoskeletons, their initial isolation represents a significant enrichment step. Cytoskeletons of *Tetrahymena* were isolated by detergent extraction and were estimated to contain 38% of the total pellicular protein (Williams *et al.*, 1979). McKeithan and Rosenbaum (1981) isolated cytoskeletons from the alga *Polytomella* in a glycerol-containing buffer after detergent lysis of the cells. They then solubilized the tubulin with cold treatment, sedimented the remaining particulate matter by centrifugation, and reassembled soluble tubulin into microtubules by warming the preparation. Holberton and Ward (1981) isolated cytoskeletons from trophozoites of the parasitic flagellated protozoan *Giardia* and estimated that tubulin comprised 20–25% of the total cytoskeletal protein. *Giardia* tubulin was purified by gel filtration chromatography in the presence of SDS (Crossley and Holberton, 1983). White *et al.* (1983) prepared cytoskeletons from amebae of the slime mold *Dictyostelium* by extraction with a low-ionic-strength and high-concentration detergent buffer. Two-dimensional gels exhibited enrichment of tubulin subunits.

The pellicular membranes of the parasitic protozoans *Leishmania* and *Trypanosoma* are known to be associated with large numbers of cytoplasmic

microtubules, and the membranes can be isolated by linear sucrose density gradient centrifugation (Dwyer, 1980; Bordier *et al.,* 1982a). Dwyer (1980) and Dwyer and D'Alesandro (1980) noted that microtubules remained attached to the membranes during isolation and tentatively identified a prominent 53,000-dalton protein as tubulin. Bordier *et al.* (1982a,b) showed that the protein so isolated was indeed tubulin by immunological cross-reaction with antibodies against porcine brain microtubules and by comparative peptide mapping of the α- and β-subunits. Russell *et al.* (1984) detergent-extracted *Giardia* pellicles and sonicated the extract before polymerizing the soluble tubulin into microtubules in glycerol. Stieger *et al.* (1984) sonicated whole trypanosomes before inducing microtubule polymerization with taxol. These studies demonstrated that the pellicular membrane–microtubule preparation is a rich source of tubulin from these protozoans.

2.7. Technical Problems

Numerous problems can beset the investigator who attempts to isolate tubulin from plants, fungi, or protists. These difficulties may not be unique to nonanimal systems, but they do seem to be more severe in these organisms than in most animal systems. Commonly encountered stumbling blocks include low cellular protein levels with concomitantly low tubulin levels, proteinaceous inhibitors such as endogenous proteinases and oxidases, and nonproteinaceous compounds that chemically modify protein structure and function. It should be emphasized, therefore, that the investigator should carefully consider the physiological suitability of several related organisms as potential sources of microtubule protein prior to his full commitment to an organism as a potential source of microtubule protein.

Gull and co-workers (Roobol *et al.,* 1980a,b) characterized numerous inhibitory activities in extracts of the slime mold *Physarum* by assaying the effects the extracts had on brain microtubule assembly *in vitro.* An endogenous GTPase activity was countered by the addition of a GTP-regenerating system to the isolation buffer, and proteolytic activities were inhibited by the proteinase inhibitor leupeptin, but not phenylmethyl sulfonyl fluoride (PMSF). Since RNA is thought to bind to MAPs and prevent their normal function in microtubule nucleation (Bryan *et al.,* 1975a,b; Nagle and Bryan, 1976), the extracts were treated with RNase. Finally, extracts were concentrated to greater than 100 mg/ml of total soluble protein before purifying microtubules by three cyclic microtubule assembly–disassembly steps in glycerol (for details see Roobol and Gull, 1982).

Fosket *et al.*(1981) also used brain microtubule assembly to investigate the nature of inhibitory activities in extracts of cultured rose (*Rosa*) cells. Dilutions of rose cell supernatants or of acetone-extracted supernatants were added to brain microtubule protein and assembled once at 30°C for 30 min. Increasing amounts of both rose extract preparations inhibited brain microtubule assembly, although the acetone-soluble extract contained less inhibitory activity. Addition of the commonly used proteolytic inhibitor PMSF to the isolation

buffer reduced, but did not abolish, proteolytic degradation of brain MAPs and increased the yield of tubulin. The finding of acetone-soluble inhibitory factors in rose extracts persuaded us to first isolate tubulin by DEAE-chromatography before attempting microtubule assembly (Morejohn and Fosket, 1982).

Antibodies raised against the individual subunits of rose tubulin (Morejohn *et al.*, 1984) were used to assay the efficacy of certain proteolytic inhibitors in plant tubulin isolations (Morejohn *et al.*, 1985). Immunoblotting of rose tubulin samples isolated by DEAE-chromatography in the presence or absence of the inhibitors demonstrated that in the absence of any added proteolytic inhibitor, the rose tubulin α subunit was completely cleaved into smaller immunoreactive polypeptide fragments, while the β subunit was partially cleaved. The addition of leupeptin hemisulfate to the isolation buffer significantly reduced the cleavage of the α subunit and completely protected the β subunit. Inclusion of both leupeptin hemisulfate and pepstatin A in the isolation buffer resulted in no detectable proteolytic cleavage of either subunit. It is likely that similar proteolytic activities have been encountered by other workers who have used plant or fungal materials. For example, Rubin and Cousins (1976) reported that a tubulinlike protein from mung bean (*Phaseolus*) exhibited variable electrophoretic mobility on SDS gels, sometimes being resolved as a doublet and other times as a single band, suggesting that the protein had been proteolytically cleaved. Partial degradation by endogenous peptidase activities of higher plant tubulins isolated by Mizuno *et al.* (1985) may account for the presence of lower-molecular-weight immunoreactive polypeptides. Davidse and Flach (1977) published electrophoretic gels of tubulin from *Aspergillus* that contained numerous low-molecular-weight polypeptides, a result reminiscent of proteolysis. That these low-molecular-weight bands were probably proteolytic fragments of tubulin can be seen in the work of Weatherbee and Morris (1984), where antibodies to pig brain tubulin were shown to bind not only to the α- and β-tubulins of *Aspergillus* on immunoblots, but also to several low-molecular-weight bands. This tubulin preparation employed only PSMF as a proteinase inhibitor.

Fortunately, effective inhibitors of such proteinases have become available. Leupeptin hemisulfate and pepstatin A have been used in a few tubulin isolations from nonanimal species and appear to preserve the expected equimolar α- and β-subunit stoichiometry on SDS gels (Kilmartin, 1981; Roobol *et al.*, 1980a,b; Mizuno *et al.*, 1981; Morejohn *et al.*, 1984, 1985).

Many potent antimitotic drugs such as colchicine, vinblastine, podophyllotoxin, taxol, and griseofulvin are naturally occurring secondary products from plants or fungi (Dustin, 1984). While these substances are specialized products of limited taxonomic distribution, most plant and fungal cells contain a large variety of secondary products, some of which may hinder tubulin isolation and assembly, even if tubulin is not a specific target for these substances. Some of the more common and potentially harmful compounds include polyphenolics, tannins, and quinones. Phenolics can bind very strongly to proteins by hydrogen bonding or may be converted to highly reactive

quinones that can covalently bond to proteins; tannins will precipitate proteins from solution. Removal of these substances by inclusion of synthetic adsorbants such as polyvinylpyrrolidone or polystyrene in the isolation buffer may lessen the possibility of modification of tubulin during purification (Loomis and Battaile, 1966; Loomis, 1974). Indeed, the inhibition of microtubule assembly *in vitro* by quinones has been documented. O'Brien *et al.* (1983) showed that the orthoquinone stypoldione from the brown alga *Stypopodium* inhibited bovine brain microtubule assembly and tubulin colchicine-binding activity *in vitro*. Pfeifer and Irons (1983) reported that both hydroquinone and Adriamycin, a polycyclic quinone, inhibited pig brain microtubule polymerization, and they proposed that tubulin was inactivated by selective alkylation of sulfhydryl groups.

We have observed in preliminary experiments that extracts of different cultured higher plant cells exhibit different levels of a "browning reaction" in which oxidation reactions occur. While no browning has been observed in extracts of cultured rose cells, extracts of cultured carrot and hibiscus cells showed browning unless homogenates were prepared with an isolation buffer containing both the reducing agent dithiothreitol and the inhibitor of polyphenol oxidase activity diethyldithiocarbamic acid (Loomis, 1974; Morejohn *et al.* 1984). Okamura (1983) has also observed browning in extracts of cultured carrot cells and was able to preserve colchicine-binding activity by preparing extracts with a buffer containing polyvinylypyrrolidone. Mizuno *et al.* (1981) reported that azuki bean epicotyl tubulin showed no colchicine-binding activity unless dithiothreitol was used throughout the purification procedure, indicating that sulfhydryl inactivation may occur in plant extracts.

Other organisms have also been found to contain inhibitors of tubulin assembly. For reasons that are not completely clear a putative tubulin preparation from the alga *Chlamydomonas* was found to inhibit the assembly of brain microtubules and was incompetent in self-assembly (Farrell and Burns, 1975). Naruse and Sakai (1981) reported that a polysaccharide from the cortex of sea urchin eggs was a potent inhibitor of brain microtubule polymerization *in vitro* and also depolymerized preformed microtubules. They demonstrated that the protein bound to a MAPs–Sepharose column and proposed that the polysaccharide inhibited polymerization by blocking the normal interaction of MAPs with tubulin. Weinert *et al.* (1982) found that mammalian brain tubulin would not polymerize in slime mold (*Dictyostelium*) extracts. They isolated a 13,000-dalton protein that inhibited brain microtubule assembly when used at a ratio of 1 mole inhibitor per 100 moles of tubulin and that apparently had no proteolytic or GTPase activities (Weinert and Cappuccinelli, 1982).

Because there is no known tubulin-rich cell type or tissue available from nonanimal species, another important consideration in choosing a system for tubulin isolation is the relative ease with which abundant amounts of starting material can be obtained. The development of inexpensive defined media for the growth of numerous plants, fungi, and protists has facilitated the axenic culture of these organisms. Sterile conditions reduce possible contamination by other eukaryotic cells (and their tubulin) and permit the growth of large

batches of cells in relatively short periods. Usually tubulins from nonanimal species have been isolated in quantities sufficient to permit self-assembly into microtubules when large batches of cells were grown under axenic conditions (Maekawa and Sakai, 1978; Roobol *et al.*, 1980a,b; Kilmartin, 1981; McKeithan and Rosenbaum, 1981; Morejohn and Fosket, 1982, Morejohn *et al.*, 1984). However, Mizuno (1985) and Mizuno *et al.* (1985) have isolated tubulins from plant organs after growing large numbers of plants in vermiculite.

3. Tubulin Dimer Structure

Ludueña *et al.* (1977) were the first to convincingly show that soluble tubulin exists as a heterodimer composed of two similar subunits designated α and β. They used a bifunctional cross-linking agent to demonstrate that most dimers in solution are of the αβ composition. The α and β monomers are held together by noncovalent interactions, and although there appears to be a monomer–dimer equilibrium in solution, the estimated strength of the monomer–monomer interaction ($K_d = 8 \times 10^{-7}$ M) is sufficiently high to indicate that the α and β subunits exist mainly in the dimeric form under conditions *in vitro* that are thought to mimic the physiological state (Detrich and Williams, 1978). Analytical centrifugation of the tubulin dimer has yielded a sedimentation coefficient of 5.8 S (Frigon and Timasheff, 1975).

The animal tubulin dimer has been shown to have distinct binding sites for physiologically significant cofactors such as the nucleotide GTP and the divalent cations calcium and magnesium. Sites exist also for the binding of MAPs and several antimicrotubule drugs. Although it is likely that all tubulins interact with the same or chemically similar nucleotides and cations, there have been no reports on the binding of cofactors to nonanimal tubulins. For treatment of the existing literature on animal tubulin binding of drugs and cofactors we refer the reader to Dustin (1984) and Ludueña (1979). For background on MAPs see Kirschner (1978) and Hill and Kirschner (1983). The interaction of nonanimal tubulins with drugs is discussed in Section 4. In this section we have summarized the structural similarities and differences between nonanimal and animal tubulins that have been detected with several routine biochemical techniques including gel electrophoresis, amino acid sequencing, peptide mapping, and immunological methods.

3.1. Amino Acid Sequences

Partial amino acid sequences of the α and β subunits of tubulin were first published by Ludueña and Woodward (1973). They separated the subunits of tubulins from chicken brain and sea urchin (*Strongylocentrotus*) sperm flagellar outer doublets on denaturing gels and sequenced in their NH_2 termini. A comparison of the sequences revealed that the α and β subunits were distinct from each other, yet shared some degree of homology. Furthermore, the amino acid sequences of chicken and sea urchin tubulins were nearly identi-

cal. They proposed that tubulin was a highly conserved protein and that the present-day α and β subunits evolved from a common ancestral protein following a gene duplication. Since their report appeared, the partial or complete sequences of tubulin subunits from several organisms have been determined either directly from purified polypeptides or by deduction from the nucleotide sequences of cloned tubulin genes. Published sequences include those of human, cow, pig, rat, chicken, alga, ciliate protozoan, and yeast (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981; Valenzuela *et al.*, 1981; Lemischka *et al.*, 1981; Wilde *et al.*, 1982; Hall *et al.*, 1983; Neff *et al.*, 1983; Toda *et al.*, 1984; Youngblom *et al.*, 1984; Silflow *et al.*, 1985). While a direct comparison of the nucleotide sequences of animal tubulins does indicate some divergence, most nucleotide substitutions either have been silent and do not give rise to a different amino acid or have resulted in the substitution of chemically similar amino acids. These studies have confirmed that tubulin protein is conserved and that α and β subunits are each ≃50,000 daltons and encoded by different genes.

Ponstingl *et al.* (1981) and Krauhs *et al.* (1981) reported comprehensive sequencing studies on porcine brain tubulin. In the course of sequencing they found six positions of amino acid heterogeneity on each subunit, indicating that each subunit preparation contained variant polypeptides. In the case of the α subunit, variant polypeptides were isolated by high-pressure liquid chromatography and sequenced (Ponstingl *et al.*, 1981). Two amino acids were found in five positions of heterogeneity, whereas three amino acids were found in a sixth position of heterogeneity. In the β subunit, six positions of heterogeneity were found to contain two amino acids, and two variant polypeptides were isolated and sequenced (Krauhs *et al.*, 1981). The results indicated that at least four variant α subunits and two variant β subunits exist in porcine brain tubules. Variant tubulins such as these reflect the expression of multiple tubulin genes from a given cell type or a combination of the expression of tubulins from nerve cells and glial cells, both of which existed in the brain preparation (see Section 3.7).

Little *et al.* (1981b) compared the sequence of porcine brain tubulin with that deduced from chicken brain tubulin cDNAs (Valenzuela *et al.*, 1981) and found ~99% homology and calculated an evolutionary mutation rate of 0.22 accepted point mutations/100 residues per 100 million years. This rate is somewhat lower than that of histones H3 and H4, but higher than that of histones H2A and H2B, suggesting that the tubulins are among the most highly conserved proteins in evolution. Even higher levels of evolutionary conservation have been predicted by Lemischka *et al.* (1981) after comparing the deduced amino acid sequences from rat and chicken α-tubulin genes (>99% homologous). Although it is clear that these tubulins are conserved, a simple comparison of the sequences of tubulins from chicken and pig, or chicken and rat, may be somewhat misleading. Since the porcine tubulin subunit was composed of at least six variant polypeptides having several positions of amino acid heterogeneity, there were potentially more differences between individual porcine variants than between the porcine and chicken

sequences. Furthermore, the amino acid sequence deduced from a human β-subunit gene (Hall *et al.*, 1983) had less homology (95%) to those of pig and chicken, even though humans and pigs as mammals are more closely related to one another than either is to chicken. The reason for this discrepancy probably can be explained by the evidence that most organisms have multiple, nonidentical copies of tubulin genes and that these genes are differentially expressed during development or according to tissue type (Cowan and Dudley, 1983).

The potentially large variation of sequence divergence of tubulin genes within a given organism has been illustrated by the work of Sullivan and Cleveland (1984). They deduced the amino acid sequence of a chicken β-tubulin by sequencing its gene and found it to be 8.9% divergent from another chicken β-tubulin. This extent of divergence is nearly as great as that (12%) between the β-tubulins from human and *Chlamydomonas* (Youngblom *et al.*, 1984). Sullivan and Cleveland have proposed that certain isotypic forms of tubulin genes have been more highly conserved between species, while other isotypic forms have diverged within a species. This may indicate that there are functionally distinct tubulins in a given organism.

The comparison of tubulin sequences from widely diverse species such as higher animals and lower eukaryotes provides more perspective on the degree to which tubulin has been conserved over evolution. A comparison of the deduced amino acid sequence of β-tubulin from a yeast (*Saccharomyces*) (Neff *et al.*, 1983) with that of chicken (Valenzuela *et al.*, 1981) provides a rather interesting picture. Where regions of the yeast and chicken sequences can be directly compared, a total of 128 of 445 possible amino acids are different and they showed 71% homology. The sequences had a consistent pattern of homology with substitutions dispersed along most of their lengths, except for the COOH-terminal ends where the yeast sequence had a run of substitutions between positions 430 and 445, and 12 additional residues beyond the chicken sequence COOH-terminal residue. The yeast β-subunit gene encoded a polypeptide of 457 amino acids rather than the 445 residues of chicken and would produce a subunit with a molecular weight of 51,073 in contrast to the predicted chicken subunit molecular weight of 49,935.

A comparison of the deduced amino acid sequences of two α-tubulins from yeast (*Schizosaccharomyces*) (Toda *et al.*, 1984) with the most similar sequence of porcine α-tubulin (Ponstingl *et al.*, 1981) also revealed regions of homology throughout, with the most highly conserved positions being 1–30 and 377–443. In contrast to the evenly dispersed substitutions in yeast and porcine β-tubulins, the α-tubulin sequences of yeast contained internal additions and deletions and two obvious runs of substitutions and deletions in positions 34–54 and 444–452. The two yeast α-tubulin genes encoded polypeptides having 455 and 449 residues rather than the 450 of porcine brain and showed 76% homology with the most similar porcine α-tubulin sequence.

The complete amino acid sequences of *Chlamydomonas* α- and β-tubulins have been deduced from the cloned genes. Youngblom *et al.* (1984) reported that both β-tubulin genes encoded polypeptides of 443 amino acids and that

their amino acid sequences are identical (mol. wt. = 49,556). The sequence is 88%, 89%, and 72% homologous to those of human, chicken, and yeast, respectively. Substitutions between *Chlamydomonas* and animal β subunits are scattered throughout the sequences, but there were three regions containing runs of substitutions, positions 199–202, 229–238, and 429–443. Silflow *et al.* (1985) found that the two α-tubulin genes of *Chlamydomonas* encoded slightly different polypeptides each with 451 amino acids (mol. wt. = 49,529 and 49,530) and only two amino acid substitutions. The amino acid sequences were 86% and 70% homologous to the α-tubulins from rat and yeast, respectively. Substitutions between *Chlamydomonas* and rat α-tubulins were also scattered throughout, and regions containing the most substitutions were positions 188–204, 378–389, and 437–448. These data indicate that the β subunits of animal and lower plant tubulins have been conserved slightly more than the α subunits and partly confirm the prediction made on the basis of comparative peptide mapping of plant and animal tubulin subunits (discussed in Section 3.3). Furthermore, the alga and animal tubulin sequences are more similar to one another than either is to those of yeast.

Helftenbein (1985) has predicted the amino acid sequence of an α-tubulin after sequencing a genomic clone from the ciliated protozoan *Stylonychia.* The protein has 438 amino acid residues and is 86.7% and 92% homologous to the α-tubulin sequences from rat and *Chlamydomonas,* respectively. This α-tubulin has an obvious cluster of amino acid substitutions and deletions in the region between residues 261 and 275 and is shorter than α-tubulins from other organisms.

These comparative sequencing studies have revealed that yeast α- and β-subunit polypeptides have regions that are very different from those of animals and lower eukaryotes. Certainly the differences between the yeast and pig brain sequences would account for the obvious differences in electrophoretic mobility that have been shown for yeast and porcine brain subunits in SDS gels (Kilmartin, 1981; Section 3.2). Ludueña and Woodward (1973) proposed that α and β subunits diverged from a common ancestral sequence. When the sequences of the α subunit of *Schizosaccharomyces* and the β subunit of *Saccharomyces* are compared with one another, ~44% of their amino acid sequences are identical, a level of homology akin to those of the α and β subunits of pig and chicken tubulins (Ponstingl *et al.* 1981; Krauhs *et al.*, 1981; Valenzuela *et al.*, 1981). This observation strongly supports the proposal of Ludueña and Woodward (1973). An overall 71–76% homology of yeast tubulin with animal tubulin indicates a much greater amount of divergence of tubulins than was previously estimated from comparisons of animal sequences only. Ultimately, the comparison of tubulin sequences from numerous non-animal species should clarify to what extent tubulins have been conserved over evolution and may serve to pinpoint regions on tubulins that are responsible for their common functions in microtubules, as well as those regions that confer subtle and distinct immunological and biochemical properties on diverse tubulins.

3.2. Electrophoretic Resolution of α *and* β *Subunits*

Bryan and Wilson (1971) originally reported that chicken brain tubulin was composed of nearly equal amounts of slightly different α- and β-monomer polypeptides and proposed that tubulin was a heterodimer. They found that reduced and carboxymethylated tubulin was resolved either as a single band on one-dimensional SDS–acrylamide gels or as two closely spaced bands of nearly equal stoichiometry on urea–acrylamide gels; the upper, slow-moving band was designated α and the lower, fast-moving band β. Immediately following their report, similar observations were published by Feit *et al.* (1971) for tubulins from porcine brain and sea urchin flagella, by Olmsted *et al.* (1971) for tubulins from neuroblastoma cells, porcine brain, and *Chlamydomonas* flagella, and by Everhart (1971) for tubulin from *Tetrahymena* cilia. The apparent molecular weights of the α and β subunits were 56,000 and 53,000, respectively, as determined by their mobilities relative to known protein standards (Feit *et al.*, 1971; Olmsted *et al.*, 1971). Bryan (1972a) used Ferguson plots of reduced and acetylated sea urchin egg tubulin in urea–acrylamide gels to show that the separation of α and β subunits was due primarily to differences in charge rather than molecular weight. In later work, Bryan (1974) demonstrated that tubulin subunits could be effectively separated on SDS–acrylamide gels if the conditions of ionic strength and pH were carefully selected. Since then the most commonly used one-dimensional electrophoretic systems for tubulin subunit analyses have been those of either continuous or discontinuous SDS–polyacrylamide design (Weber and Osborn, 1969; Laemmli, 1970; Yang and Criddle, 1970; Studier, 1973).

The comparative electrophoretic mobilities of tubulin subunits from diverse species are generally similar, yet several studies have demonstrated that tubulin subunits from nonanimal species do not precisely comigrate with those from animals in any electrophoretic system. This was first observed by Olmsted *et al.* (1971), who noted that tubulins from porcine brain and neuroblastoma cells comigrated as single bands, but *Chlamydomonas* flagellar outer doublet tubulin was separated into α- and β-subunit bands in the same urea–acrylamide gel system. They showed that the subunits of all three tubulins could be separated in SDS–urea/acrylamide gels, but while the neuroblastoma and brain tubulin subunits comigrated exactly, the alga subunits had different mobilities from those of the animals, with the α-subunit mobility being the most different.

The subunits of tubulins from several algae and higher plants have been readily resolved on SDS–polyacrylamide gels. Tubulins from the flagellated algae *Chlamydomonas*, *Chlorogonium*, and *Polytomella* exhibit subunits that are usually more closely spaced that those of most animals. The β subunits migrate very near to or slightly ahead of animal β subunits, and the α subunits are generally found to migrate faster than those of animals (Olmsted *et al.*, 1971; McKeithan and Rosenbaum, 1981; Little *et al.*, 1981a, 1982a,b, 1983; Morejohn *et al.*, 1984). The tubulins from ferns (*Marselia* and *Pteridium*) and cultured

cells of higher plants (*Rosa, Nicotiana, Daucus,* and *Hibiscus*) exhibit electrophoretic mobilities similar, but not identical, to those of algae on SDS–polyacrylamide gels. In general, the β subunits either comigrate with animal β subunits or run slightly faster, and the α subunits run at a position intermediate to the α and β subunits of animals and close to algal α subunits (Ludueña *et al.*, 1980; Little *et al.*, 1981a; Morejohn and Fosket, 1982; Yadav and Filner, 1983, Morejohn *et al.*, 1984). Figure 3 shows a comparative electrophoretic analysis of

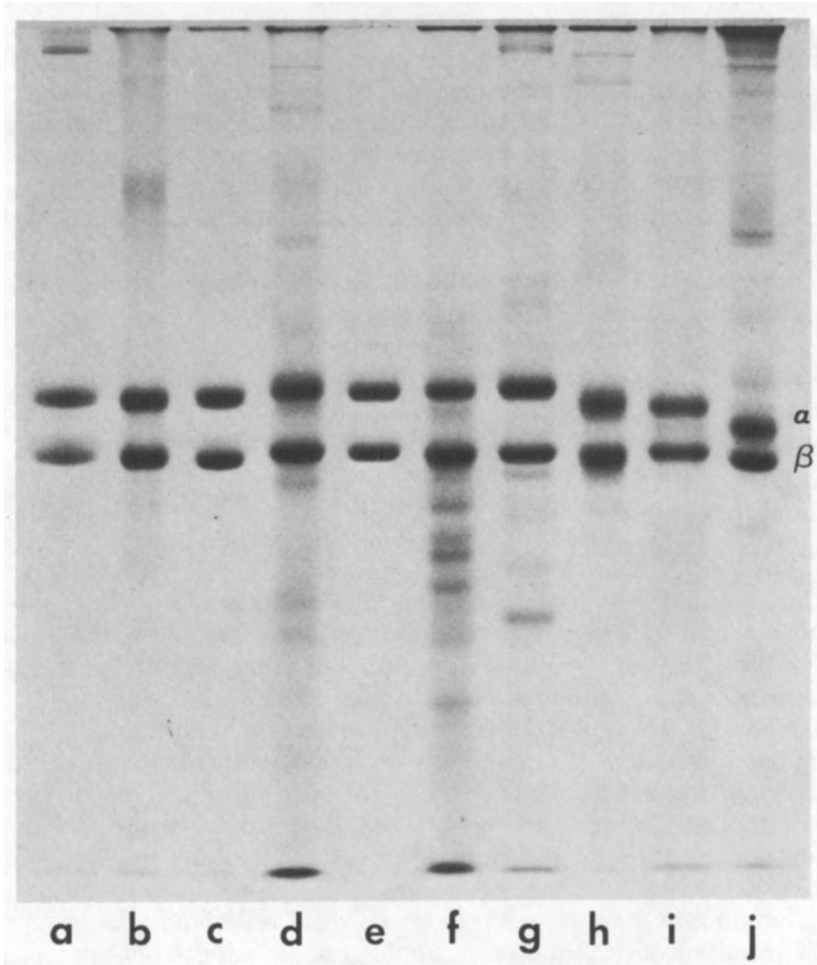


Figure 3. Electrophoretic separation of tubulin subunits. Carboxymethylated tubulins were run on an SDS/7% polyacrylamide gel and stained with Coomassie brillian blue. (a) Bovine brain tubulin; (b) bovine kidney tubulin; (c) dogfish brain tubulin; (d) sea urchin egg tubulin; (e) sea urchin sperm flagellar outer doublet tubulin; (f) worm sperm flagellar outer doublet tubulin; (g) worm sperm flagellar central pair tubulin; (h) squid brain tubulin; (i) fern sperm flagellar outer doublet tubulin; (j) alga flagellar tubulin. Reproduced by permission from the authors (Little *et al.*, 1981a) and from the *Journal of Molecular Biology* **149**:95–107, 1981. Copyright 1981 by Academic Press Inc. (London) Ltd.

various plant and animal tubulins (Little *et al.*, 1981a). However, there have been recent reports that the α subunit of higher plant tubulins can run significantly faster than the β subunit. This apparently inverted electrophoretic mobility pattern was observed by Mizuno *et al.* (1985), Mizuno (1985), and Hussey and Gull (1985). These results are not surprising because although the subunits of higher plants and *Chlamydomonas* nearly comigrate (Little *et al.*, 1981a; Morejohn *et al.*, 1984), the *Chlamydomonas* α subunit has a molecular weight (49,530) lower than that (49,556) of its β subunit (Youngblom *et al.*, 1984; Silflow *et al.*, 1985). It appears that the mobility of plant tubulin subunits can shift, presumably as a result of the particular electrophoretic system that is employed.

The electrophoretic separation of protozoan α- and β-tubulins is apparently more difficult than for other tubulins, and their mobilities are clearly different from those of brain tubulin. Maekawa and Sakai (1978) used two different SDS–gel systems to compare the mobilities of tubulins from *Tetrahymena* and pig brain. In three of four possible treatments the *Tetrahymena* α and β subunits were not as well separated from each other as those of brain tubulin. After reduction and carboxymethylation of *Tetrahymena* subunits, they were separated most on Weber and Osborn (1969) gels; the β subunit nearly comigrated with that of brain, while the *Tetrahymena* α subunit ran faster than the brain α subunit. Inefficient separations of tubulin subunits from the protozoans *Naegleria, Giardia,* and *Leishmania* have been observed (Kowit and Fulton, 1974; Holberton and Ward, 1981; Bordier *et al.*, 1982a,b). In the case of the *Leishmania* tubulin, very long (22-cm) SDS–urea–gradient polyacrylamide gels were used to resolve separate bands, neither of which exactly comigrated with brain subunit bands (Bordier *et al.*, 1982a,b). *Giardia* tubulin subunits were difficult to resolve on discontinuous polyacrylamide SDS gels, but readily separated on continuous polyacrylamide gels containing a low ionic strength buffer (Holberton and Ward, 1981). Little *et al.* (1983) were able to effectively separate the tubulin subunits of *Tetrahymena* and the heliozoan *Echinosphaerium* on Yang and Criddle (1970) gels and showed that both subunits of *Echinosphaerium* migrated considerably faster than those from several other species including those of *Tetrahymena.* Adoutte *et al.* (1984) observed the α subunits of *Paramecium* and *Tetrahymena* to run faster than the β subunits in electrophoretic gels. Similar results have been reported for the tubulin subunits from the trypanosome *Crithidia* (Russell and Gull, 1984).

Among all tubulins, those of the slime molds display a pattern most consistently. Clayton *et al.* (1980) compared the mobilities of tubulin subunits from *Physarum* with those from sheep brain and *Tetrahymena* cilia in gels of SDS–acrylamide and urea–SDS–acrylamide. When *Physarum* subunits were first separated in an SDS gel, individually cut out, and rerun in separate lanes of a urea–SDS gel, the relative positions of each subunit completely reversed. That is, the slow-moving and fast-moving bands were able to exchange positions depending on the particular gel conditions. To determine which band actually corresponded to those of brain tubulin α and β subunits, Clayton *et al.* (1980) and Clayton and Gull (1982) peptide-mapped both *Physarum* bands

and found that in SDS–polyacrylamide gels, the upper, slow-moving band was the β subunit and the lower, fast-moving band the α subunit. That this electrophoretic behavior is not an isolated case among the slime molds has been shown by White *et al.* (1983). They demonstrated with peptide mapping and immunoblotting that the slow-moving subunit of *Dictyostelium* tubulin is the β and the fast-moving subunit is the α in SDS–polyacrylamide gels.

Tubulin subunits from yeasts have been readily resolved on both continuous and discontinuous SDS–polyacrylamide gel systems. The α subunits of *Aspergillus* and bovine brain tubulins comigrated, and the *Aspergillus* β subunit ran faster than the brain β subunit in Weber and Osborn (1969) gels, whereas both subunits from these species comigrated in Yang and Criddle (1970) gels (Ludueña *et al.*, 1980). The α and β subunits of *Saccharomyces* tubulin showed slower mobilities than those of pig brain in a modified Laemmli (1970) gel (Kilmartin, 1981), a pattern that is likely to be a reflection of their differences in molecular weight (Neff *et al.*, 1983; Toda *et al.*, 1984). The subunits of yeast tubulins run in the conventional order in SDS gels, rather than in the inverted pattern of slime mold tubulins, since comparative peptide mapping of the ascomycete *Aspergillus* and pig brain tubulins has shown the expected subunit orientation in the SDS dimension of a two-dimensional electrophoretic gel (Sheir-Neiss *et al.*, 1978). Toda *et al.* (1984) reported immunoblots of *S. pombe* tubulin subunits with a conventional orientation of bands.

The numerous studies cited here illustrate that the adequate separation of α and β subunits requires that the conditions of electrophoresis be optimized for the particular tubulin of interest. Clayton *et al.* (1980) found that the relative mobilities of tubulin subunits from *Physarum, Tetrahymena,* and brain were strongly influenced by the type of SDS used in the gel. Subsequently, it was determined that SDS preparations from different vendors contained different amounts of detergent hydrocarbon chains and that a preparation (Sigma) with chains of different sizes was more effective for separation of tubulin subunits than that (Fisons) with a single chain (Best *et al.*, 1981). We have obtained similar results with plant tubulins and different SDS preparations. Very little separation of plant tubulin subunits was observed when using highly purified BioRad SDS, but adequate separation was obtained with Sigma SDS. We also have found that the low ionic strength Tris buffer of the electrophoretic method of Studier (1973) contributed to increased subunit separation of tubulin (Morejohn, unpublished results). The apparent molecular weights (51,000–57,000) of subunits from diverse tubulins on several electrophoretic gel systems are up to 10% larger than those (49,500–51,000) estimated from sequencing studies. This fairly large disparity in estimated molecular weight probably results from an atypical interaction of the strongly acidic tubulin with SDS when compared with commonly used protein standards.

Tubulin subunits can also be resolved in isoelectric focusing (IEF) gels and two-dimensional gels that separate by IEF in the first dimension and under denaturing conditions (e.g., SDS) in the second dimension (O'Farrell, 1975). Both subunits of most tubulins run in the acidic range with the iso-

electric point (pI) of β-tubulin being more acidic (pI = 5.3–5.5) than the pI of α-tubulin (pI = 5.5–5.8). While the tubulins from nonanimal species also run in the acidic range in IEF, there are examples of anomalous mobilities of both subunits. Kilmartin (1981) showed that the α subunit of *Saccharyomyces* tubulin was more basic and the β subunit more acidic than the corresponding subunits of porcine brain tubulin. Mizuno *et al.* (1981) reported that the α subunit of azuki bean tubulin was more acidic than the β subunit, but another possibility was that the subunits were inverted in the SDS dimension and therefore improperly designated. The α-tubulins of rose and corn cells, however, ran in the usual more basic position relative to β-tubulin, indicating that the reversal of subunit pI in higher plant tubulins is not universal (Morejohn, Ranum, and Silflow, unpublished results). This is supported by the work of Hussey and Gull (1985) on *Phaseolus*. Thomashow *et al.* (1983) also found that the *in vitro* translated subunits of trypanosome tubulin ran in an apparently reversed order. The α subunit was more acidic (pI = 4.8) than the β subunit (pI = 5.2). Little *et al.* (1983) found that carboxymethylation of pig brain, sea anemone, and *Physarum* tubulins produced a more acidic pI for α-tubulins than for their β-tubulins, but had the opposite effect on the subunits from the heliozoan *Echinosphearium* and the protozoan *Tetrahymena*. White *et al.* (1983) reported that both the α and β subunits of *Dictyostelium* tubulin were much more basic (pIs 6.2–6.7) than those of brain tubulin. These numerous electrophoretic studies have uncovered subtle differences between nonanimal and animal tubulins that reflect their distinct charges and/or molecular weights. The use of IEF in one- and two-dimensional gels for the identification of tubulin isotypes is discussed in Section 3.5.

3.3. Peptide Mapping Patterns

Stephens (1970) and Fine (1971) reported two-dimensional tryptic peptide maps of tubules from sea urchin sperm flagellar outer doublets and sympathetic ganglion neurons, respectively, that demonstrated that the α and β subunits were similar, but not identical. Ludueña and Woodward (1973) compared the cyanogen bromide peptide maps of α and β subunits from chicken brain tubulin and sea urchin sperm flagellar outer doublet tubulin and also observed that the map of each α-tubulin was very different from the map of each β-tubulin. They also showed that the fragment patterns of the two α subunits were very similar to each other and that those of the β subunits were also very similar. These studies established that the technique of peptide mapping was useful not only for the study of tubulin structure, but also for the comparison of tubulins from diverse species.

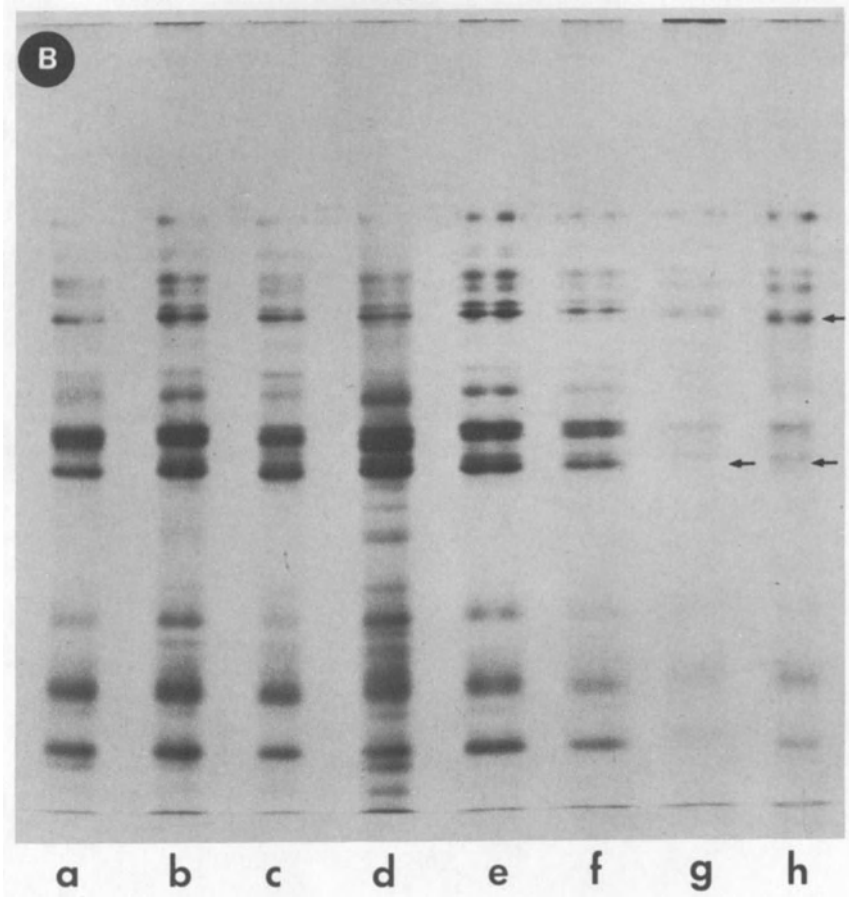
Clayton *et al.* (1980) were the first to demonstrate with comparative peptide mapping that the tubulins from a lower eukaryote slime mold (*Physarum*) and mammalian brain were structurally different. They used *Staphylococcus aureus* V8 protease in the limited proteolysis peptide mapping method of Cleveland *et al.* (1977b) to show that the β subunits of *Physarum* and sheep brain were similar, but that the α subunits were quite different. Little and

Ludueña and their collegues (Little *et al.*, 1981a, 1982a, 1983, 1984) undertook a comprehensive series of comparative peptide mapping studies on tubulins from phylogenetically diverse species. They compared the one-dimensional mapping patterns generated by *S. aureus* V8 protease cleavage of tubulin subunits from 28 species including higher plants, algae, protozoans, a slime mold, and numerous vertebrate and invertebrate animals. The β--subunit fragment patterns of numerous species were rather similar, but the α-subunit patterns showed obvious differences suggesting that the β subunits have been more highly conserved than the α subunits (Fig. 4). Other reports of comparative peptide mapping of protozoan, higher plant, and mammalian tubulins with *S. aureus* protease have generally supported the notion that β subunits have been more highly conserved than α subunits (Morejohn and

Figure 4. Peptide maps of tubulin subunits. α or β subunits were digested with *S. aureus* protease in a 5% stacking gel and resolved by electrophoresis on a 15% gel. α subunits are shown in A and β subunits are shown in B. (a) Bovine brain tubulin; (b) bovine kidney tubulin; (c) squid brain tubulin; (d) sea urchin egg tubulin; (e) sea urchin sperm flagellar outer doublet tubulin; (f) worm

Fosket, 1982; Bordier *et al.*, 1982a,b; Yadav and Filner, 1983; Stieger *et al.*, 1984; Mizuno *et al.*, 1985). However, recent sequencing studies (Section 3.1) indicate that both the α and β subunits of nonanimal tubulins are nearly equally diverged from their animal counterparts, a result that is inconsistent with most of the numerous peptide mapping studies. It would appear that the common use of a single protease (usually *S. aureus* V8) in comparative peptide mapping has revealed differences between diverse α subunits, but not those differences between diverse β subunits.

Little *et al.* (1982a, 1984) traced possible phylogenetic relationships between organisms by comparing their tubulin peptide mapping patterns and assigning each α-tubulin to either of two different structural groups, type I (animal) or type II (nonanimal). While the peptide maps of α-tubulins in each



sperm flagellar central pair tubulin; (g) fern sperm flagellar outer doublet tubulin; (h) alga flagellar tubulin. Arrows indicate small differences in the alga and fern maps from those of the animals. Reproduced by permission from the authors (Little *et al.*, 1981a) and from the *Journal of Molecular Biology* **149**:95–107, 1981. Copyright 1981 by Academic Press, Inc. (London) Ltd.

group were not identical, they were rather similar. The type I pattern could be further subdivided into two subtypes, axonemal and cytoplasmic, but the type II pattern could not be subdivided in this way. Little *et al.* (1982a,b, 1984) proposed that nonanimal tubulins in functionally different microtubules (cytoplasmic or axonemal) are structurally similar, but that during the evolution of metazoans, duplication and modification of tubulin genes gave rise to structurally and functionally specialized animal tubulins.

Structural differences or similarities between diverse tubulins have not always been revealed by cleavage with a single protease or by one-dimensional peptide mapping. Clayton and Gull (1982) compared the peptide-mapping patterns of tubulins from the slime mold *Physarum* and sheep brain after cleavage with four different proteases. They found that *S. aureus* protease and papain produced α-tubulin mapping patterns that reflected obvious differences between the tubulins, but that trypsin and chymotrypsin produced slime mold and sheep brain α-tubulin fragments patterns that were almost indistinguishable. Cleavage of the β-tubulins by *S. aureus* protease produced very similar patterns, but digestion by trypsin revealed the β subunits to be less homologous. Little *et al.* (1983) used both *S. aureus* protease one-dimensional mapping and tryptic two-dimensional mapping to compare the tubulin from heliozoan (*Echinosphaerium*) axopodia with those from cow brain, *Physarum,* and sea anemone (*Tealia*) sperm and *Tetrahymena* ciliary axonemes. The one-dimensional patterns of *Echinosphaerium, Tetrahymena,* and *Physarum* α subunits resembled each other, but not those of the animal α subunits. The β-tubulin map of *Echinosphaerium* was very different from all the others. Two-dimensional patterns showed that the *Echinosphaerium* α and β subunits were even more different from those of *Tetrahymena* and *Physarum* than had been revealed by one-dimensional mapping.

Sheir-Neiss *et al.* (1978) used *S. aureus* protease in one-dimensional peptide maps to identify *Aspergillus* tubulin subunits by comparison with those from pig brain. Similar results were reported by Weatherbee and Morris (1984). Interestingly, the maps of Sheir-Neiss *et al.* (1978) showed more similarities between the fungal and brain α subunits than between the β subunits. They noted that cleavage of the subunits by chymotrypsin showed the fungal and brain tubulins to be even more divergent. This finding and the work of Clayton and Gull (1982) imply that the use of a single enzyme in comparative mapping of widely diverse tubulins may not accurately predict relative levels sequence homology. While comparative peptide mapping studies have provided valuable information on the structural relationships of diverse tubulins in lieu of their amino acid sequences, it is recommended that more than one protease be used to either identify tubulins or estimate levels of homology between tubulins from vastly divergent organisms.

3.4. Immunology

Numerous monoclonal or polyclonal antibody preparations against tubulins from various organisms and their tissues and organelles have been

described. The degree to which different antibody preparations cross-react with diverse tubulins can vary significantly. For example, a broad range of cross-reactivity has been observed for a rat monoclonal antibody (YL ½) against the α subunit of yeast tubulin (Kilmartin *et al.*, 1982). This antibody binds to the tyrosinolated form of the α subunit of tubulin or microtubules in a variety of mammalian cells and in a higher plant (Kilmartin *et al.*, 1982; Cumming *et al.*, 1984; Wehland *et al.*, 1984). On the other hand, White *et al.* (1983) reported that an antibody against *Tetrahymena* tubulin failed to react with *Dictyostelium* tubulin. Fulton and Kowit (1975) prepared an antiserum to *Naegleria* flagella tubulin that did not react significantly with *Naegleria* cytoplasmic tubulin. Apparently the binding specificity of antibody preparations is variable because immunized animals respond differently, the immunogens are variably antigenic according to their origin and method of preparation, and investigators have used different host mammals and different injection schedules. It is important, therefore, to prepare or obtain antibodies that are particularly suited to the purpose of the investigation at hand (Rousset *et al.*, 1983).

Antibodies have been used to decorate microtubules in the flagellated protozoa *Trypansoma* and *Giardia,* the slime mold *Dictyostelium,* the yeast *Saccharomyces,* the fungus *Aspergillus,* the alga *Polytomella,* the mosses *Sphagnum, Polytrichum,* and *Physcomitrella,* and various higher plants such as onion, carrot, tobacco, hibiscus, *Leucojum, Hemanthus, Clivia,* and *Tradescantia* (Brown *et al.*, 1976; Franke *et al.*, 1977; Lloyd *et al.*, 1979, 1980; Powell *et al.*, 1980; Wick *et al.*, 1981; van der Valk *et al.*, 1981; DeMey *et al.*, 1982; Schmit *et al.*, 1983; Miller *et al.*, 1983; White *et al.*, 1983; Gallo and Anderton, 1983; Cumming and Williamson, 1984; Torian *et al.*, 1984; Clapham and Östergren, 1984; Gambino *et al.*, 1984; Hahne and Hoffmann, 1984; Kilmartin and Adams, 1984). An example of the high-resolution image produced by immunogold staining of the mitotic spindle in *Hemanthus* endosperm is shown in Fig. 5.

Quantitation of tubulin or microtubules in extracts of higher plant tissues has been reported with antibodies against animal tubulins. Koehn and Olsen (1978) used antibodies against bovine brain tubulin to compare the relative amounts of tubulin in different soybean tissues by radioimmunoassays. They found more immunoreactive material in rapidly dividing regions than in elongating regions. Polyclonal antibodies to calf brain tubulin were used to develop a radioimmunoassay for cotton tubulin (Rikin *et al.*, 1982). This assay was used in a study of the effects of chilling and antimicrotubule drugs on cotton tissues (Rikin *et al.*, 1983).

The cross-reactivities of polyclonal antibodies against the α or β subunits of rose tubulin were examined by immunoblotting (Morejohn *et al.*, 1984). Neither antibody cross-reacted with the other subunit of rose tubulin, but both antibodies cross-reacted strongly with the corresponding tubulin subunits of carrot, hibiscus, and *Chlamydomonas* (Fig. 6). A comparison of the cross-reactivities of these antibodies with tubulins from more diverse organisms, including sea urchin, rabbit, and cow, revealed that most of the antigenic determinants were specific to plant tubulins (Fig. 7). This study demon-

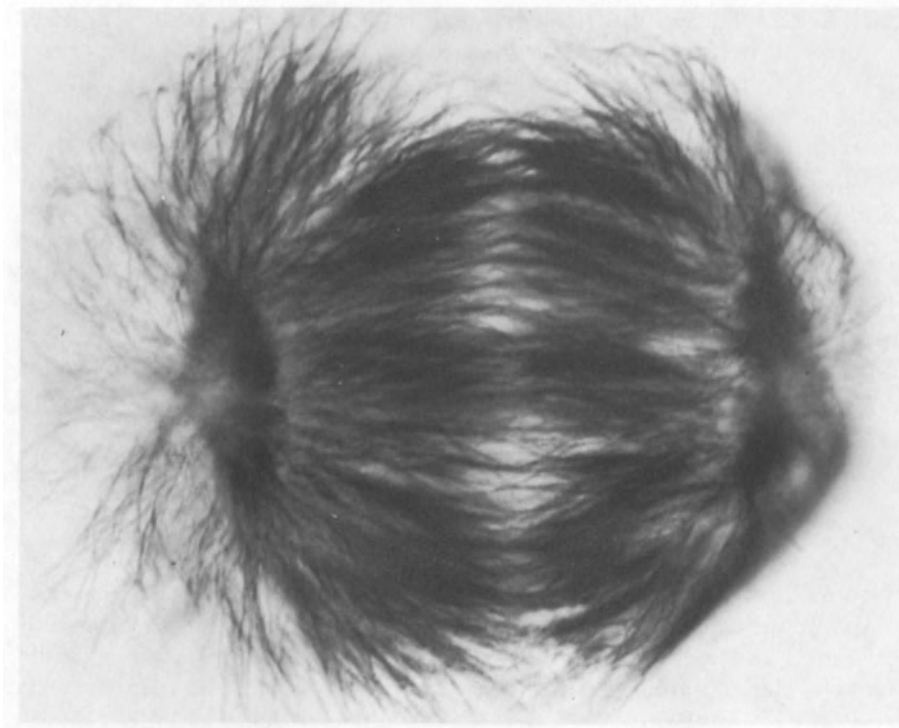


Figure 5. Mitotic spindle of *Hemanthus* endosperm cell. The microtubules in this anaphase cell were resolved on the light microscope after indirect immunogold staining (DeMey *et al.*, 1982). Micrograph supplied by Dr. J. Molè-Bajer.

strated significant structural differences between plant and animal tubulins that exist at the level of primary amino acid sequence and/or posttranslational modification. A monoclonal antibody against mung bean tubulin has been shown to bind to the α subunit of mung bean and carrot tubulins, but not to tubulin from sheep brain (Mizuno *et al.*, 1985). This result is consistent with the observation that plant and animal tubulins are immunologically different.

Adoutte *et al.* (1984) observed with comparative immunoblotting and immunocytochemistry that antibodies against higher animal tubulins bound to those of protists, while antibodies against tubulins from *Paramecium* or *Tetrahymena* reacted with protist tubulin, but not higher animal tubulins. They proposed that the protist antibodies recognized ancient determinants that are not present on higher animal tubulins. Investigations on posttranslational modifications and sequencing of nonanimal tubulins should reveal the nature of the immunological differences that exist between diverse tubulins.

3.5. Subunit Microheterogeneity

Indirect evidence for heterogeneity of tubulins came from early studies that showed that microtubules in different organelles of the same species were

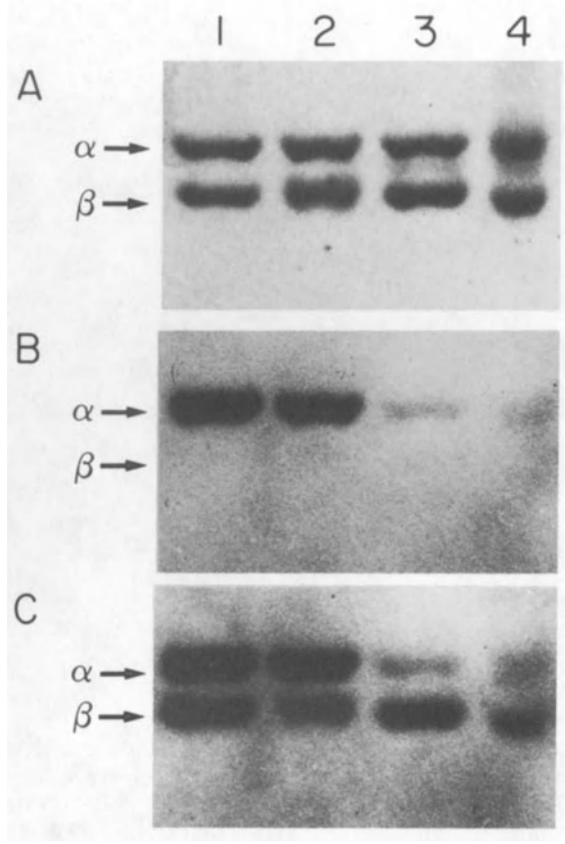


Figure 6. Cross-reactivities of rose tubulin antibodies with carrot, hibiscus, and alga tubulins. (A) SDS/7.5% polyacrylamide gel containing 2 μg of rose (lane 1), carrot (lane 2), hibiscus (lane 3), and alga (*Chlamydomonas*) flagellar tubulins (lane 4). (B) Autoradiograph of blot probed with 1:100 dilution of α-IgG. (C) Autoradiograph of the same blot shown in B reprobed with 1:50 dilution of β-IgG. Autoradiographs B and C were exposed for 3.25 and 3.5 hr, respectively. Positions of α and β subunits are shown on the left. Reproduced from Morejohn *et al.* (1984).

differentially sensitive to temperature range, pH range, ionic strength, or antimicrotubule drugs (Behnke and Forer, 1967; Tilney and Gibbons, 1968; Linck, 1973; Brinkley and Cartwright, 1975). Immunological differences between tubulins in different organelles of sea urchin were reported by Fulton *et al.* (1971). They demonstrated that an antiserum to flagellar axonemes cross-reacted differentially with tubulins from the mitotic apparatus, cilia, and flagella and proposed that different microtubules were composed of similar, but nonidentical, tubulins. Similarly, Kowit and Fulton (1974) found that an antiserum against *Naeglaria* flagellar outer doublet tubulin did not bind to cytoplasmic tubulin in myxamebae. Feit *et al.* (1971) observed that the α and β subunits of mouse brain tubulin became split into several finer bands when run in IEF gels, indicating that each subunit was composed of microheterogeneous forms of tubulin. Soon afterward, Witman *et al.* (1972) reported that *Chlamydomonas* flagellar tubulin subunits were resolved into five bands in IEF gels, suggesting that more than one type of tubulin dimer made up the flagellum. Bibring *et al.* (1976) obtained strong evidence that different microtubule-containing organelles in sea urchin were composed of microheterogeneous forms of the α subunit. Ciliary and mitotic α subunits were composed of different species as shown by both electrophoresis in different

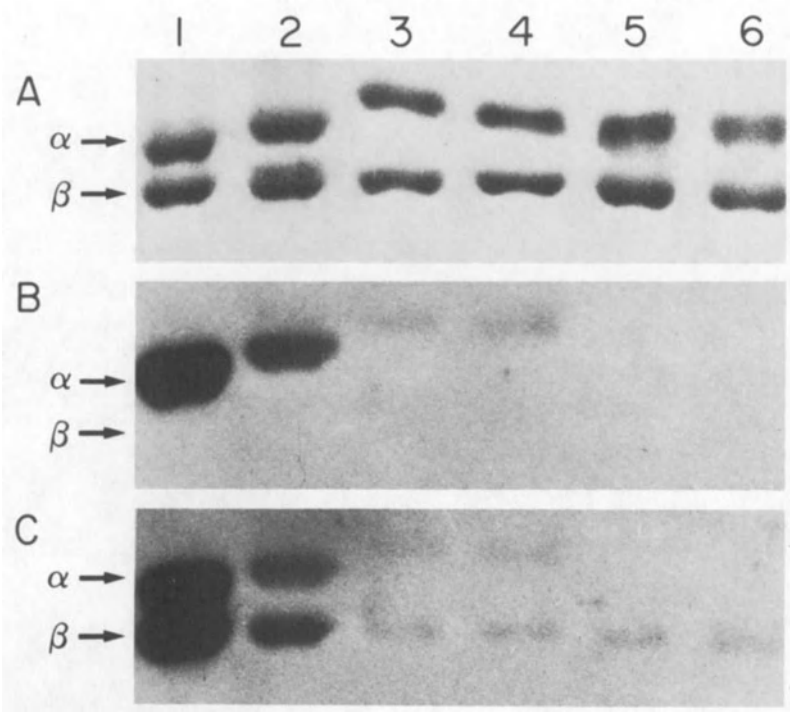


Figure 7. Reactivity of rose tubulin IgGs with tubulins from phylogenetically distant species. (A) SDS/7.5% polyacrylamide gel containing α and β subunits of tubulins (3μg) from various species. (B) Autoradiograph of blot probed with a 1:100 dilution of α-IgG. (C) Autoradiograph of the same blot shown in B after reprobing with a 1:50 dilution of β-IgG antibody. Autoradiographs were exposed for 4 hr. (1) Rose cell tubulin, (2) alga flagellar tubulin, (3) sea urchin sperm flagellar outer doublet tubulin, (4) sea urchin egg tubulin, (5) rabbit brain tubulin, (6) bovine brain tubulin. Positions of α and β subunits are designated on the left. Reproduced from Morejohn *et al.* (1984).

gel systems and cyanogen bromide peptide mapping. In a more comprehensive study of organelle-specific tubulin in sea urchin Stephens (1978) compared the amino acid compositions and peptide-mapping patterns of subunits from sperm flagellar A tubules, B subfibers, and central pair microtubules, embryo ciliary A tubules and B subfibers, and egg cytoplasmic microtubules. He found small differences in the tubulins from these different locations and proposed that local regions of variability in tubulin dimers might provide functional specificity to different microtubules. On the basis of the chemical and immunological evidence for microheterogeneity of tubulin within a given organism, Fulton and Simpson (1976) proposed the multitubulin hypothesis, which stated that an organism would have multiple, nonidentical copies of tubulin genes for the purpose of encoding different organelle-specific tubulins. This proposal did not rule out the possibility that any of the tubulins could be modified posttranslationally.

Since these early investigations, numerous studies have demonstrated microheterogeneity from a given organism or its tissues and cells. Microheterogeneity has been revealed with various techniques. including high-resolution one- or two-dimensional electrophoresis, peptide mapping, chemical cross-linking, protein or gene sequencing, and immunological and mutant methodologies (for example, Krauhs *et al.*, 1981; George *et al.*, 1981; Gallo and Anderton, 1983; Thompson *et al.*, 1984; Weatherbee and Morris, 1984; Schedl *et al.*, 1984a; Sullivan and Cleveland, 1984; Field *et al.*, 1984; Field and Lee, 1985; Birkett *et al.*, 1985; Little and Ludueña, 1985; and references therein). Among nonanimal tubulins, microheterogeneity has been observed in *S. pombe, Physarum, Dictyostelium, Polytomella, Chlamydomonas, Phaseolus, Aspergillus,* and *Crithidia* (Lefebvre *et al.*, 1980; McKeithan and Rosenbaum, 1981; Clayton *et al.*, 1983; White *et al.*, 1983; Toda *et al.*, 1984; Russell *et al.*, 1984; Weatherbee and Morris, 1984; Hussey and Gull, 1985). Information on the origin and significance of subunit microheterogeneity from studies on both nonanimal and animal systems demonstrates that there are at least two possible explanations for the existence of tubulin isotypes. First, different isotypes can be products of nonidentical genes, provided that the organism of interest has multiple tubulin genes. Second, tubulin isotypes may arise as a result of the posttranslational modification of a tubulin gene product. Both possible sources of microheterogeneity have been demonstrated in several diverse organisms, some of which are discussed in Sections 3.6 and 3.7.

3.6. Posttranslational Modifications

Types of tubulin posttranslational modification that have been reported are phosphorylation, tyrosinolation, glycosylation, and acetylation. This area of microtubule biochemistry is interesting because posttranslational modifications could potentially confer rapid and reversible changes to the properties of microtubule dynamics *in vivo*. There have been relatively few accounts of the glycosylation of tubulin, and those cases involved putative membrane-associated tubulins in scallop cilia (Stephens, 1977) and *Tetrahymena* cilia (Dentler, 1980).

Tyrosine can be specifically removed from the COOH-terminal end of the α-subunit of brain tubulin by the enzyme tyrosinotubulin carboxypeptidase (Hallak *et al.*, 1977; Argaraña *et al.*, 1978; Kumar and Flavin, 1981). Detyrosinolated α-tubulin can be tyrosinolated at the COOH-terminal glutamate by the enzyme tubulin–tyrosine ligase (TTL) (Barra *et al.*, 1974; Raybin and Flavin, 1975, 1977a; Rodriquez and Borisy, 1979; Schroder *et al.*, 1985). The reaction of TTL *in vitro* occurs preferentially with soluble tubulin rather than microtubules, while tyrosinotubulin carboxypeptidase prefers microtubules as substrates (Thompson, 1977, 1982; Thompson *et al.*, 1979; Kumar and Flavin, 1981).

Raybin and Flavin (1977b) reported that the tyrosinolation state of brain tubulin had no effect on microtubule assembly *in vitro*. However, Kumar and Flavin (1982) reported that brain tubulin tyrosinolation was important in

several parameters of microtubule assembly. They found that microtubules polymerized from tyrosinolated tubulin were significantly shorter in the presence of MAP2 and that tyrosinolated microtubules contained 20–30% more MAPs. Also, the rate and extent of polymerization of MAP-free, tyrosinolated tubulin in the presence of substoichiometric concentrations of taxol was nearly twofold greater than with detyrosinolated tubulin. Nath *et al.* (1981) showed that colchicine at concentrations that depolymerize microtubules in rabbit leukocytes stimulated tyrosinolation, and that taxol treatment inhibited tyrosinolation. These *in vivo* results suggest that tyrosinolation is regulated according to the size of the unassembled tubulin pool. Gundersen *et al.* (1984) used specific antibodies against either tyrosinolated or detyrosinolated α-tubulins to examine the distribution of these forms of tubulin in microtubules in monkey kidney cells. They found that the two types of tubulin were predominantly located in different populations of microtubules, and that there were many more microtubules containing the tyrosinolated form. The physiological significance of the α-tubulin tyrosylation-detyrosylation cycle *in vivo* is not known, although it has been proposed that it may influence neuronal cell morphology and membrane excitability by modulating microtubule assembly–disassembly dynamics (Deanin *et al.*, 1977; Matsumoto *et al.*, 1979). Ponstingl *et al.*, (1979) speculated that the tyrosinolation–detyrosinolation cycle may shift the conformational state of the COOH-terminal end of α-tubulin, a highly acidic region that may represent a functional domain for the binding of polycationic MAPs.

Preston *et al.* (1979) examined the phylogenetic distribution of TTL activity in animals and found activity in vertebrates, while Kobayashi and Flavin (1981) demonstrated TTL activity in invertebrates. Attempts to identify TTL activity in extracts of *Tetrahymena, Physarum,* and *Saccharomyces* have failed (Raybin and Flavin, 1975, 1977b; Roobol and Gull, 1982). Because sequencing studies of α-tubulin genes from several organisms, including yeast and *Chlamydomonas,* have shown tyrosine to be encoded at their COOH termini, it is possible that detyrosinolation by tubulinotyrosine carboxypeptidase would be a more appropriate enzymatic assay to examine across phylogenetic lines. One exception is the α-tubulin in the ciliated protozoan *Stylonychia,* where a COOH-terminus tyrosine is not encoded by the gene (Helftenbein, 1985). Interestingly, Stieger *et al.* (1984) demonstrated that tyrosine can be reversibly added *in vivo* to the α-tubulin in the protozoan *Trypanosoma.* Wehland *et al.* (1984) reported that all the microtubule arrays in onion meristematic cells were labeled with a monoclonal antibody specific to the tyrosinolated form of tubulin. This result does not necessarily imply that all dimers in these microtubules were tyrosinolated or that the cells contain TTL. Future studies with antibodies specific for the nontyrosinolated COOH terminus of α-tubulin from plants may resolve this question.

Numerous studies have demonstrated the phosphorylation of mammalian tubulin both *in vivo* (Bryan, 1972a; Eipper, 1972) and *in vitro* (Piras and Piras, 1975; Goldenring *et al.*, 1983). *In vivo* labeling with ^{32}P in mammalian cells has shown that one phosphate group is added to a β-tubulin

serine residue per tubulin dimer (Eipper, 1972, 1974). *In vitro* labeling of sea urchin sperm axoneme outer doublet tubulin also resulted in the incorporation of one phosphate group per β-tubulin, but in addition, 1.2–1.4 phosphates were incorporated per α-tubulin (Stephens, 1975). Piperno and Luck (1976) noted that the *in vitro* labeling of *Chlamydomonas* flagellar axoneme tubulin occurred and that both serine and threonine residues of α-tubulin were phosphorylated. Lu and Elzinga (1978) reported that a serine in the COOH-terminal region of a brain β-tubulin was phosphorylated. A calmodulin-dependent kinase from rat brain has been shown to phosphorylate brain tubulin *in vitro* (Goldenring *et al.*, 1983). The kinase phosphorylated threonine and serine residues on the β subunit and predominantly serine residues on the α subunit. If MAPs interact with the residues that are phosphorylated on tubulin, then reversible phosphorylation could alter microtubule assembly/dissassembly kinetics *in vivo*.

Reversible acetylation of tubulin used in flagella has been demonstrated. McKeithan and Rosenbaum (1981) examined tubulins from flagella or the cytoskeleton of *Polytomella* by two-dimensional electrophoresis and found that cytoskeletal tubulin contained a major species of α subunit (α_1) and flagellar tubulin contained mostly α_3 (Fig. 8). Brunke *et al.* (1982) and McKeithan *et al.* (1983) observed a similar situation in *Chlamydomonas*. McKeithan *et al.* (1983) provided evidence that the cytoplasmic α_1 is posttranslationally modified into the α_3 during flagellar regeneration in both *Polytomella* and *Chlamydomonas*. L'Hernault and Rosenbaum (1983, 1985a) showed that the modification was reversibly coupled with the regeneration or resorption of flagella in *Chlamydomonas*. L'Hernault and Rosenbaum (1985b) demonstrated that the reversible modification was the result of the addition and removal of acetate on the

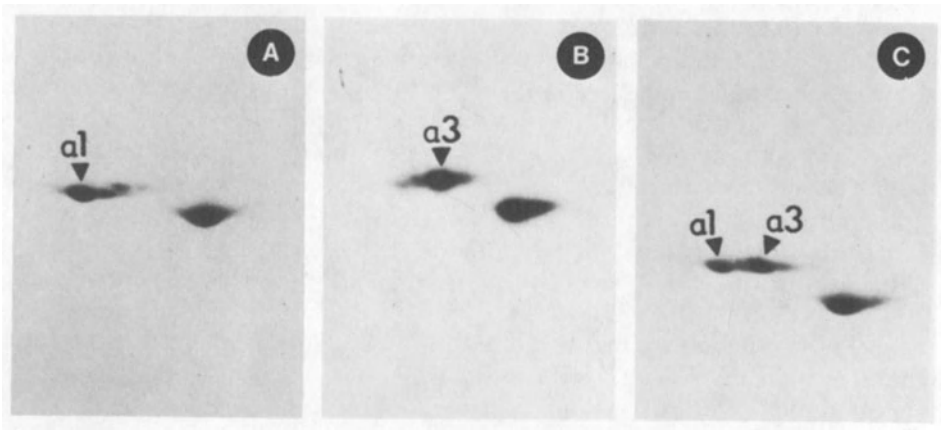


Figure 8. Two-dimensional gels of *Polytomella* tubulins. Gels show different amounts of α-tubulin isoforms in different preparations. (A) Cytoplasmic tubulin; (B) flagellar tubulin; (C) mixture of cytoplasmic and flagellar tubulins. Reproduced by permission from the authors (McKeithan and Rosenbaum, 1981). Copyright 1981 by The Rockefeller University Press, New York.

epsilon-amino group of a lysine residue in the α subunit. The finding that *Chlamydomonas* α-tubulin genes encode slightly different polypeptides may indicate that the acetylation reaction occurs preferentially with one of the gene products (Silflow *et al.*, 1985).

That this reversible posttranslational modification is not unique to flagellated algae has been shown by studies on the flagellated form of *Physarum* myxamoebae and on the trypanosome *Crithidia.* (Russell *et al.*, 1984; Russell and Gull, 1984; Green and Dove, 1984). While acetylation has not been shown directly in these organisms, the typical α_1/α_3 electrophoretic patterns are associated with the cytoplasmic and flagellar tubulins as in the algae. The purpose of such a modification is not known, but one possibility is that tubulin in the cell body would interact with different MAPs than exist in the flagellum. In this way, a given gene product may have its chemical properties modified for specific microtubule-dependent functions in different organelles.

3.7. Multiple Tubulin Genes

Molecular cloning studies have provided evidence of small, heterodispersed miltigene families in animals. Estimates of the number of genes per haploid equivalent of genomic DNA indicate that each subunit may be encoded by several genes with 15–20 in human and rat, four to nine in chicken, 9–13 in sea urchin, and four in *Drosophila* (Cleveland *et al.*, 1980; Sánchez *et al.*, 1980; Alexandraki and Ruderman, 1981; Lemischka and Sharp, 1982; Ruderman and Alexandraki, 1983). Sequence analysis of many of the human tubulin genes indicates that several are pseudogenes and, thus, are not expressed (Lee *et al.*, 1983; and references therein). Tubulin genes have been shown to be expressed differentially according to particular developmental stages or tissue types in these organisms (Lopata *et al.*, 1983; Kalfayan and Wensink, 1982; Kemphues *et al.*, 1982; Hall and Cowen, 1985). Heterogeneity of the tubulins expressed in a given species has been shown also by gene-sequencing studies (Lee *et al.*, 1983; Sullivan and Cleveland, 1984).

Small multigene families have been reported for several nonanimal species. Gene copy number estimates are one α-tubulin and possibly two β-tubulins in *Tetrahymena,* two each in *Chlamydomonas,* one or two each in different yeasts, three to four each in *Physarum,* 14–17 each in *Trypanosoma,* 15 each in *Leischmania,* and two α-tubulins in *Stylonychia* (Silflow and Rosenbaum, 1981; Thomashow *et al.*, 1983; Seebeck *et al.*, 1983; Neff *et al.*, 1983; Landfear *et al.*, 1983; Callahan *et al.*, 1984; Schedl *et al.*, 1984b; Toda *et al.*, 1984; Helftenbein, 1985). One interesting difference in tubulin gene organization has been found in both *Trypanosoma* and *Leishmania;* both organisms contain tandemly repeated sequences rather than dispersed organization. *Trypanosoma* contains linked and alternating α- and β-tubulin sequences, while *Leishmania* has tandem α-tubulin sequences that are not linked to tandem β-tubulin sequences (Thomashow *et al.*, 1983; Landfear *et al.*, 1983; Seebeck *et al.*, 1983). Weatherbee and Morris (1984) demonstrated that *Aspergillus* contains at least two

structural genes for each tubulin subunit after analysis of electrophoretic variants in benomyl-resistant mutants.

There is evidence that at least some of the tubulin microheterogeneity found in fungi and protists is the result of the expression of different tubulin genes. Toda *et al.* (1984) provided evidence of two α-tubulin isotypes in gels, indicating that each of the two α-tubulin genes (which had different sequences) gave rise to a different isotype. Sheir-Neiss *et al.* (1978) isolated *Aspergillus* mutants resistant to benomyl, nocodazole, and thiabendazole and genetically mapped several mutations to the *ben*A locus. Analysis of the tubulins from these mutants demonstrated that most contained abnormalities in β-tubulin isotypes since these polypeptides had altered electrophoretic mobilities in two-dimensional gels. Construction and analysis of diploids heterozygous for *ben*A showed that the variant isotypes did not arise from posttranslational modification. Similar analyses have been reported for mutations in the α-tubulin gene (*tub*A locus) (Morris *et al.*, 1979). Because each of these genes produces two electrophoretically different forms of tubulin polypeptide, differential levels of posttranslational modification of each primary gene product are possible (Weatherbee and Morris, 1984).

In *Chlamydomonas* the two β-tubulin genes encode identical polypeptides, but the two α-tubulin genes encode polypeptides that differ by two amino acids (Youngblom *et al.*, 1984; Silflow *et al.*, 1985). The two α-tubulins differ by a single charge, and it has been proposed that one of these α subunits is preferentially posttranslationally modified into the flagellar α_3 by acetylation (Silflow *et al.*, 1985). This possibility lends some support to the multitubulin hypothesis of Fulton and Simpson (1976).

Heterogeneity of tubulin subunits has been documented in *Physarum*. Clayton *et al.* (1983) identified myxamebal tubulin isoforms on two-dimensional gels. Differential expression of isoforms was observed by Burland *et al.* (1983) and Schedl *et al.* (1984a) in myxamebae and plasmodia of *Physarum*, and evidence was presented that at least four of the isoforms arise from different genes. Posttranslational modification of at least one α-tubulin isoform in myxamebae was correlated with flagellar development (Green and Dove, 1984), an observation very similar to that reported in *Chlamydomonas* and *Polytomella*. Subsequent genetic analyses of *Physarum* mutants resistant to benzimidizoles demonstrated that the number of genes (three to four) for each subunit was similar to the number of different isoforms detected (Schedl *et al.*, 1984b; Burland *et al.*, 1983). For a treatment of the implications of this work regarding the multitubulin hypothesis, see Schedl *et al.* (1984b).

Investigation of tubulin genes in higher plants is underway. On the basis of Southern blotting analysis of genomic DNA and restriction endonuclease mapping of genomic or cDNA clones, the number of tubulin subunit genes is estimated to be between two and five each in soybean (Guiltinan, Cyr, and Fosket, unpublished data) and in maize (Morejohn and Hunsperger, unpublished data). Tubulins isolated from cultured cells of soybean and maize exhibit two to three isotypes for each subunit when run on two-dimensional gels (Morejohn, unpublished data; Cyr, Bustos, and Fosket, unpublished

data), suggesting that higher plants also express microheterogeneous forms of tubulin that arise from slightly different genes. That tubulin genes are differentially expressed in higher plant development seems likely and is being studied as well. Recently, Hussey and Gull (1985) showed four tubulin isotypes for each subunit of *Phaseolus* tubulin in root and shoot tips.

3.8. Membrane-Associated Tubulin

Taylor and co-workers initially found that all the colchicine-binding protein of cultured animal cells was tubulin and virtually all of it was present as a soluble protein in the high-speed supernatant of homogenates (Taylor, 1965; Borisy and Taylor, 1967a,b). Subsequent work with different animal tissues and organs revealed that a significant fraction of the total colchicine-binding activity was associated with the particulate fraction. In brain extracts, less than 50% of the colchicine-binding activity occurred in the soluble fraction, and a substantial portion of the colchicine-binding activity was associated with various particulate fractions such as microsomes and nerve endings, which were shown to contain no microtubules (Feit and Barondes, 1970; Lagnado *et al.*, 1971; Bhattacharyya and Wolff, 1975). Similarly, Stadler and Franke (1974) reported that only 25% of the colchicine-binding activity of rat and mouse liver occurred in the 100,000-g supernatant, with most activity being found in various membrane fractions. Colchicine binding to the soluble and membrane fractions exhibited very different kinetics. Subsequent studies have proven conclusively that this particulate colchicine-binding activity is due to membrane-bound tubulin (Blitz and Fine, 1974; Kornguth and Sunderland, 1975; Bhattacharyya and Wolff, 1975; Estridge, 1977; Kelly and Cottman, 1978; Gozes and Littauer, 1979; Zisapel *et al.*, 1980; Rubin *et al.*, 1982; Kelly *et al.*, 1983; Pfeffer *et al.*, 1983).

Stephens (1977) reported that the gill ciliary membranes of the scallop contain tubulin as a major membrane protein while tubulin is not associated with the sperm flagellar membrane. Subsequent work by Stephens (1981) showed significant chemical differences between axonemal tubulin and ciliary membrane tubulin. Stephens (1983) solubilized scallop gill ciliary membranes with detergent and demonstrated that after the detergent was removed, the membranes reconstituted themselves, and that tubulin was incorporated into the reconstituted membranes to the same extent that it was present in the membranes upon first isolation. Although the ciliary membrane tubulin is chemically different from cytoplasmic tubulins, other investigators have found that brain cytoplasmic tubulins also associate with phospholipid vesicle membranes at the lipid phase transition temperature (Caron and Berlin, 1979; Klausner *et al.*, 1981; Kumar *et al.*, 1981). Feit and Shay (1980) suppressed microtubule formation in brain extracts with colchicine and observed that the tubulin underwent an alternative form of assembly into structures that resembled membranes. Bernier-Valentin *et al.* (1983) studied rat brain tubulin binding to isolated cellular membranes from the adrenal medulla and

liver. They found that the overall affinity constant for the tubulin–membrane interaction was $1.5–3.0 \times 10^7\ M^{-1}$. They also showed the interaction to be specific, saturable, and both time- and temperature-dependent.

Membrane-bound tubulin has not been extensively investigated in plants, fungi, or protists. Dentler (1980) provided compelling data to indicate that the membranes of *Tetrahymena* cilia contained a glycosylated form of tubulin. Comparative electrophoretic mobilities and two-dimensional peptide maps of axonemal and membrane tubulins demonstrated their similar structural properties.

Hart and Sabnis (1973, 1976a,b) observed colchicine binding to the particulate fractions of *Heracleum* extracts, but also demonstrated that these fractions bound lumicolchicine. Hotta and Shepard (1973) investigated the colchicine binding of lily meiocyte nuclear membranes. Substantial colchicine-binding activity was associated with the nuclear membranes, and the activity could be solubilized with detergent. The colchicine-binding activity was associated with the nuclear membrane only during meiotic prophase in this species. All the colchicine-binding activity was associated with the soluble portion of lily somatic cell extracts. That tubulin may become transiently bound to the nuclear envelope during some phase of the somatic division cycle can be drawn from the work of Wick and Duniec (1983). These workers used indirect immunofluorescence staining and found that a diffuse reaction with *Allium* nuclear membranes occurred in late mitotic interphase, the time when the preprophase band of microtubules started to form. Tubulin has been shown to be an integral protein of coated vesicles in mammalian cells (Pfeffer *et al.*, 1983; Kelly *et al.*, 1983). Doohan and Palevitz (1980) noted that microtubules remained associated with plasma membranes and with coated vesicles after they lysed *Allium* protoplasts. The presence of tubulin in membranes may provide a mechanism for the association of microtubules with membranes.

4. Antimicrotubule Drugs

A number of different compounds are able to block microtubule-dependent processes in cells. These compounds include plant alkaloids such as colchicine, vinblastine, vincristine, podophyllotoxin, taxol, and maytansine, the antifungal and anthelmintic benzimidazole derivatives, and several herbicides. Many of these drugs stop mitosis by destroying the microtubules of the mitotic spindle (Gelfant, 1963; Kihlman, 1966; Deysson, 1968;, 1975; Dustin, 1984). Colchicine has been the most universally used and thoroughly studied of these compounds. It binds to tubulin dimers to destroy the mitotic spindle and cytoplasmic microtubules in animals by a substoichiometric poisoning of microtubule assembly (Olmsted and Borisy, 1973; Wilson *et al.*, 1974, and see Section 4.2). The story is much less complete for the other antimitotic agents or for the effects of any of these compounds, including colchicine, on the tubulin and microtubules of plants, fungi, and protists. In this section we will

consider those antimitotic agents whose actions indicate interesting similarities or differences between tubulins from animals, plants, protists, and fungi. We will consider the effects of colchicine, taxol, antimicrotubule herbicides, and benzimidazole derivatives because there is too little information available on the actions of most of the other putative antimicrotubule agents on nonanimal species to draw any firm conclusions at the present time.

4.1. Colchicine Effects in Vivo

Nanomolar to micromolar concentrations of colchicine block mitosis in cultured mammalian cells (Levan, 1954; Taylor 1965; Stubblefield and Klevecz, 1965; Kleinfeld and Sisken, 1966; Brinkley *et al.*, 1967). Taylor (1965) reported that 0.05 μM colchicine was sufficient to arrest mitosis in cultured human cells (strain KB). Cells exposed to 0.1 μM colchicine for 6–8 hr were irreversibly blocked in metaphase, although the drug had no effect on RNA, DNA, or protein synthesis in this cell line. Brinkley *et al.* (1967) obtained similar results with cultured Chinese hamster cells, except that the effects of 0.15 μM colcemid were reversible. While spindle and cytoplasmic microtubules of animal cells are highly sensitive to low levels of colchicine, other microtubule arrays may be less so. Tilney and Gibbons (1968) observed that colchicine concentrations as low as 10 μM caused the complete disappearance of cytoplasmic microtubules from sea urchin gastrulae, but that the ciliary axonemal microtubules persisted even at colchicine concentrations as high as 10^{-2} M.

Colchicine also acts as an antimitotic and antimicrotubule agent in various protists, although its effectiveness varies greatly with the organism. Ciliary regeneration and cell division were blocked in *Stentor* by colchicine at concentrations of 10^{-6}–10^{-8} M (Margulis *et al.*, 1975), while mitosis in *Tetrahymena* is stopped by colchicine only with concentrations in the millimolar range (Wunderlich and Speth, 1970; Kuzmich and Zimmerman, 1972). At millimolar concentrations colchicine also inhibited RNA, DNA, and protein synthesis. Tilney (1968) observed the rapid loss of axopodial microtubules in a colchicine-treated heliozoan, but only at millimolar concentrations.

Microtubules in fungi and slime molds also exhibit various degrees of resistance to colchicine. Quinlan *et al.* (1981) found that colchicine at 0.1 mM did not inhibit the growth of *Physarum* myxamoebae. Colchicine inhibited cell multiplication in the slime molds *Dictyostelium* and *Polyphondylium* only at concentrations in excess of 26 mM (Cappuccinelli and Ashworth, 1976; Williams, 1980). Similarly, in yeasts colchicine or its derivative colcemid inhibits cell division only at millimolar concentrations (Lederberg and Stetten, 1970; Haber *et al.*, 1972). The microtubules of other fungi seem to be even less sensitive to colchicine. Slifkin (1967) showed that concentrations of colchicine up to 2.6 mM had no effect on nuclear division in the water mold *Saprolegnia.* Heath (1975b) reported that colchicine would inhibit *Saprolegnia* colony growth at millimolar concentrations and above, but ultrastructural studies revealed that the cytoplasmic and nuclear microtubules of this organism were

unaffected by any of the colchicine concentrations tested. That is, the growth-inhibiting levels of colchicine were lethal to this organism without disrupting its spindle or cytoplasmic microtubules.

Typically, plant mitosis is 1000-fold less sensitive to colchicine than animal mitosis (Levan, 1938). For example, Deysson (1968) found that 0.25 mM colchicine was necessary to completely block *Allium* root mitosis at metaphase, but that the same effect could be observed in cultured HeLa cells with 5 nM colchicine. Most plant species exhibit mitotic arrest in the presence of colchicine, and mitosis readily resumes after the drug is washed out of the cells. If the duration of the treatment is long, restitution nuclei may form and subsequently divide as polyploids after the colchicine block is removed (Levan, 1938; Eigsti and Dustin, 1955). Millimolar levels of colchicine usually are required to induce mitotic arrest and to destroy the spindle microtubules of higher plant cells (Pickett-Heaps, 1967; Burgess and Northcote, 1969; Palevitz and Hepler, 1974; Hardham and Gunning, 1980). However, mitosis in *Colchicum* roots is completely resistant to colchicine (Levan and Steinegger, 1947). Colchicine also has been shown to block mitosis in a fern (Ludueña *et al.*, 1980) and in several algal species (for example, Wanka, 1968; Turner, 1970; Marchant and Pickett-Heaps, 1974; Hogetsu and Shibaoka, 1978) when the drug is provided to these cells in the millimolar concentration range.

A characteristic long-term response of higher plant roots and shoots to colchicine treatment is a radial enlargement of the cells (Levan, 1938; Eigsti and Dustin, 1955). This response appears to be brought about initially by the disappearance of cortical microtubules. Interphase plant cells exhibit an array of microtubules in their cortical cytoplasm in close proximity to the plasma membrane (Ledbetter and Porter, 1963; Hardham and Gunning, 1978; Gunning *et al.*, 1978). The cortical microtubules are oriented at right angles to the direction of growth in elongating cells, and they tend to be parallel with the most recently deposited cellulose microfibrils in the cell wall. It has been proposed that microtubules play a role in determining cellulose microfibril orientation (Hepler and Fosket, 1971; Hepler and Palevitz, 1974; Heath, 1974; Marchant, 1979). This hypothesis is based in part on the observation that colchicine, at concentrations that partially or completely eliminate cytoplasmic microtubules, also disrupts the pattern of cellulose microfibril deposition. Numerous studies have demonstrated that colchicine disrupts plant cortical microtubules (Pickett-Heaps, 1967; Nooden, 1971; Hepler and Fosket, 1971; Brown and Bouck, 1974; Robinson *et al.*, 1976; Hardham and Gunning, 1978, 1980). The concentrations of colchicine required for this effect typically are in the millimolar range, and some studies have reported that not all of the cortical microtubules disappeared, even after prolonged exposure to high levels of the drug (Nooden, 1971; Palevitz and Hepler, 1976; Hardham and Gunning, 1978; Mita and Shiboaka, 1983). The hypothesis that microtubules determine the pattern of wall deposition is weakened by the fact that the high concentrations of colchicine inhibit physiological processes such as respiration (Nooden, 1971; Sloan and Camper, 1981). The hypothesis is supported by studies using antimicrotubule herbicides at low

concentrations (Robinson and Herzog, 1977; Quader *et al.*, 1978). Furthermore, not all plant systems seem to require such high levels of colchicine for the destruction of the cortical microtubules. Durham and Jones (1982) reported that 1 μM colchicine was sufficient to inhibit hormone-induced lettuce hypocotyl cell elongation, to disrupt cortical microtubules and cause radial enlargement.

4.2. Colchicine Effects in Vitro

Several methods have been devised to examine the kinetics of colchicine binding to tubulin. Much of this work has utilized radiolabeled colchicine, where the free and bound colchicine can be separated by gel filtration or following adsorption of the tubulin–colchicine complex to DEAE–cellulose filter discs (Borisy and Taylor, 1967a,b; Borisy, 1972; Wilson and Bryan, 1974). Tubulin–colchicine complex formation may also be monitored by fluorescence since colchicine becomes fluorescent upon binding to tubulin (Bhattacharyya and Wolff, 1974). Analysis of colchicine binding to tubulin from many animal sources shows that the binding reaction is slow and favored at elevated temperatures, and that tubulin has a single class of high-affinity colchicine-binding sites. Affinity constants typically are $1–40 \times 10^6\ M^{-1}$ depending on the source of tubulin and the method of measurement (Wilson and Meza, 1973; Bhattacharyya and Wolff, 1974; Owellen *et al.*, 1972; Wilson, 1975; Sherline *et al.*, 1975; McClure and Paulson, 1977). There have been a few reports of more than one affinity class of binding sites on animal tubulins. Usually a single high-affinity class is observed at low colchicine concentrations, and another low-affinity class is seen at higher colchicine concentrations (Schmitt and Atlas, 1976; Williams *et al.*, 1983; Ringel and Sternlicht, 1984; Ray *et al.*, 1984).

The colchicine-binding site decays with first-order kinetics in the absence of colchicine. To solubilize sea urchin sperm axoneme outer doublet tubulin, Wilson and Meza (1973) heated the preparation to 50°C. They calculated that the colchicine-binding site decayed during solubilization with a half-life at 5.4 min. More typically, the rat brain tubulin colchicine-binding site decays with a half-life of 5.2 hr at 37°C (Sherline *et al.*, 1975). A number of solution components will stabilize the colchicine-binding site. These include sucrose, GTP, dithiothreitol, vinblastine, microtubule-associated proteins, organic acids, and even colchicine (Solomon *et al.*, 1973; Wilson *et al.*, 1974; Sherline *et al.*, 1975; McClure and Paulson, 1977; Hamel and Lin, 1981). When the binding data are corrected for decay, animal tubulins typically have molar binding stoichiometries at 0.5–0.8 (Wilson *et al.*, 1974; Sherline *et al.*, 1975). Colchicine binds to the dimeric form of tubulin, to vinblastine–tubulin paracrystals (Bryan, 1972b), and to 36 S oligomeric tubulin rings (Penningroth and Kirschner, 1977). It does not bind to tubulin in intact microtubules (Wilson and Meza, 1973).

The binding of a number of colchicine analogs to animal tubulin has been helpful in elucidating the nature of the tubulin–colchicine interaction. For

example, podophyllotoxin, which has a trimethoxybenzene ring like the A ring of colchicine (Fig. 9), also binds to tubulin, but in contrast to colchicine its binding is rapid and does not induce a conformational change in tubulin (Wilson, 1975; Cortese *et al.*, 1977; Andreu and Timasheff, 1982a,b). Colchicine also contains a tropolone ring (C ring), and the binding of tropolone and tropolone methyl ether to tubulin has been investigated. In contrast to colchicine, these compounds bind weakly, but do induce a conformational change in tubulin (Bhattacharyya and Wolff, 1974; Cortese *et al.*, 1977; Andreu and Timasheff, 1982a,b). Lumicolchicine, an ultraviolet (UV)-induced derivative of colchicine, has an altered C ring (Fig. 9) and binds to tubulin with low affinity (McClure and Paulson, 1977). The binding of both tropolone methyl ether and podophyllotoxin is inhibited competitively by colchicine, indicating that both these ligands bind to the colchicine-binding site. However, the podophyllotoxin binding is not inhibited by tropolone, nor is the tropolone binding inhibited by podophyllotoxin. Taken together these results suggest that the colchicine binding site consists of two adjacent subsites, one with an affinity for a trimethoxybenzene moeity (A ring) and a second with an affinity for tropolone (C ring).

The location of the colchicine-binding site on the tubulin dimer has been the subject of some controversy. Analysis of colchicine-resistant mutants in cultured Chinese hamster cells has demonstrated that mutations in either α-tubulin (Keates *et al.*, 1981) or β-tubulin (Cabral *et al.*, 1980) have resulted in a reduced affinity of tubulin for the drug. Schmitt and Atlas (1976) used an affinity label, bromocolchicine, to identify the subunit containing the binding site. They concluded that the high-affinity site was on the α subunit since this linkage was blocked by low concentrations of colchicine. Ludueña (1979) has criticized these conclusions, pointing out that bromocolchicine is an alkylating agent and that it would bind to readily accessible sulfhydryl groups on either α- or β-tubulin. Ringel and Steinlicht (1984) suggested that the colchicine site was at the α/β interface of the dimer—a hypothesis that is consistent with the data.

Previously, Ludueña *et al.* (1977) demonstrated that colchicine suppressed the alkylation of both α- and β-tubulin by iodoacetamide. In an expansion of their work on the alkylation of tubulin, Ludueña and Roach (1981a) observed that the bifunctional cross-linking agent *N,N'*-ethylene-

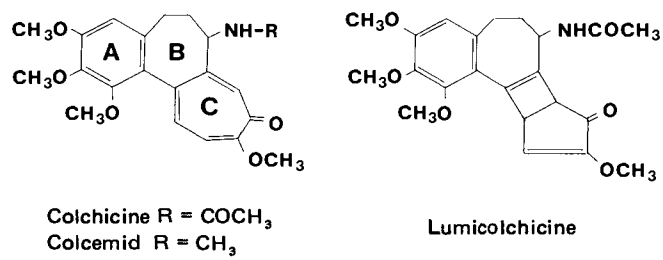


Figure 9. Structures of colchicine, colcemid, and lumicolchicine.

bis(iodoacetamide) (EBI) reacted with β-tubulin to convert it to a form they designated β*, which had an altered electrophoretic motility. Ludueña and Roach (1981b) further demonstrated that the alkylation of both α- and β-tubulin was blocked by colchicine and podophyllotoxin, but that the drugs were especially effective inhibitors of B* formation. Ludueña *et al.* (1982) demonstrated that there were two main forms of β-tubulin in chordate brain tissue, designated β_1 and β_2. EBI reacted with the cysteines only in β_1 to form β*. Recently, Little and Ludueña (1985) isolated and sequenced tryptic fragents of β_1, β_2, and β*. The β_1 and β_2 subunits exhibited at least 11 differences, which included the substitution of a serine for cysteine at position 239 in the β_2. They showed that EBI cross-linked the two cysteine residues at positions 239 and 354 in β_1 tubulin and that these residues are ~9 Å apart in the intact dimer. They proposed that colchicine binds to the β_1-containing dimer in the region between cysteines 239 and 354. They suggested that since yeast lacks the cysteine at position 239 (Neff *et al.*, 1983), this could explain its lowered affinity for colchicine (Kilmartin, 1981). However, the β-tubulin from *Chlamydomonas* has cysteines at both these positions (Youngblom *et al.*, 1984), but shows very low colchicine-binding activity (Flanagan and Warr, 1977). This suggests that the colchicine-binding sites on nonanimal tubulins interact differently with the ligand and that amino acids in addition to these cysteines may form the colchicine-binding site.

Colchicine blocks microtubule assembly in animals both *in vivo* and *in vitro*. The mechanism by which colchicine produces these effects is still under investigation. Although there is a stoichiometry of colchicine binding to tubulin dimers approaching one, colchicine concentrations well below that of tubulin dimers will block microtubule assembly *in vitro* (Olmsted and Borisy, 1973). Margolis and Wilson (1978, 1981) demonstrated that colchicine itself did not bind to the free ends of microtubules. Rather, colchicine first binds to the tubulin dimer and the tubulin–colchicine complexes then bind to the growing ends of the microtubules. They postulated that microtubules in equilibrium with a dimer pool *in vitro* will have structural polarity and that each microtubule has a net assembly end and a net disassembly end. The addition of the tubulin–colchicine complex to the net assembly end would effectively "cap" it, preventing further addition of dimers to it; net disassembly would continue so that the "capped" microtubule would disassemble. Sternlicht and Ringel (1979) reported that the tubulin–colchicine complex was able to copolymerize with tubulin, but that the affinity of unliganded tubulin for the tubulin–colchicine complex was reduced. The complex was postulated to bind to both ends of the microtubule and to reduce the probability of further assembly, while the disassembly reaction would continue unaffected. Bergen and Borisy (1983) have obtained data confirming Sternlicht and Ringel's hypothesis.

Regarding tubulins from plants, fungi, and protists, much less is known about their colchicine-binding properties. In some cases, it has been difficult to demonstrate colchicine binding in crude extracts of these organisms, and in fact Borisy and Taylor (1967a) reported that colchicine binding was undetect-

able in extracts of *Tetrahymena* and *Dictyostelium*. Burns (1973) also was unable to detect colchicine binding in extracts of yeast, *Chlamydomonas*, *Tetrahymena*, pea root tips, or corn coleoptiles. In contrast, Flanagan and Warr (1977) observed low levels of colchicine binding in the high-speed supernatants of *Chlamydomonas*. The significance of these observations was enhanced by their subsequent finding that the extent of this binding was reduced in mutants that exhibited a degree of resistance to colchicine (Flanagan and Warr, 1978). Cappuccinelli and Hames (1978) not only demonstrated colchicine binding in the high-speed supernatants of *Dictyostelium* myxamebae, but also showed that this activity had many of the characteristics of the animal tubulin–colchicine interaction. Specifically, binding was enhanced by vinblastine and organic acids, the complex bound to DEAE Sephadex, and it was insensitive to the UV-converted derivative lumicolchicine.

Davidse and Flach (1977) demonstrated that colchicine bound to tubulin from *Aspergillus*, but the apparent binding constant was very low (2.5×10^3 M^{-1} at 4°C). Colchicine inhibited the binding of the antimicrotubule fungicide methyl benzimidazol-2-yl carbamate to *Aspergillus* tubulin in a competitive manner. Baum *et al.* (1978) found low-level binding of either colcemid (Fig. 9) or lumicolcemid to proteins from *Saccharomyces,* and Kilmartin (1981) demonstrated that millimolar concentrations of colchicine were needed to inhibit *Saccharomyces* tubulin polymerization *in vitro*. Roobol *et al.* (1980a) observed very low binding ratios of colchicine and isolated *Physarum* tubulin, and they reported that microtubule polymerization was not inhibited by 1×10^{-4} M colchicine. Maekawa and Sakai (1978) showed very low-level binding of colchicine to isolated *Tetrahymena* tubulin, and Maekawa (1978) estimated the dissociation constant of the colchicine-tubulin interaction to be $K_d = 3.7 \times 10^{-3}$ M.

Hart and Sabnis (1973, 1976a,b, 1977) observed weak colchicine-binding activity in extracts of several higher plant species after concentrating the protein fraction by ammonium sulfate precipitation. Although this binding activity had some of the properties of colchicine binding to animal tubulin (it bound to DEAE–Sephadex, decayed with time, and was enhanced by vinblastine), it also exhibited some unique properties, most notably a greater affinity for lumicolchicine than colchicine (Sabnis, 1981). Colchicine-binding proteins (putative tubulins) have been reported in a number of higher plant species. These studies used a combination of ammonium sulfate precipitation and DEAE–Sephadex or –cellulose chromatography to isolate proteins from *Phaseolus* seedlings (Rubin and Cousins, 1976), cultured carrot cells (Okamura, 1980, 1983), and azuki bean seedlings (Mizuno *et al.*, 1981). Okamura (1980) used Scatchard analysis to show that carrot extracts had a single class of colchicine-binding sites with a binding constant of 3.5×10^4 M^{-1} at 30°C. He also showed that the binding was enhanced by vinblastine and unaffected by lumicolchicine, and that it was unstable and decayed with first-order kinetics. Inclusion of several components in the binding buffer that are known to stabilize colchicine binding in animal systems increased the estimated binding constant to 1.6×10^5 M^{-1} at 37°C (Okamura, 1983). Podophyllotoxin did not

inhibit colchicine binding to carrot extracts (Okamura *et al.*, 1984). Colchicine- and colcemid-binding was reported in supernatants of *Mimosa* (Mukherjee and Biswas, 1982). Colchicine binding was enhanced by sulfate and tartrate and was not readily reversible, but colcemid binding was reversible.

The colchicine-binding properties of isolated plant tubulins have been recently examined. Morejohn *et al.* (1984) isolated tubulins from three higher plant species and showed that the binding activities were not only different from each other, but also much lower than bovine brain tubulin colchicine-binding activity. Morejohn *et al.* (1986a) examined the colchicine-induced inhibition of taxol-induced initiation of rose microtubule polymerization. Colchicine inhibited assembly weakly ($K_i = 1.4 \times 10^{-4}$ M) when compared with bovine brain tubulin ($K_i = 8.8 \times 10^{-7}$ M) at 24°C. The binding constant for the colchicine–rose tubulin interaction was estimated to be $K = 9.7 \times 10^2$ M^{-1}, indicating that the resistance of polymerization to colchicine results from a low-affinity binding of colchicine to tubulin. The maximum binding stoichiometry was not dissimilar from that of brain tubulin and was estimated to be 0.47 mole colchicine bound per mole tubulin. Neither podophyllotoxin nor tropolone inhibited the binding.

There are several possible reasons for the low effectiveness of colchicine in disrupting the microtubules of plants, fungi, and protists. Colchicine may be metabolized or compartmentalized by the cells of these organisms, or it may not be taken up readily. These cells also may contain inhibitors of colchicine binding, possibly in the form of small molecules that interact with tubulin at the colchicine-binding site. Alternatively, the tubulins of these organisms may lack a high-affinity colchicine-binding site. Circumstantial evidence exists to show that any of these mechanisms of resistance is possible. Certainly the colchicine-resistant mutants of cultured vertebrate cells in some cases exhibit reduced colchicine uptake (Minor and Roscoe, 1975), while in other cases the mutation has altered the affinity of tubulin for the drug (Ling *et al.* 1979; Cabral *et al.*, 1980). At the present time it would be premature to generalize about the basis for colchicine resistance in plants, fungi, and protists. However, where it has been examined, colchicine binding affinities for nonanimal tubulins are markedly reduced.

4.3. Antimicrotubule Herbicides

The literature demonstrating that certain herbicides act as antimicrotubule agents has been reviewed (Parka and Soper, 1977; Fedtke, 1982) and will only be summarized here. There are three main classes of herbicides that have been shown to have antimicrotubule activities. They are the dinitroaniline herbicides such as trifluralin and oryzalin, the phenyl carbamate herbicides such as isopropyl-*N*-phenyl carbamate (IPC), chlorisopropyl-*N*-phenyl carbamate (CIPC), and ethyl-*N*-phenylcarbamate (EPC), and the phosphoric amide herbicides such as amiprophos-methyl (APM). Structures of some of these are shown in Fig. 10. The dinitroaniline and phosphoric amide herbicides produce colchicinelike effects in various higher plants and algae;

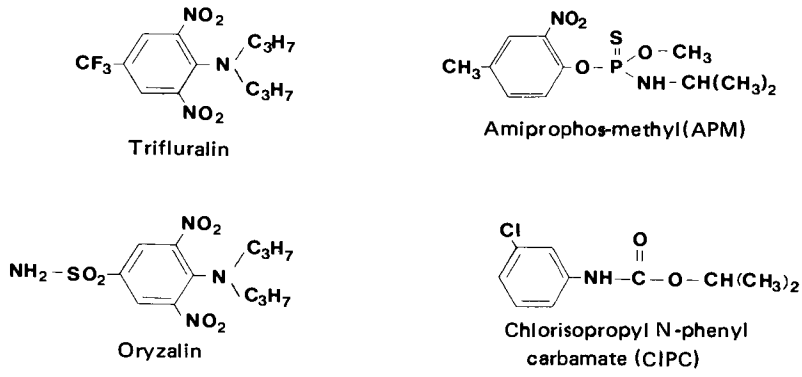


Figure 10. Structures of various antimicrotubule herbicides.

that is, they block mitosis at metaphase and depolymerize spindle microtubules and cortical cytoplasmic microtubules with the subsequent disruption of the orientation of the newly deposited wall cellulose microfibrils. They also block the regeneration of flagella in deflagellated algal cells (refer to Fedtke, 1982). The extreme sensitivity of some plant microtubule systems to these dinitroaniline and phosphoric amide herbicides is dramatically illustrated by the recent work of Bajer and Molè-Bajer (1985), who found that the rate of chromosome movement in *Hemanthus* endosperm cells was slowed by 50% with 10 nM oryzalin or APM and that there was nearly complete destruction of the spindle microtubules in this system after a few minutes' exposure to 100 nM herbicide. The carbamate herbicides produce somewhat different effects in higher plants. Although the carbamate herbicides cause mitotic arrest, the microtubules are not completely destroyed, but rather, they become disoriented. For instance, multipolar mitoses were observed in IPC-treated *Hemanthus* endosperm (Hepler and Jackson, 1969), and Coss *et al.* (1975) suggested that the carbamate herbicides disrupt microtubule-organizing centers. Confirmation of these effects has been reported in the moss *Physcomitrella* (Doonan *et al.*, 1985) and onion (Clayton and Lloyd, 1984).

Although Hess and Bayer (1977) demonstrated that trifluralin bound to *Chlamydomonas* flagellar tubulin some time ago, it was not completely accepted that these antimicrotubule herbicides had tubulin and/or microtubules as their primary targets. There are several reasons for this. First, these herbicides at high micromolar concentrations exert a diverse array of effects on higher plants, including inhibition of RNA and protein synthesis (refer to Parka and Soper, 1977). Second, unlike colchicine, the antimitotic and antimicrotubule effects of these herbicides are generally confined to nonanimal organisms. Antimitotic herbicides have been shown to disrupt or prevent microtubule formation not only in higher plants (Hess and Bayer, 1974), but also in algae (Brown and Bouck, 1974; Robinson and Herzog, 1977; Kiermayer and Fedtke, 1977; Hess, 1979; Quader and Filner, 1980). Margulis *et al.* (1975) reported that trifluralin was highly effective in blocking cilary re-

generation in *Stentor*, and CIPC has been found to be a potent antimicrotubule agent in *Dictyostelium* (White *et al.*, 1981). There are very few reports of antimitotic effects of herbicides in animal systems. Magistrini and Szollosi (1980) found that IPC prevented the reformation of the second meiotic spindle after it was disrupted by a cold treatment in mouse oocytes, although they noted that the herbicide had no effect on previously formed microtubules. Trifluralin did not affect the growth of cultured normal or transformed sheep lung cells (Hess and Bayer, 1977). Morejohn and Fosket (1984a) and Bajer and Molè-Bajer (1985) found no effect of APM or oryzalin on mitosis in cultured PTK_2 or *Xenopus* cells. Both IPC and trifluralin have been tested for their ability to block brain tubulin assembly *in vitro*. IPC was completely without effect (Coss *et al.*, 1975), while trifluralin was reported to have no effect on brain tubulin assembly *in vitro* in one study (Bartels and Hilton, 1973) and to produce a partial inhibition of assembly at a relatively high concentration in another study (Robinson and Herzog, 1977). Third, Hess and Bayer (1977) could not detect trifluralin binding to extracts of cotton roots, although the microtubules in the cotton root cells were known to be readily depolymerized by trifluralin (Hess and Bayer, 1977). Similarly Upadhyaya and Nooden (1980) could not detect oryzalin binding in a corn root tip extract.

Finally, the antimitotic herbicides were shown to affect calcium uptake from isolated plant mitochondria (Hertel *et al.*, 1980). A half-maximal effect was observed at APM and oryzalin concentrations of 2×10^{-5} M, while trifluralin was slightly more effective. They proposed that the herbicides increased passive efflux of calcium from mitochondria. Colchicine, on the other hand, did not affect mitochondrial calcium uptake. Thus, the depolymerization of plant microtubules in herbicide-treated plant cells was proposed to be an indirect effect of elevated cytoplasmic calcium levels. At the present time it is difficult to evaluate this hypothesis since the effect of the herbicides on cytoplasmic calcium compartmentalization in intact cells has not been examined. It is an attractive hypothesis to explain the antimicrotubule action of the herbicides only in the absence of data showing that the herbicides bind to tubulin.

Recent work, however, has made it clear that these herbicides do bind to higher plant and algal tubulins and inhibit assembly of microtubules *in vitro*. Strachan and Hess (1983) examined the binding of oryzalin to *Chlamydomonas* flagellar tubulin by means of a DEAE–cellulose assay. Scatchard analysis of the binding data demonstrated a single class of binding sites ($K = 2.08 \times 10^5$ M^{-1} at 25°C) and an estimated maximum stoichiometry near one. Morejohn *et al.* (1986b) obtained similar data for the binding of oryzalin to rose tubulin. Analysis of the binding data again indicated a single class of binding sites with an affinity constant $K = 1.19 \times 10^5$ M^{-1}. However, they obtained a binding stoichiometry of 0.15, suggesting that not all of the tubulin dimers bound oryzalin. The latter result may indicate that the higher plant tubulin possesses binding-site heterogeneity not found in the *Chlamydomonas* tubulin, or that binding activity was lost during tubulin isolation. Morejohn *et al.* (1986b) also

demonstrated that oryzalin inhibited the rapid phase of taxol-induced rose tubulin assembly *in vitro,* with a K_i = 2.6 μM. APM also inhibited the initiation of taxol-induced rose tubulin assembly *in vitro* (Morejohn and Fosket, 1984a), and we have calculated a K_i = 1.57 μM. Neither herbicide affected taxol-induced bovine brain tubulin assembly as shown by turbidity measurements and sedimentation analysis. Both the brain and rose tubulins were isolated by anion exchange methods, and the assemblies were carried out under comparable conditions.

The yield of taxol-induced rose tubulin and brain tubulin polymers in the presence or absence of herbicides or colchicine is summarized in Table 1. The data show that APM and oryzalin are more effective inhibitors of rose tubulin polymerization than either CIPC or colchicine, and that only colchicine inhibits bovine brain tubulin polymerization. It is interesting to note that CIPC and colchicine have similar efficacies in rose tubulin polymerization inhibition assays.

Data on the nature of the interaction of the carbamate herbicides with plant tubulins are fragmentary at the present time. The fact that higher plant tubulin can be enriched from extracts with EPC–affinity chromatography (Mizuno *et al.*, 1981, 1985; Mizuno, 1985) demonstrates that this carbamate herbicide binds to plant tubulin. Our preliminary work with CIPC and microtubule polymerization (Table 1) shows that it inhibits rose, but not brain,

Table 1. Inhibition of Taxol-Induced Microtubule Polymerization by Antimicrotubule Herbicides and Colchicine[a]

Compound	Concentration (μM)	Rose tubulin polymer yield (% of control)	Brain tubulin polymer yield (% of control)
Amiprophos-methyl[b]	2.5	77	—
	5.0	30	—
	10.0	6	—
	100.0	—	100
Oryzalin[c]	1.0	108	—
	5.0	51	—
	7.0	6	98
Chlorisopropyl *N*-phenyl carbamate	200.0	73	103
Colchicine[d]	10.0	—	10
	100.0	93	—
	500.0	63	—
	1000.0	53	—

[a]Tubulin (10 μM) was added to a solution containing taxol (40 μM for rose; 27 μM for brain) and a given concentration of each tested compound at 24°C. After 1 hr of polymerization, samples were sedimented at 48,000 g for 1 hr, and polymer pellets were assayed for protein. Data are expressed as % of control samples that contained no inhibiting drug.
[b]Data taken from Morejohn and Fosket, 1984a.
[c]Data taken from Morejohn *et al.*, 1986b.
[d]Data taken from Morejohn and Fosket, 1984b.

tubulin assembly *in vitro*. Since carbamate herbicides have quite distinct effects on microtubules *in vivo*, as opposed to the dinitroaniline and phosphoric amide herbicides, carbamate herbicide binding to tubulin in cells may affect its interaction with MAPs, or microtubule-organizing centers, as well as dimer–dimer interactions.

It is fairly easy to reconcile the apparent divergent views on the mode of action of the dinitroaniline and phosphoric amide herbicides. The inability of Upadhyaya and Nooden (1980) and Hess and Bayer (1977) to detect dinitroaniline herbicide binding to tubulin in higher plant extracts is not surprising considering the low specific activity of the radiolabeled herbicides and the likelihood that the tubulin in these extracts was degraded in the absence of effective inhibitors of proteolysis (see Section 2.7). Although the dinitroaniline herbicides may have secondary effects related to their lipophyllic properties (they may partition into membranes), the only explanation for their antimicrotubule properties *in vitro* is their binding to tubulin. This is the most likely explanation for their antimitotic and antimicrotubule effects *in vivo* as well. Certainly, Bajer and Molè-Bajer (1985) have observed effects on *Hemanthus* mitosis at concentrations of APM and oryzalin that are 100-fold lower than the threshold required to release calcium from isolated plant mitochondria. The work of Hess and Bayer (1977), Strachan and Hess (1983), Morejohn and Fosket (1984a), and Morejohn *et al.* (1986b) has revealed a previously unexpected major difference in the pharmacological properties of plant and animal tubulins. Furthermore, the herbicide-binding sites may represent functionally important parts of the tubulin molecule *in vivo*.

4.4. Benzimidazole Derivatives

A number of different benzimidazole derivatives are selective fungicides or anthelmentics. Structures of two of these compounds are shown in Fig. 11. The fungicides include benomyl, thiabendazole, and the benomyl metabolite methyl benzimidazol-2-yl carbamate (MBC). Benzimidazole derivatives acting as selective anthelmintics include fenbendazole, mebendazole, and parbendazole. Still other benzimidazole derivatives, such as nocodazole, have antitumor properties. Most of these compounds have been shown to have antimicrotubule properties not only in target organisms, but also in host organisms. Benomyl and MBC act as antimitotic agents in fungi, slime molds, higher plants, and mammalian cells (Hammerschlag and Sisler, 1973; Styles and Garner, 1974; Seiler, 1975; Davidse, 1975; Richmond and Phillips, 1975; DeBrabander *et al.*, 1975; Howard and Aist, 1977, 1980; Quinlan *et al.*, 1980; Welker and Williams, 1980; Umesono *et al.*, 1983; Kitanishi *et al.*, 1984). The anthelmintic benzimidazole derivatives destroy both cytoplasmic and mitotic spindle microtubules in mammalian cells and in parasitic roundworms (Borgers *et al.*, 1975; Friedman and Platzer, 1978; Ireland *et al.*, 1979; Havercroft *et al.*, 1981). The antitumor benzimidazole derivatives are highly effective antimitotic agents in mammalian cells (DeBrabander *et al.*, 1975).

While the benzimidazole derivatives can act as antimicrotubule agents in

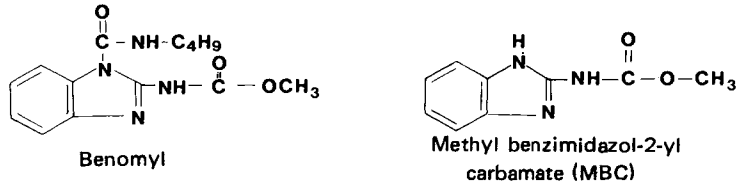


Figure 11. Structures of benomyl and methyl benzimidazol-2-yl carbamate (MBC).

a wide spectrum of organisms, their effectivenss as fungicides and anthelmintics is dependent upon a degree of selectivity in blocking the growth of fungi and/or roundworms. There is some evidence for a differential sensitivity of comparable microtubule arrays in different organisms. Borgers *et al.* (1975) reported that mebendazole completely destroyed the microtubules of the roundworms *Ascaris, Syngamus,* and *Taenia* without affecting the microtubules of their mammalian hosts when the anthelmintic was given orally. Similarly, the fungicide benomyl may be used to treat fungal infestation of higher plants, with little adverse effects on the plant (for example, Shields *et al.,* 1984). Robinson and Herzog (1977) noted that MBC did not disrupt the cytoplasmic microtubules of the alga *Oocystis,* although it did alter the organization of the wall cellulose microfibrils. They also reported that benzimidazole and nocodazole were without effect on either the orientation of the wall cellulose microfibrils or the cytoplasmic microtubules.

Several independent investigations have shown that various benzimidazole derivatives do interact with tubulin. Hoebeke *et al.* (1976) demonstrated that nocodazole blocked rat brain tubulin assembly, although it did not depolymerize microtubules after they had been formed *in vitro.* Friedman and Platzer (1978) examined 11 benzimidazole derivatives for their effects on bovine brain tubulin assembly. The various compounds differed considerably in their ability to block the assembly reaction. The mean inhibitory concentrations of nocodazole, oxibendazole, parbendazole, mebendazole, and fenbendazole were in the μM range, whereas those of benomyl, cambendazole, and carbendazin were approximately 10-fold higher. They noted that the order of effectiveness of these compounds in killing the nematode *Caenorhabditis* was similar to the order of activity of the compounds in blocking bovine brain assembly. Ireland *et al.* (1979) also reported that benzimidazole derivaties inhibited mammalian brain tubulin assembly *in vitro.* However, they noted that some of them were effective only in relatively high concentrations. Parbendazole, nocodazole, fenbendazole, and oxibendazole inhibited assembly by 50% when present at concentrations in the micromolar range. In contrast, millimolar levels of thiabendazole and benomyl were required to produce the same effect.

Friedman and Platzer (1980) examined the inhibition of colchicine binding to *Ascaris* tubulin or bovine brain tubulin by mebendazole and fenbendazole. Noncompetitive binding to *Ascaris* tubulin was found for mebendazole

($K_i = 1.9 \times 10^{-8}$ M) and fenbendazole ($K_i = 6.5 \times 10^{-8}$ M), while competitive binding to bovine brain tubulin was seen with mebendazole ($K_i = 7.3 \times 10^{-6}$ M) and fenbendazole ($K_i = 1.7 \times 10^{-5}$ M). They reasoned that the 250- to 400-fold differences in inhibition constants may explain the selective toxicity of these compounds toward nematodes. Kohler and Bachmann (1981) reported that mebendazole competitively inhibited the binding of colchicine to *Ascaris* tubulin ($K_i = 4.22 \times 10^{-6}$ M) and to porcine brain tubulin ($K_i = 8 \times 10^{-6}$ M). This result indicated that the two antimicrotubule drugs bind to the same site with slightly different affinities. Dawson *et al.* (1984) found rather analogous effects of various benzimidazoles on the *in vitro* polymerization of tubulin from the nematode *Ascardia* and sheep brain. These compounds inhibited assembly in both systems, but the effects were more pronounced in *Ascardia*.

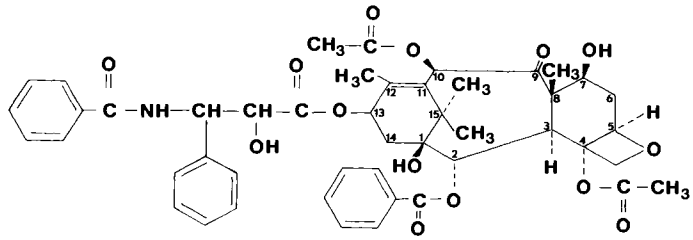
In yeasts benzimidazoles arrest cell division (Quinlan *et al.*, 1980), and Kilmartin (1981) has shown that MBC and nocodazole are effective inhibitors of yeast microtubule assembly *in vitro* at micromolar concentrations. Davidse and Flach (1977) showed that MBC binds to tubulin from *Aspergillus* with a $K = 1.6 \times 10^6$ M^{-1} and that the binding was competitively inhibited by colchicine and nocodazole. The binding of nocodazole to brain tubulin has been carefully investigated (Lee *et al.*, 1980), and many, but not all of the effects are similar to those of colchicine. Low concentrations of various benzimidazole compounds have been shown to depolymerize microtubules and arrest mitosis in the slime molds *Dictyostelium, Polyphondylium,* and *Physarum* (Welker and Williams, 1980; Williams, 1980; Kitanishi *et al.*, 1984). Quinlan *et al.* (1981) found that micromolar concentrations of parbendazole and nocodazole inhibited the growth of *Physarum* myxamoebae and purified myxamoebal microtubule protein polymerization *in vitro*.

The aggregate of the information from studies on benzimidazole derivatives suggests that these compounds bind with different affinities to tubulins from diverse species. The differential affinities are paralleled by the concentrations of drugs required to inhibit microtubule polymerization *in vivo* and *in vitro*. There is also a good indication from the competitive binding studies that both colchicine and benzimidazoles bind to or near the same geographical site on tubulins from widely divergent species. However, the affinity of these antifungal benzimidazole compounds for plant tubulin has not been tested.

4.5. Taxol

Of all the compounds that have been shown to have effects on microtubules, taxol is perhaps the most unique. Taxol is a taxane alkaloid produced by plants of the genus *Taxus,* and it inhibits microtubule-dependent processes by stabilizing microtubules and promoting microtubule polymerization. The structure of taxol is given in Fig. 12.

The effects of taxol on organisms spanning diverse phylogenic lines have been reported in some detail. Taxol inhibits animal cell replication at low



Taxol

Figure 12. Structure of taxol.

concentrations and has some potential as an antitumor drug (Wani *et al.*, 1971). Taxol inhibits the division of cultured mouse fibroblasts and HeLa cells at low micromolar concentrations by arresting cells in G_2 or M of the cell cycle (Schiff and Horwitz, 1980). Nuclear migration in eggs of *Xenopus* and sea urchin is arrested, and microtubule asters are formed (Heidemann and Gallas, 1980; Schatten *et al.*, 1982). Taxol promotes microtubule formation and stabilization in cells treated with depolymerizing drugs or low temperature (Schiff and Horwitz, 1980). Taxol stabilizes microtubules in isolated sand dollar mitotic spindles to depolymerization by calcium, and the stabilization can be reversed by washing the spindles in taxol-free medium (Salmon and Wolniak, 1983).

Mitosis is slowed, but not arrested, in *Hemanthus* endosperm cells treated with taxol (Bajer *et al.*, 1982). The *Hemanthus* spindle becomes reorganized, new microtubules appear in the spindle and cytoplasm, and microtubules become cold-resistant after taxol treatment (Bajer *et al.*, 1982; Molè-Bajer and Bajer, 1983). Microtubules were polymerized and cell division occurred after treatment of microtubule-free, division-arrested hibiscus protoplasts with 1–10 μM taxol (Hahne and Hoffmann, 1984). Herth (1983) observed in the chrysoflagellate alga *Poterioochromonas* that low concentrations (>1μM) of taxol stabilized microtubules, and at slightly higher levels (2.4–4.8 μM) of taxol new microtubules were formed. At 6 μM taxol stationary phase cells became abnormally round as a consequence of the formation of a hoop of marginal microtubules and flagella were stumplike (Herth, 1983). Hausmann *et al.* (1983) found taxol to stabilize microtubules and increase the number of microtubules in the heliozoan *Actinophrys*. Also, the normal geometric pattern of microtubules in the axopodal axonemes was disrupted (Hausmann *et al.*, 1983). Taxol blocked replication, presumably at cytokinesis, in the protozoan *Trypanosoma* (Baum *et al.*, 1981). Taxol blocks mitosis in myxameboe of *Physarum*, but no effects of taxol up to 200 μM were observed on the plasmodial stage (Wright *et al.*, 1982; Wright and Oustrin, 1983). However, Lataste *et al.* (1984) claimed 50% inhibition of plasmoidal growth at 10 μM taxol.

In vitro taxol promotes brain tubulin polymerization, lowers the critical concentration of tubulin required for microtubule assembly, and permits as-

sembly at low temperature, or in the absence of GTP and MAPs (Schiff *et al.*, 1979; Kumar, 1981; Schiff and Horwitz, 1981; Thompson *et al.*, 1981; Parness *et al.*, 1983). Maximum levels of brain tubulin polymerization were obtained at nearly equal concentrations of taxol and tubulin (Kumar, 1981; Schiff and Horwitz, 1981). Brain tubulin polymerization is more resistant to the effects of calcium, colchicine, or other drugs when induced by taxol, although inhibition of assembly can be obtained at nearly 1-to-1 M ratios of tubulin and colchicine or podophyllotoxin (Kumar, 1981; Caplow and Zeeberg, 1982). Taxol-stabilized microtubules are relatively resistant to depolymerization by cold, calcium, podophyllotoxin, or vinblastine (Schiff *et al.*, 1979; Kumar, 1981; Parness and Horwitz, 1981; Schiff and Horwitz, 1981).

Taxol-binding studies have shown that the drug binds directly to microtubules *in vivo* and *in vitro* (Parness and Horwitz, 1981; Manfredi *et al.*, 1982). Quantitation of the binding reaction indicates a specific and high-affinity interaction with microtubules. Parness and Horwitz (1981) reported a $K_d = 8.7 \times 10^{-7}$ M and a maximum binding stoichiometry of 0.6 mole taxol/mole tubulin in purified microtubules at 37°C. A binding stoichiometry approaching a value of 1 has been taken to mean that each dimer in microtubules possesses one taxol site. Manfredi *et al.* 1982) estimated $K_d = 2 \times 10^{-7}$ M for taxol binding to microtubules in cultured cells (line J774.2). While taxol binds to sea urchin flagellar outer doublet microtubules and microtubules reassembled from outer double tubulin, their maximum stoichiometries for binding were quite different (Parness *et al.*, 1983). Outer doublet microtubules and singlet microtubules maximally bind 0.25 and 1.32 mole taxol/mole tubulin, respectively. This finding suggests that taxol sites are more or less accessible in different polymeric structures and that the possible number of binding sites on the dimer may exceed one on diverse tubulins. Apparently taxol does not displace MAPs from microtubules where this possibility has been examined (Kumar, 1981; Vallee, 1982; Caplow and Zeeberg, 1982).

Taxol binding to microtubules both *in vivo* and *in vitro* is inhibited by drugs that depolymerize microtubules, and there is evidence that they do not complete for binding to the same sites, but rather compete for binding to different forms of tubulin, the dimer or the polymer (Kumar, 1981; Parness and Horwitz, 1981; Manfredi *et al.* 1982). However, Carlier and Pantaloni (1983) found that the intrinsic GTPase activity of the soluble tubulin–colchicine complex was enhanced by taxol in a concentration-dependent fashion. They demonstrated that taxol binds to this form of dimeric tubulin with a $K_d = 1.3 \times 10^{-7}$ M at 37°C and agreed that taxol and colchicine do not bind to the same site on the soluble dimer.

Possible mechanisms for the taxol-induced modulation of microtubule dynamics *in vitro* have been pursued in only a couple of studies. Kumar (1981) claimed that the rate of dimer treadmilling through taxol microtubules was five-fold lower than MAP-containing microtubules at steady state. However, Caplow and Zeeberg (1982) found that the rates of treadmilling in taxol microtubules versus MAP-containing microtubules were the same and proposed that a reduction in the dimer dissociation rate constants at both microtubule ends would account for decreases in both molecular rate constants at

steady state. Apparently there is some question as to the time when taxol microtubules have reached steady state (dynamic equilibrium with the soluble dimers).

Structure/activity studies on taxol and/or its derivatives have been examined with animal, slime mold, and plant systems. Parness *et al.* (1982) studied the cytotoxicity to cultured animal cells and microtubule assembly promotion of 10 taxol derivatives. They found that a large hydrocarbon substituent esterified to C-13 of the taxane ring was necessary for cytotoxicity and *in vitro* polymerization activity, but that acetates esterified to C-7 and C-10 abolished the assembly promotion activity.

The effects of various taxol derivatives on cold-induced disassembly of *Physarum* and porcine brain microtubules were compared *in vitro* (Lataste *et al.* 1984), and quite different activities were seen. They confirmed that the substituent at C-13 was important in animal microtubule stability, but that it was not important in *Physarum* microtubule stability. Among 16 different derivatives examined all were effective at <10 μM in the *Physarum* assays and only five were active at <10μM in the porcine assays. Although these subtle differences were obvious, the tubulins from mammals and slime molds must contain rather similar binding sites for the taxane ring.

The requirements for taxol in the polymerization of tubulins from cultured plant cells and bovine brain were compared (Morejohn and Fosket, 1984b). The concentrations of taxol producing maximum turbidity levels and polymer yields were only slightly different for each system. Polymerization saturations of rose and brain microtubules were observed at ratios of taxol to tubulin of 2:1 and 1:1, respectively. Subsequent studies with maize microtubules indicated that 1:1 ratios of taxol and maize tubulin produce maximal polymer yield according to sedimentation analysis (Morejohn, unpublished results). These results indicate further that taxol sites have been conserved over evolution, although some heterogeneity may exist at or near these sites in different tubulins.

4.6. Other Agents

A number of other compounds act as antimicrotubule agents, but in most cases too little is known of their specificities in cells of plants, fungi, and protists to warrant an in-depth review here. Such compounds include griseofulvin, podophyllotoxin, vinblastine, melatonin, caffeine, and various herbicides. For information on the effects of these and other compounds, the reader is referred to the reviews of Parka and Soper (1977), Ludueña (1979), and Dustin (1984). For background on the antagonistic or synergistic effects of antimicrotubule drugs and plant hormones on microtubules in plant cells, see Gunning and Hardham (1982).

5. Summary and Future Directions

In this chapter we have catalogued numerous aspects of the similarities and differences between tubulins from diverse species. The current body of

information on the structures of tubulins from animals, plants, fungi, and protists demonstrates that their biochemical properties are subtly different. Certainly, a comparison of the sequences of diverse tubulins has shown that these proteins are highly conserved, and yet minor changes in sequences have conferred striking variations in their immunological and pharmacological properties. It is reasonable to speculate that future studies on diverse tubulins and microtubules will uncover many more differences between them.

In many areas of tubulin research the future directions are rather obvious. Chief among these is the continued characterization of the drug-binding properties of tubulins. Such studies have the potential to yield important insights on the structure of the tubulin dimer and how it has evolved, as well as on the nature of the dimer–polymer interaction phenomenon. This area is particularly intriguing inasmuch as drug-binding sites could represent important regulatory domains that operate microtubule dynamics *in vivo*. The most fruitful approach to the localization of drug-binding sites will combine methods of ligand binding, chemical cross-linking, sequencing, and possibly site-directed mutagenesis.

In vitro microtubule assembly studies will continue to be important. It is clear that nonanimal tubulins will polymerize *in vitro* under conditions that promote animal tubulin assembly. However, where it has been examined carefully, different animal tubulins have been shown to have slightly different requirements for optimum polymerization/depolymerization. Likewise, it will be important to determine the optimum conditions of pH, temperature, and ionic strength for nonanimal microtubule systems *in vitro*. To date, there have been virtually no reports of the isolation of MAPs from nonanimals. The profound effects of MAPs on microtubule assembly and disassembly properties *in vitro* make these proteins prime candidates for the regulation of microtubule dynamics in cells. Because MAPs from diverse species are not highly conserved, this area of research could be expected to reveal very interesting and unique properties of nonanimal microtubule systems.

Fundamental to our understanding of how microtubules function in cells will be the continued characterization of the modes of expression of tubulin genes at the transcriptional, translational, and posttranslational levels. Because tubulin isotypes can arise from different genes that are differentially expressed in development, the possibility of function-specific tubulin dimers in microtubules is very real. Correlations between the composition and biochemistry of tubulin dimers from different tissues or cell types in a given organism and the modes of expression of the corresponding tubulin genes may provide information on the multitubulin hypothesis. That is, do particular genes encode structurally unique tubulins with specific functions?

Many other lines of research must be pursued. They are too numerous to be adequately addressed here, but the following questions may summarize some of these directions. Is membrane-associated tubulin very different from microtubule tubulin? What does membrane-associated tubulin do? Are some isotypes of tubulin preferentially modified by posttranslational means? What purposes do various types of posttranslational modification serve? Do plant

hormones bind to microtubule proteins directly, or are their effects on microtubules indirect (see Gunning and Hardham, 1982)? Why does the number of tubulin genes vary between different organisms? We anticipate that these and numerous other aspects of microtubule research will continue to be studied in diverse systems for some time to come.

ACKNOWLEDGMENTS. We thank the numerous investigators who provided us with preprints and reprints of their work. We thank the following persons for providing figure materials for this review: Dr. John Kilmartin (Fig. 2), Dr. Tim McKeithan (Fig. 8), Dr. Melvyn Little (Figs. 3 and 4), and Dr. Judwiga Molè-Bajer (Fig. 5). For help with the organization of the manuscript and her valuable comments we thank Dr. Brooke S. Kirby. The expert assistance of Ms. Gail Hall in the preparation of the typescript is gratefully acknowledged. The preparation of this review was supported by grants to D.E.F. from the National Science Foundation (PCM 80–16035) and from the Monsanto Co. L.C.M. was supported by a National Science Foundation Plant Biology Postdoctoral fellowship.

References

Adoutte, A., Claisse, M., and Cance, J., 1984, Tubulin evolution: An electrophoretic and immunological analysis, *Origins Life* **13:**177.

Alexandraki, D., and Ruderman, J. V., 1981, Sequence heterogeneity, multiplicity, and genomic organization of α- and β-tubulin genes in sea urchin *Mol. Cell. Biol.* **1:**1125.

Amos, L., 1979, Structure of microtubules, in: *Microtubules* (K. Roberts and J. S. Hyams, eds.), pp. 1–64, Academic Press, London.

Andreu, J. M., and Timasheff, S. N., 1982a, Interaction of tubulin with single ring analogues of colchicine, *Biochemistry* **21:**534.

Andreu, J. M., and Timasheff, S. N., 1982b, Conformational states of tubulin liganded to colchicine, tropolone methyl ether, and podophyllotoxin, *Biochemistry* **21:**6465.

Argaraña, C. E., Barra, H. S., and Caputto, R., 1978, Release of [^{14}C] tyrosine from tubulinyl-[^{14}C]tyrosine by brain extract. Separation of a carboxypeptidase from tubulin-tyrosine ligase, *Mol. Cell. Biochem.* **19:**17.

Asnes, C. F., and Wilson, L., 1979, Isolation of bovine brain microtubule protein without glycerol: Polymerization kinetics change during purification cycles, *Analyt. Biochem.* **98:**64.

Bajer, A., and Molè-Bajer, J., 1985, Drugs with colchicine-like effects that specifically disassemble plant but not animal microtubules, *Ann. NY Acad. Sci.* (in press).

Bajer, A. S., Cypher, C., Molè-Bajer, J., and Howard, H. M., 1982, Taxol-induced anaphase reversal: Evidence that elongating microtubules can exert a pushing force in living cells, *Proc. Natl. Acad. Sci. USA* **79:**6569.

Bamburg, J. R., Shooter, E. M., and Wilson, L., 1973, Assay of microtubule protein in embryonic chick dorsal root ganglia, *Neurobiology* **3:**162.

Barra, H. S., Arce, C. A., Rodriguez, J. A., and Caputto, R., 1974, Some common properties of the protein that incorporates tyrosine as a single unit into microtubule proteins, *Biochem. Biophys. Res. Commun.* **60:**1384.

Bartels, P. G., and Hilton, J. L., 1973, Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules, *Pest. Biochem. Physiol.* **3:**462.

Baum, P., Thorner, J., and Honig, L., 1978, Identification of tubulin from the yeast *Saccharomyces cerevisiae, Proc. Natl. Acad. Sci. USA* **75:**4962.

Baum, S. G., Wittner, G., Nadler, J. P., Horwitz, S. B., Dennis, J. E., Schiff, P. B., and Tanowitz, H. B., 1981, Taxol, a microtubule stabilizing agent, blocks the replication of *Trypanosoma cruzi*, *Proc. Natl. Acad. Sci. USA* **78:**4571.

Behnke, O., and Forer, A., 1967, Evidence for four classes of microtubules in individual cells, *J. Cell Sci.* **2:**169.

Bensch, K. G., Marantz, R., Wisnewski, H., and Shelanski, M., 1969, Induction *in vitro* of microtubular crystals by vinca alkaloids, *Science* **165:**495.

Bergen, L. G., and Borisy, G. G., 1983, Tubulin-colchicine complex inhibits microtubule elongation at both plus and minus ends, *J. Biol. Chem.* **258:**4190.

Bernier-Valentin, F., Aunis, D., and Rousset, B., 1983, Evidence for tubulin-binding sites on cellular membranes: Plasma membranes, mitochondrial membranes, and secretory granule membranes, *J. Cell Biol.* **97:**209.

Best, D., Warr, P. J., and Gull, K., 1981, Influence of the composition of commercial sodium dodecyl sulfate preparations on the separation of α-and β-tubulin during polyacrylamide gel electrophoresis, *Analyt. Biochem.* **114:**281.

Bhattacharyya, B., and Wolff, J., 1974, Promotion of fluorescence upon binding of colchicine to tubulin, *Proc. Natl. Acad. Sci. USA* **71:**2627.

Bhattacharyya, B., and Wolff, J., 1975, Membrane-bound tubulin in brain and thyroid tissue, *J. Biol. Chem.* **250:**7639.

Bibring, T., Baxandall, J., Denslow, S., and Walker, B., 1976, Heterogeneity of the alpha subunit of tubulin and the variability of tubulin within a single organism, *J. Cell Biol.* **69:**301.

Binder, L. I., and Rosenbaum, J. L., 1978, The *in vitro* assembly of flagellar outer doublet tubulin, *J. Cell Biol.* **79:**500.

Birkett, C. R., Foster, K. E., Johnson, L., and Gull, K., 1985, Use of monoclonal antibodies to analyse the expression of a multi-tubulin family, *FEBS Lett.* **187:**211.

Blitz, A. L., and Fine, R. E., 1974, Muscle-like contractile proteins and tubulin in synaptosomes, *Proc. Natl. Acad. Sci. USA* **71:**4472.

Bordier, C., Garavito, R. M., and Armbruster, B., 1982a, Biochemical and structural analysis of microtubules in the pellicular membrane of *Leishmania tropica*, *J. Protozool.* **29:**560.

Bordier, C., Garavito, R. M., and Armbruster, B. L., 1982b, Membrane microtubules of *Leishmania tropica*, in: *Microtubules in Microorganisms* (P. Cappuccinelli and N. R. Morris, eds.), pp. 377–389, Marcel Dekker, New York.

Borgers, M., DeNollin, S., Verheyen, A., DeBrabander, M., and Thienpont, D., 1975, Effects of new anthelmintics on the microtubular system of parasites, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 497–508, North Holland, Amsterdam.

Borisy, G. G., 1972, A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters, *Analyt. Biochem.* **50:**373.

Borisy, G. G., and Taylor, E. W., 1967a, The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein, *J. Cell Biol.* **34:**525.

Borisy, G. G., and Taylor, E. W., 1967b, The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus, *J. Cell Biol.* **34:**535.

Borisy, G. G., and Olmsted, J. B., 1972, Nucleated assembly of microtubules in porcine brain extracts, *Science* **177:**1196.

Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., and Johnson, K. A., 1975, Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly *in vitro*, *Ann. NY Acad. Sci.* **253:**107.

Brinkley, B. R., and Cartwright, J., 1975, Cold-labile and cold-stable microtubules in the mitotic spindle of mammalian cells, *Ann. NY Acad. Sci.* **253:**428.

Brinkley, B. R., Stubblefield, E., and Hsu, T. C., 1967, The effects of Colcemid inhibition and reversal on the fine structure of the mitotic apparatus of Chinese hamster cells *in vitro*, *J. Ultrastruct. Res.* **19:**1.

Brown, D. L., and Bouck, G. B., 1974, Microtubule biogenesis and cell shape in *Ochromonas* III. Effects of the herbicidal mitotic inhibitor isopropyl N-phenylcarbamate on shape and flagellum regeneration, *J. Cell Biol.* **61:**514.

Brown, D. L., Massalski, A., and Patenaude, R., 1976, Organization of the flagellar apparatus and

associated cytoplasmic microtubules in the quadriflagellate alga *Polytomella agilis*, *J. Cell Biol.* **69:**106.

Brunke, K. J., Collis, P. S., and Weeks, D. P., 1982, Posttranslational modification dependent upon organelle assembly, *Nature (London)* **297:**316.

Bryan, J., 1971, Vinblastine and microtubules I. Induction and isolation of crystals from sea urchin oocytes, *Exp. Cell Res.* **66:**129.

Bryan, J., 1972a, Vinblastine and microtubules II. Characterization of two protein subunits from the isolated crystals, *J. Mol. Biol.* **66:**157.

Bryan, J., 1972b, Definitions of three classes of binding sites in isolated microtubule crystals, *Biochemistry* **11:**2611.

Bryan, J., 1974, Biochemical properties of microtubules, *Fed. Proc.* **33:**152.

Bryan, J., and Wilson, L., 1971, Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. USA* **68:**1762.

Bryan, J., Nagle, B. W., and Doenges, K. H., 1975a, Inhibition of tubulin assembly by RNA and other polyanions: Evidence for a required protein, *Proc. Natl. Acad. Sci. USA* **72:**3570.

Bryan, J., Nagle, B. W., and Meza, I., 1975b, Inhibition of tubulin assembly by RNA and other polyanions. A mechanism, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 91–101, North Holland, Amsterdam.

Burgess, J., and Northcote, D. H., 1969, Action of colchicine and heavy water on the polymerization of microtubules in wheat root meristem, *J. Cell Sci.* **5:**433.

Burns, R. G., 1973, ^{3}H-Colchicine binding. Failure to detect any binding to soluble proteins from various lower organisms, *Exp. Cell Res.* **81:**285.

Burland, T. G., and Gull, K., 1984, Molecular and cellular aspects of the interaction of benzimidazole fungicides with tubulin and microtubules, in: *Mode of Action of Antifungal Agents* (A. P. J. Trinci and J. F. Ryley, eds.), pp. 299–320, British Mycological Society, London.

Burland, T. G., Gull, K., Schedl, T., Boston, R. S., and Dove, W. F., 1983, Cell type-dependent expression of tubulins in *Physarum*, *J. Cell Biol.* **97:**1852.

Burton, P. R., 1981, Polymorphic assemblies of tubulin, in: *Cell and Muscle Motility*, Vol. 1 (R. M. Dowben and J. M. Shay, eds.), pp. 289–333, Plenum Press, New York.

Cabral, F., Sobel, M. E., and Gottesman, M. M., 1980, CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered β-tubulin, *Cell* **20:**29.

Callahan, R. C., Shalke, G., and Gorovsky, M. A., 1984, Developmental rearrangements associated with a single type of expressed α-tubulin gene in *Tetrahymena*, *Cell* **36:**441.

Caplow, M., and Zeeberg, B., 1982, Dynamic properties of microtubules at steady state in the presence of taxol, *Eur. J. Biochem.* **127:**319.

Cappuccinelli, P., and Ashworth, J. M., 1976, The effect of inhibitors of microtubule and microfilament function on the cellular slime mold *Dictyostelium discoideum*, *Exp. Cell Res.* **103:**387.

Cappuccinelli, P., and Hames, B. D., 1978, Characterization of colchicine-binding activity in *Dictyostelium discoideum*, *Biochem. J.* **169:**499.

Cappuccinelli, P., Martinotti, G., and Hames, B. D., 1978, Identification of cytoplasmic tubulin in *Dictyostelium discoideum*, *FEBS Lett.* **91:**153.

Carlier, M. F., and Pantaloni, D., 1983, Taxol effect on tubulin polymerization and associated guanosine 5′-triphosphate hydrolysis, *Biochemistry* **22:**4814.

Caron, J. M., and Berlin, R. D., 1979, Interaction of microtubule protein with phospholipid vesicles, *J. Biol. Chem.* **81:**665.

Clapham, D. H., and Östergren, G., 1984, Immunocytochemistry of tubulin at meiosis in *Tradescantia* by a protein-A gold method, *Hereditas* **101:**137.

Clayton, L., and Gull, K., 1982, Tubulin and the microtubule organizing centres of *Physarum polycephalum* myxamoebae, in: *Microtubules in Microorganisms* (P. Cappuccinelli and N. R. Morris, eds.), pp. 179–199, Marcel Dekker, New York.

Clayton, L., and Lloyd, C. V., 1984, The relationship between the division plane and spindle geometry in *Allium* cells treated with CIPC and griseofulvin: an antitubulin study, *Eur. J. Cell Biol.* **34:**248.

Clayton, L., Pogson, C. I., and Gull, K., 1979, Microtubule proteins in the yeast *Saccharomyces cerevisiae*, *FEBS Lett.* **106:**67.

Clayton, L., Quinlan, R. A., Roobol, A., Pogson, C. I., and Gull, K., 1980, A comparison of tubulins from mammalian brain and *Physarum polycephalum* using SDS-polyacrylamide gel electrophoresis and peptide mapping, *FEBS Lett.* **115**:301.

Clayton, L., Pogson, C. I., and Gull, K., 1983, Ultrastructural and biochemical characterization of the cytoskeleton of *Physarum polycephalum* myxamoebae, *Protoplasma* **118**:181.

Cleveland, D. W., Hwo, S-Y., and Kirschner, M. W., 1977a, Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin, *J. Mol. Biol.* **116**:207–225.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K., 1977b, Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis, *J. Biol. Chem.* **252**:1102.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirschner, M. W., 1980, Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β-and γ-actin genes using specific cloned cDNA probes, *Cell* **20**:85.

Correia, J. J., and Williams, Jr., R. C., 1983, Mechanisms of assembly and disassembly of microtubules, *Ann. Rev. Biophys. Bioeng.* **12**:211.

Cortese, F., Bhattacharyya, B., and Wolff, J., 1977, Podophyllotoxin as a probe for the colchicine binding site of tubulin, *J. Biol. Chem.* **252**:1134.

Coss, R. A., Bloodgood, R. A., Brower, D. L., Pickett-Heaps, J. D., and McIntosh, R., 1975, Studies on the mechanism of action of isopropyl *N*-phenylcarbamate, *Exp. Cell Res.* **92**:394.

Cowan, N. J., and Dudley, L., 1983, Tubulin isotypes and the multigene tubulin families, *Int. Rev. Cytol.* **85**:147.

Crossley, R., and Holberton, D. V., 1983, Characterization of proteins from the cytoskeleton of *Giardia lamblia*, *J. Cell Sci.* **59**:81–103.

Cumming, R., and Williamson, J., 1984, Differential labelling of trypanosome microtubules using tubulin subunit monoclonal antibodies, *Cell Biol. Int. Rep.* **8**:2.

Cumming, R., Burgoyne, R. O., and Lytton, N. A., 1984, Immunocytochemical demonstration of α-tubulin modification during axonal maturation in the cerebellar cortex, *J. Cell Biol.* **98**:347.

David-Pfeuty, T., Erickson, H. P., and Pantaloni, D., 1977, Guanosinetriphosphatase activity of tubulin associated with microtubule assembly, *Proc. Natl. Acad. Sci. USA* **74**:5372.

Davidse, L. C., 1975, Antimitotic activity of methyl benzimidazol-2-yl carbamate in fungi and its binding to cellular protein, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 483–495, North Holland, Amsterdam.

Davidse, L. C., and Flach, W., 1977, Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*, *J. Cell Biol.* **72**:174.

Dawson, P. J., Gutteridge, W. E., and Gull, K., 1984, A comparison of the interactions of anthelmintic benzimidazoles with tubulin isolated from mammalian tissue and the parasitic nematode *Ascardia galli*, *Biochem. Pharmacol.* **33**:1069.

Deanin, G. G., Thompson, W. C., and Gordon, M. W., 1977, Tyrosyltubulin ligase activity in brain, skeletal muscle, and liver of developing chick, *Dev. Biol.* **57**:230.

DeBrabander, M., Van De Veire, R., Aerts, F., Guens, G., Borgers, M., Desplenter, L., and deCree, J., 1975, Oncodazole (R17934): A new anti-cancer drug interfering with microtubules. Effects on neoplastic cells cultured *in vitro* and *in vivo*, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 509–521, North Holland, Amsterdam.

DeMey, J., Lambert, A. M., Bajer, A. S., Moeremans, M., and DeBrabander, M., 1982, Visualization of microtubules in interphase and mitotic plant cells of *Haemanthus* endosperm with the immuno-gold staining method, *Proc. Natl. Acad. Sci. USA* **79**:1898.

Dentler, W. L., 1980, Microtubule-membrane interactions in cilia. I. Isolation and characterization of ciliary membranes from *Tetrahymena pyriformis*, *J. Cell. Biol.* **84**:364.

Dentler, W. L., Granett, S., and Rosenbaum, J. L., 1975, Ultrastructural localization of the high molecular weight proteins associated with *in vitro*-assembled brain microtubules, *J. Cell Biol.* **65**:237.

Detrich, H. W., III, and Williams, R. C. Jr., 1978, Reversible dissociation of the dimer of tubulin from bovine brain, *Biochemistry* **17**:3900.

Deysson, G., 1968, Antimitotic substances, *Int. Rev. Cytol.* **24**:99–148.
Deysson, G., 1975, Microtubules and antimitotic substances, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 427–451, North Holland, Amsterdam.
Doohan, M. E., and Palevitz, B. A., 1980, Microtubules and coated vesicles in guard-cell protoplasts of *Allium cepa* L., *Planta* **149**:389.
Doonan, J. H., Cove, D. J., and Lloyd, C. W., 1985, Immunofluorescence microscopy of microtubules in intact cell lineages of the moss, *Physcomitrella patens*. I. Normal and CIPC-treated tip cells, *J. Cell Sci.* **75**:131.
Durham, D. J., and Jones, R. L., 1982, The effect of colchicine and gibberellic acid on growth and microtubules in excised lettuce hypocotyls, *Planta* **154**:204.
Dustin, P., 1984, *Microtubules*, Springer-Verlag, Berlin.
Dwyer, D. M., 1980, Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes, *J. Protozool.* **27**:176.
Dwyer, D. M., and D'Alesandro, P. A., 1980, Isolation and characterization of pellicular membranes from *Trypanosoma lewisi* bloodstream forms, *J. Protozool.* **66**:377.
Eigsti, O. J., and Dustin, Jr., P., 1955, *Colchicine in Agriculture, Medicine, Biology and Chemistry*, Iowa State University Press., Ames.
Eipper, B. A., 1972, Rat brain microtubule protein: Purification and determination of covalently bound phosphate and carbohydrate, *Proc. Natl. Acad. Sci. USA* **68**:2028.
Eipper, B. A., 1974, Properties of rat brain tubulin, *J. Biol. Chem.* **249**:1407.
Estridge, M., 1977, Polypeptides similar to the α-and β-subunits of tubulin are exposed on the neuronal surface, *Nature (London)* **268**:60.
Everhart, Jr., L. P., 1971, Heterogeneity of microtubule proteins from *Tetrahymena* cilia, *J. Mol. Biol.* **61**:745.
Farrell, K. W., and Burns, R. G., 1975, Inability to detect *Chlamydomonas* microtubule assembly *in vitro:* Possible implications to the *in vivo* regulation of microtubule assembly, *J. Cell Sci.* **17**:669.
Farrell, K. W., and Wilson, L., 1978, Microtubule reassembly *in vitro* of *Strongylocentrotus purpuratus* sperm tail outer doublet tubulin, *J. Mol. Biol.* **121**:393.
Fedtke, C., 1982, *Biochemistry and Physiology of Herbicide Action*, Springer-Verlag, Berlin.
Feit, H., and Barondes, S. A., 1970, Colchicine-binding activity in particulate fractions of mouse brain, *J. Neurochem.* **17**:1355.
Feit, H., and Shay, J. W., 1980, The assembly of tubulin into membranes, *Biochem. Biophys. Res. Commun.* **94**:324–331.
Feit, H., Slusarek, L., and Shelanski, M. L., 1971, Heterogeneity of tubulin subunits, *Proc. Natl., Acad. Sci. USA* **68**:2028.
Field, D. J., Collins, R. A., and Lee, J. C., 1984, Heterogeneity of vertebrate brain tubulins, *Proc. Natl. Acad. Sci. USA* **81**:4041.
Field, D. J., and Lee, J. C., 1985, Isoelectric focusing and two-dimensional electrophoresis of tubulin using immobilized pH gradients under denaturing conditions, *Analyt. Biochem.* **144**:584.
Fine, R. E., 1971, Heterogeneity of tubulin, *Nature New Biol.* **233**:283.
Flanagan, D. J., and Warr, J. R., 1977, Colchicine binding of a high-speed supernatant of *Chlamydomonas reinhardtii, FEBS Lett.* **80**:14.
Flanagan, D. J., and Warr, J. R., 1978, Colchicine binding of cell extracts from colchicine-resistant mutants of *Chlamydomonas reinhardtii, Exp. Cell Res.* **116**:474.
Fong, D., and Chang, K.-P., 1981, Tubulin biosynthesis in the developmental cycle of a parasitic protozoan, *Leishmania mexicana:* Changes during differentiation of the motile and nonmotile stages, *Proc. Natl. Acad. Sci. USA* **78**:7624.
Fosket, D. E., Morejohn, L. C., and Westerling, K. E., 1981, Control of growth by cytokinin: An examination of tubulin synthesis during cytokinin-induced growth in cultured cells of Paul's scarlet rose, in: *Metabolism and Molecular Activities of Cytokinins* (J. Guern and C. Péaud-Lenoël, eds.), pp. 193–211, Springer-Verlag, Berlin.
Franke, W. W., Seib, E., Osborn, M., Weber, K., Herth, W., and Falk, H., 1977, Tubulin-containing structures in the anastral mitotic apparatus of endosperm cells of the plant *Leucojum aestivum* as revealed by immunofluorescence microscopy, *Cytobiologie* **15**:24.

Friedman, P. A., and Platzer, E. G., 1978, Interaction of anthelmintic benzimidazoles derivatives with bovine brain tubulin, *Biochim. Biophys. Acta* **544**:605.

Friedman, P. A., and Platzer, E. G., 1980, Interaction of anthelmintic benzimidazoles with *Ascaris suum* embryonic tubulin, *Biochim. Biophys. Acta* **630**:271.

Frigon, R. P., and Timasheff, S. N., 1975, Magnesium-induced self-association of calf brain tubulin I. Stoichiometry, *Biochemistry* **14**:4559.

Fulton, C., and Kowit, J., 1975, Programmed synthesis of flagellar tubulin during cell differentiation in *Naegleria*, *Ann. NY Acad. Sci.* **253**:318.

Fulton, C., and Simpson, P. A., 1976, Selective synthesis and utilization of flagellar tubulin. The multi-tubulin hypothesis, in: *Cold Spring Harbor Conference on Cell Proliferation*, Book C (R. Golden, T. Pollard, and J. Rosenbaum, eds.), pp. 987–1005, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Fulton, C., Kane, R. E., and Stephens, R. E., 1971, Serological similarity of flagellar and mitotic microtubules, *J. Cell Biol.* **50**:762.

Gallo, J-M., and Anderton, B. H., 1983, A subpopulation of trypanosome microtubules recognized by a monoclonal antibody to tubulin, *EMBO J.* **2**:479.

Gambino, J., Bergen, L. G., and Morris, N. R., 1984, Effects of mitotic and tubulin mutations on microtubule architecture in actively growing protoplasts of *Aspergillus nidulans*, *J. Cell Biol.* **99**:830.

Gaskin, F., Cantor, C. R., and Shelanski, M. L., 1974, Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules, *J. Mol. Biol.* **89**:737.

Gelfant, S., 1963, Inhibition of cell division: A critical and experimental analysis, *Int. Rev. Cytol.* **14**:1.

George, H. J., Misra, L., Field, D. J., and Lee, J. C., 1981, Polymorphism of brain tubulin, *Biochemistry* **20**:2402.

Gibbons, I. R., 1965, Chemical dissection of cilia, *Arch. Biol.* **76**:317.

Goldenring, J. R., Gonzalez, B., McGuire, Jr., S., and DeLorenzo, R. J., 1983, Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associated proteins, *J. Biol. Chem.* **258**:12632.

Gozes, I., and Littauer, U. Z., 1979, The α-subunit of tubulin is preferentially associated with brain presynaptic membrane, *FEBS Lett.* **99**:86.

Green, L. L., and Dove, W. F., 1984, Tubulin proteins and RNA during the myxamoeba-flagellate transformation of *Physarum polycephalum*, *Mol. Cell. Biol.* **4**:1706.

Gundersen, G. G., Kalnoski, M. A., and Bulinski, J. C., 1984, Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*, *Cell* **38**:779.

Gunning, B. E. S., and Hardham, A. R., 1982, Microtubules, *Ann. Rev. Plant Physiol.* **33**:651.

Gunning, B. E. S., Harding, A. R., and Hughes, J. E., 1978, Evidence for initiation of microtubules in discrete regions of the cell cortex in *Azolla* root tip cells, and an hypothesis on the development of cortical arrays of microtubules, *Planta* **143**:161.

Haber, J. E., Peloquin, J. E., Halvorson, H. O., and Borisy, G. G., 1972, Colcemid inhibition of cell growth and the characterization of a colcemid-binding activity in *Saccharomyces cerevisiae*, *J. Cell Biol.* **55**:355.

Hahne, G., and Hoffmann, F., 1984, Dimethyl sulfoxide can initiate cell divisions of arrested callus protoplasts by promoting cortical microtubule assembly, *Proc. Natl. Acad. Sci. USA* **81**:5449.

Hall, J. L., and Cowan, N. J., 1985, Structural features and restricted expression of a human α-tubulin gene, *Nucl. Acids Res.* **13**:207.

Hall, J. L., Dudley, L., Dobner, P. R., Lewis, S. A., and Cowan, N. J., 1983, Identification of two human β-tubulin isotypes, *Mol. Cell. Biol.* **3**:854.

Hallak, M. E., Rodriquez, J. A., Barra, H. S., and Caputto, R., 1977, Release of tyrosine from tyrosinated tubulin. Some factors that affect this process and the assembly of tubulin, *FEBS Lett.* **73**:147.

Hamel, E., and Lin, C. M. 1981, Stabilization of the colchicine-binding activity of tubulin by organic acids, *Biochim. Biophys. Acta* **675**:226.

Hammerschlag, R. S., and Sisler, H. D., 1973, Benomyl and methyl-2-benzimidazole-carbamate (MBC): Biochemical, cytological and chemical aspects of toxicity to *Ustilago maydis* and *Saccharomyces cerevisiae*, *Pest. Biochem. Physiol.* **3**:42.

Hardham, A. R., and Gunning, B. E. S., 1978, Structure of cortical microtubule arrays in plant cells, *J. Cell Biol.* **77**:14.

Hardham, A. R., and Gunning, B. E. S., 1980, Some effects of colchicine on microtubules and cell division in roots of *Azolla pinnata*, *Protoplasma* **102**:31.

Hart, J. W., and Sabnis, D. D., 1973, Colchicine-binding protein from phloem and xylem of a higher plant, *Planta* **109**:147.

Hart, J. W., and Sabnis, D. D., 1976a, Binding of colchicine and lumicolchicine to components in plant extracts, *Phytochemistry* **15**:147.

Hart, J. W., and Sabnis, D. D., 1976b, Colchicine binding activity in extracts of higher plants, *J. Exp. Bot.* **27**:1353.

Hart, J. W., and Sabnis, D. D., 1976c, Colchicine and plant microbubules: A critical evaluation, *Curr. Adv. Plant Sci.* **26**:1095.

Hart, J. W., and Sabnis, D. D., 1977, Microtubules, in: *The Molecular Biology of Plant Cells* (H. Smith, ed.), pp. 160–181, University of California Press, Berkeley.

Hausmann, K., Linnenbach, M., and Patterson, D. J., 1983, The effects of taxol on microtubular arrays: *In vitro* effects on heliozoan axonemes, *J. Ulstruct. Res.* **82**:212.

Havercroft, J. C., Quinlan, R. A., and Gull, K., 1981, Binding of parbendazole to tubulin and its influence on microtubules in tissue culture cells as revealed by immunofluorescence microscopy, *J. Cell Sci.* **49**:195.

Heath, I. B., 1974, A unified hypothesis for the role of membrane bound enzyme complexes and microtubules in plant cell wall synthesis, *J. Theor. Biol.* **48**:445.

Heath, I. B., 1975a, Colchicine and colcemid binding components of the fungus *Saprolegnia ferax*, *Protoplasma* **85**:177.

Heath, I. B., 1975b, The effect of antimicrotubule agents on the growth and ultrastructure of the fungus *Saprolegnia ferax* and their ineffectiveness in desruption hyphal microtubules, *Protoplasma* **85**:147.

Heidemann, S. R., and Gallas, P. T., 1980, The effect of taxol on living eggs of *Xenopus laevis*, *Dev. Biol.* **80**:489.

Helftenbein, E., 1985, Nucleotide sequence of a macronuclear DNA molecule coding for α-tubulin from the ciliate *Stylonychia lemnae*. Special codon usage: TAA is not a translation termination codon, *Nucl. Acids Res* **13**:415.

Hepler, P. K., and Fosket, D. E., 1971, The role of microtubules in vessel member differentiation in *Coleus*, *Protoplasma* **72**:213.

Hepler, P. K., and Jackson, W. T., 1969, Isopropyl *N*-phenylcarbamate affects spindle microtubule orientation in dividing endosperm cells of *Haemanthus katherinae* Baker, *J. Cell Sci.* **5**:727.

Hepler, P. K., and Palevitz, B. A., 1974, Microtubules and microfilaments, *Ann. Rev. Plant Physiol.* **25**:309.

Hertel, C., Quader, H., Robinson, D. G., and Marme, D., 1980, Anti-microtubular herbicides and fungicides affect Ca^{++} transport in plant mitochondria, *Planta* **149**:336.

Herth, W., 1983, Taxol effects cytoskeletal microtubules, flagella and spindle structure of the Chrysoflagellate alga *Poterioochromonas*, *Protoplasma* **115**:228.

Hess, F. D., 1979, The influence of the herbicide trifluralin on flagellar regeneration in *Chlamydomonas*, *Exp. Cell Res.* **119**:99.

Hess, F. D., and Bayer, D. E., 1974, The effect of trifluralin on the ultrastructure of dividing cells of the root meristem of cotton (*Gossypium hirsutum* L. "Acala 4–42"), *J. Cell Sci.* **15**:429.

Hess, F. D., and Bayer, D. E., 1977, Binding of the herbicide trifluralin to *Chlamydomonas* flagellar tubulin, *J. Cell Sci.* **24**:351.

Hill, T. L., and Kirschner, M. W., 1983, Regulation of microtubule and actin filament assembly–disassembly by associated small and large molecules, *Int. Rev. Cytol.* **84**:185.

Himes, R. H., Burton, P. R., Kersey, R. H., and Pierson, G. B., 1976, Brain tubulin polymerization in the absence of "microtubule-associated proteins," *Proc. Natl. Acad. Sci. USA* **73**:4397.

Hinman, N. D., Morgan, J. L., Seeds, N. W., and Cann, J. L., 1973, Isolation of brain tubulin by affinity chromatography, *Biochem. Biophys. Res. Commun.* **52:**752.

Hoebeke, J., Van Nijen, G., and DeBrabander, M., 1976, Interaction of oncodazole (R 17935), a new antitumoral drug, with rat brain tubulin, *Biochem. Biophys. Res. Commun.* **69:**319.

Hogetsu, T., and Shibaoka, H., 1978, Effects of colchicine on cell shape and on microfibril arrangement in the cell wall of *Closterium acerosum*, *Planta* **140:**15.

Holberton, D. V., and Ward, A. P., 1981, Isolation of the cyoskeleton from *Giardia*. Tubulin and the low-molecular-weight protein associated with microribbon structures, *J. Cell Sci.* **47:**139.

Hotta, Y., and Shepard, J., 1973, Biochemical aspects of colchicine action on meiotic cells, *Mol. Gen. Genet.* **122:**243.

Howard, R. J., and Aist, J. R., 1977, Effects on MBC on hyphal tip organization, growth and mitosis of *Fusarium accuminatum*, and their antoganism by D_2O, *Protoplasma* **92:**195.

Howard, R. J., and Aist, J. R., 1980, Cytoplasmic microtubules and fungal morphogenesis: Ultrastructural effects of methyl benzimidazole-2-yl carbamate determined by freeze-substitution of hyphal tip cells, *J. Cell Biol.* **8**755.

Hussey, P. J., and Gull, K., 1985, Multiple isotypes of α- and β-tubulin in the plant *Phaseolus vulgaris*, *FEBS Lett.* **181:**113.

Ireland, C. M., Gull, K., Gutteridge, W. E., and Pogson, C. I., 1979, The interaction of benzimidazole carbamates with mammalian microtubule proteins, *Biochem. Pharmacol.* **28:**2680.

Kalfayan, L., and Wensink, P. C., 1982, Developmental regulation of *Drosophila* α-tubulin genes, *Cell* **29:**91.

Kantharaj, G. R., Mahadevan, S., and Padmanabhan, G., 1985, Tubulin synthesis and auxin-induced root initiation in *Phaseolus*, *Phytochemistry* **24:**23.

Keates, R. A. B., 1981, Stabilization of microtubule protein in glycerol solutions, *Can. J. Biochem.* **59:**353.

Keates, R. A. B., Sarangi, F., and Ling, V., 1981, Structural and functional alterations on microtubule proteins from Chinese hamster ovary cell mutants, *Proc. Natl. Acad. Sci. USA* **78:**5638.

Kelly, P. T., and Cottman, C. W., 1978, Characterization of tubulin and actin and identification of a distinct postsynaptic density polypeptide, *J. Cell Biol.* **79:**173.

Kelly, W. G., Passaniti, A., Woods, J. W., Daiss, J. L., and Roth, T. F., 1983, Tubulin as a molecular component of coated vesicles, *J. Cell Biol.* **97:**1191.

Kemphues, K. J., Kaufman, T. C., Raff, R. A., and Raff, E. C., 1982, The testes-specific β-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis, *Cell* **31:**655.

Kiermayer, O., and Fedtke, C., 1977, Strong anti-microtubule action of amiprophos-methyl (APM) in *Micrasterias*, *Protoplasma* **92:**163.

Kihlman, B. A., 1966, *Actions of Chemicals on Dividing Cells*, Prentice Hall, Englewood-Cliffs, NJ.

Kilmartin, J. V., 1981, Purification of yeast tubulin by self-assembly *in vitro*, *Biochemistry* **20:**3629.

Kilmartin, J. V., and Adams, A. E. M., 1984, Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*, *J. Cell Biol.* **98:**922.

Kilmartin, J. V., Wright, B., and Milstein, C., 1982, Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line, *J. Cell Biol.* **93:**576.

Kirschner, M. W., 1978, Microtubule assembly and nucleation, *Int. Rev. Cytol.* **54:**1.

Kitanishi, T., Shibaoka, H., and Fukui, H., 1984, Disruption of microtubules and retardation of development of *Dictyostelium* with ethyl *N*-phenylcarbamate and thiabendazole, *Protoplasma* **120:**185.

Klausner, R. D., Kumar, D., Weinstein, J. N., Blumenthal, R., and Flavin, M., 1981, Interaction of tubulin with phospholipid vesicles I. Association with vesicles at the phase transition. *J. Biol. Chem.* **256:**5879.

Kleinfeld, R. G., and Sisken, J. E., 1966, Morphological and kinetic aspects of mitotic arrest by and recovery from colcemid, *J. Cell Biol.* **31:**369.

Kobayashi, T., and Flavin, M., 1981, Tubulin tyrosylation in invertebrates, *Comp. Biochem. Physiol.* **69B:**387.

Koehn, L. A., and Olsen, R. W., 1978, Radioimmune assay of tubulin applied to oligomers, synaptic membranes, and plants, *Biochem. Biophys. Res. Commun.* **80:**391.

Kohler, P., and Bachmann, R., 1981, Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes, *Mol. Biochem. Parasitol.* **4**:325.

Kornguth, S. E., and Sunderland, E., 1975, Isolation and partial characterization of a tubulin-like protein from human and swine synaptosomal membranes, *Biochim. Biophys. Acta.* **393**:100.

Kowit, J. D., and Fulton, C., 1974, Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmune assay for tubulin, *J. Biol. Chem.* **249**:3638.

Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, W., and Ponstingl, H., 1981, Complete amino acid sequence of β-tubulin from porcine brain, *Proc. Natl. Acad. Sci. USA* **78**:4156.

Kumar, N., 1981, Taxol-induced polymerization of purified tubulin. Mechanism of action, *J. Biol. Chem.* **256**:10435.

Kumar, N., and Flavin, M., 1981, Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin, *J. Biol. Chem.* **256**:7678.

Kumar, N., and Flavin, M., 1982, Modulation of some parameters of assembly of microtubules *in vitro* by tyrosinolation of tubulin, *Eur. J. Biochem.* **128**:215.

Kumar, N., Klausner, R. D., Weinstein J. N., Blumenthal, R., and Flavin, M., 1981, Interaction of tubulin with phospholipid vesicles II. Physical changes of the protein, *J. Biol. Chem.* **256**:5886.

Kuriyama, R., 1976, *In vitro* polymerization of flagellar and ciliary outer fiber tubulin into microtubules, *J. Biochem.* **80**:153.

Kuzmich, M. J., and Zimmerman, A. M., 1972, Colcemid action on the division schedule of synchronized *Tetrahymena*, *Exp. Cell Res.* **72**:441.

Laemmli, U. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)* **227**:680.

Lagnado, J. R., Lyons, C., and Wickremasinghe, G., 1971, The subcellular distribution of colchicine binding protein ("microtubule protein") in rat brain, *FEBS Lett.* **15**:254.

Lai, E. Y., Walsh, C., Wardell, D., and Fulton, C., 1979, Programmed appearance of translatable flagellar tubulin mRNA during cell differentiation in *Naegleria*, *Cell* **17**:867.

Landfear, S. M., McMahon-Pratt, D., and Wirth, D. F., 1983, Tandem arrangement of tubulin genes in the protozoan parasite *Leishmania enriettii*, *Mol. Cell Biol.* **3**:1070.

Langford, G. M., 1981, Comparative study of shark and beef brain microtubules, *Biosystems* **14**:247.

Lataste, H., Senilh, V., Wright, M., Guénard, D., and Potier, P., 1984, Relationships between the structures of taxol and baccatine III derivatives and their *in vitro* action on the disassembly of mammalian brain and *Physarum* amoebal microtubules, *Proc. Natl. Acad. Sci. USA* **81**:4090.

Launay, J. F., Vanier, M. T., and Gendry, P., 1983, Purification of tubulin from rat pancreas, *Biochem. Biophys. Res. Commun.* **111**:253.

Ledbetter, M. C., and Porter, K. R., 1963, A "microtubule" in plant cell fine structure, *J. Cell Biol.* **19**:239.

Lederberg, S., and Stetten, G., 1970, Colcemid sensitivity of fission yeast and the isolation of colcemid-resistant mutants, *Science* **168**:485.

Lee, J. C., and Timasheff, S. N., 1975, The reconstitution of microtubules from purified brain tubulin, *Biochemistry* **14**:5183.

Lee, J. C., and Timasheff, S. N., 1977, *In vitro* reconstitution of calf brain microtubules: Effects of solution variables. *Biochemistry* **16**:1754.

Lee, J. C., Field, D. J., and Lee, L. L. Y., 1980, Effects of nocodazole on structures of calf brain tubulin, *Biochemistry* **19**:6209.

Lee, M. G-S., Lewis, S. A., Wilde, C. D., and Cowan, N. J., 1983, Evolutionary history of a multigene family: An expressed human β-tubulin gene and three processed pseudogenes, *Cell* **33**:477.

Lefebvre, P. A., Silflow, C. D., Weiben, E. D., and Rosenbaum, J. L., 1980, Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella, *Cell* **20**:469.

Lemischka, I. R., and Sharp, P. A., 1982, The sequences of an expressed rat α-tubulin gene and a pseudogene with an inserted repetitive element, *Nature (London)* **300**:330.

Lemischka, I. R., Farmer, C., Racaniello, V. R., and Sharp, P. A., 1981, Nucleotide sequence and evolution of a mammalian α-tubulin messenger RNA, *J. Mol. Biol.* **151**:101.

Levan, A., 1938, The effect of colchicine on root mitoses in *Allium*, *Hereditas* **24**:471.

Levan, A., 1954, Cochicine-induced c-mitosis in two mouse ascites tumors, *Hereditas* **40**:1.

Levan, A., and Steinegger, E., 1947, The resistance of *Colchicum* and *Bulbocodium* to the c-mitotic action of colchicine, *Hereditas* **33**:552.

L'Hernault, S. W., and Rosenbaum, J. L., 1983, *Chlamydomonas* α-tubulin is posttranslationally modified in the flagella during flagellar assembly, *J. Cell Biol.* **97**:258.

L'Hernault, S. W., and Rosenbaum, J. L., 1985a, Reversal of the posttranslational modification on *Chlamydomonas* flagellar α-tubulin occurs during flagellar resorption, *J. Cell Biol.* **100**:457.

L'Hernault, S. W., and Rosenbaum, J. L., 1985b, *Chlamydomonas* α-tubulin is posttranslationally modified by acetylation on the epsilon-amino group of a lysine, *Biochemistry* **24**:473.

Linck, R. W., 1973, Chemical and structural differences between cilia and flagella from the lamellibranch mollusc, *Aequipecten irradions*, *J. Cell Sci.* **12**:951.

Ling, V., Aubin, J. E., Chase, A., and Sarangi, F., 1979, Mutants of Chinese hamster ovary (CHO) cells with altered colcemid-binding affinity, *Cell* **18**:423.

Little, M., and Ludueña, R. F., 1985, Structural differences between brain β1- and β2-tubulins: implications for microtubule assembly and colchicine binding, *EMBO J.* **4**:51.

Little, M., Ludueña, R. F. Langford, G. M., Asnes, C. F., and Farrell, K., 1981a, Comparison of proteolytic cleavage patterns of α-tubulins and β-tubulins from taxonomically distant species, *J. Mol. Biol.* **149**:95.

Little, M., Krauhs, E., and Ponstingl, H., 1981b. Tubulin sequence conservation, *Biosystems* **14**:239.

Little, M., Ludueña, R. F., Keenan, R., and Asnes, C. F., 1982a, Tubulin evolution: Two major types of α-tubulin, *J. Mol. Evol.* **19**:80.

Little, M., Krauhs, E., Ponstingl, H., Ludueña, R. F., and Rice, N., 1982b, Tubulin subunits from higher and lower eukaryotes—A structural comparison, in: *Microtubules in Microorganisms* (P. Cappuccinelli and N. R. Morris, eds.), pp. 1–14, Marcel Dekker, New York.

Little, M., Quinlan, R. A., Hoffman, E. J., and Ludueña, R. F., 1983, Identification and characterization of axopodial tubulins from *Echinosphaerium nucleofilum*, *Eur. J. Cell Biol.* **31**:55.

Little, M., Ludueña, R. F., Morejohn, L. C., Asnes, C., and Hoffman, E. J., 1984, The tubulins of animals, plants, fungi and protists- implications for metazoan evolution, *Origins Life* **13**:169.

Lloyd, C. W., Slabas, A. R., Powell, A. J., MacDonald, G., and Badley, R. A., 1979, Cytoplasmic microtubules of higher plant cells visualised with anti-tubulin antibodies, *Nature (London)* **279**:239.

Lloyd, C. W., Slabas, A. R., Powell, A. J., and Lowe, S. B., 1980, Microtubules, protoplasts and plant cell shape. An immunofluroescent study, *Planta* **147**:500.

Loomis, W. D., 1974, Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles, in: *Methods in Enzymology*, Vol. 31 (S. Fleischer and L. Packer, eds.), pp. 528–531, Academic Press, New York.

Loomis, W. D., and Battaile, J., 1966, Plant phenolic compounds and the isolation of plant enzymes, *Phytochemistry* **5**:423.

Lopata, M. A., Havercroft, J. C., Chow, L. T., and Cleveland, D. W., 1983, Four unique genes required for β-tubulin expression in vertebrates, *Cell* **32**:713.

Lu, R. C., and Elzinga, M., 1978, The primary structure of tubulin. Sequences of the carboxyl terminus and seven other cyanogen bromide peptides from the α-chain, *Biochim. Biophys. Acta* **537**:320.

Ludueña, R. F., 1979, Biochemistry of tubulin, in: *Microtubules* (K. Roberts and J. S. Hyams, eds.), pp. 65–116, Academic Press, London.

Ludueña, R. F., and Roach, M. C., 1981a, Interaction of tubulin with drugs and alkylating agents. 1. Alkylation of tubulin by iodo ^{14}C acetamide and *N,N'*-ethylenebis (iodoacetamide), *Biochemistry* **20**:4437.

Ludueña, R. F., and Roach, M. C., 1981b, Interaction of tubulin with drugs and alkylating agents.

2. Effects of colchicine, podophyllotoxin and vinblastine on the alkylation of tubulin, *Biochemistry* **20**:4444.

Ludueña, R. F., and Woodward, D. O., 1973, Isolation and partial characterization of α- and β-tubulin from outer doublets of sea urchin sperm and microtubules of chick-embryo brain, *Proc. Natl. Acad. Sci. USA* **70**:3594.

Ludueña, R. F., Shooter, E. M., and Wilson, L., 1977, Structure of the tubulin dimer, *J. Biol. Chem.* **252**:7006.

Ludueña, R. F., Myles, D. G., and Pfeffer, T. A., 1980, Isolation and partial characterization of flagellar tubulin from *Marsilea vestita*, *Exp. Cell Res.* **130**:455.

Ludueña, R. F., Roach, M. D., Trcka, P. P., Little, M., Palanivelu, P., Binkley, P., and Prasad, V., 1982, β_2-tubulin, a form of cordate brain tubulin with a lesser reactivity toward an assembly-inhibiting sulfhydryl-directed cross-linking reagent, *Biochemistry* **21**:4787.

Maccioni, R. B., and Seeds, N. W., 1977, Stoichiometry of GTP hydrolysis and tubulin polymerization, *Proc. Natl. Acad. Sci. USA* **72**:462.

Maekawa, S., 1978, Dissociation constant of *Tetrahymena* tubulin–colchicine complex, *J. Biochem.* **84**:641.

Maekawa, S., and Sakai, H., 1978, Characterization and *in vitro* polymerization of *Tetrahymea* tubulin, *J. Biochem.* **83**:1065.

Magistrini, M., and Szollosi, D., 1980, Effects of cold and of isopropyl-*N*-phenylcarbamate of the second meiotic spindle of mouse oocytes, *Eur. J. Cell Biol.* **22**:699.

Manfredi, J. J., Parness, J., and Horwitz, S. B., 1982, Taxol binds to cellular microtubules, *J. Cell Biol.* **94**:688.

Marchant, H. J., 1979, Microtubules, cell wall deposition and the determination of plant cell shape, *Nature* **278**:167.

Marchant, H. J., and Pickett-Heaps, J. D., 1974, The effect of colchicine on colony formation in the algae *Hydrodictyon pediastrum* and *sorastrum*, *Planta* **116**:291.

Margolis, R. L., and Wilson, L., 1978, Opposite end assembly and disassembly of microtubules at steady state *in vitro*, *Cell* **13**:1.

Margolis, R. L., and Wilson, L., 1981, Microtubule treadmills-possible molecular machinery, *Nature (London)* **293**:705.

Margulis, L., Banerjee, S., and Kelleher, J. K., 1975, Assay for antitubulin drugs in live cells: Oral regeneration in *Stentor coeruleus*, in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds), pp. 453–470, North Holland, Amsterdam.

Matsumoto, G., Kobayashi, T., and Sakai, H., 1979, Restoration of the excitability of squid giant axon by tubulin-tyrosine ligase and microtubule proteins, *J. Biochem.* **86**:1155.

McClure, W. O., and Paulson, J. C., 1977, The interaction of colchicine and some related alkaloids with rat brain tubulin, *Mol. Pharmacol.* **13**:560.

McKeithan, T. W., and Rosenbaum, J. L., 1981, Multiple forms of tubulin in the cytoskeletal and flagellar microtubules of *Polytomella*, *J. Cell Biol.* **91**:352.

McKeithan, T. W., Lefebvre, P. A., Silflow, C. D., and Rosenbaum, J. L., 1983, Multiple forms of tubulin in *Polytomella* and *Chlamydomonas:* Evidence for a precursor of flagellar tubulin. *J. Cell Biol.* **96**:1056.

Miller, C. C. J., Duckett, J. D., Sheterline, P., and Carothers, Z. B., 1983, Immunofluorescence microscopy of the flagella nd multilayered structure in two mosses, *Sphagnum palustre* L. and *Polytrichum juneperinum* Hedw., *J. Cell Sci.* **61**:71.

Minor, P. D., and Roscoe, D. H., 1975, Colchicine resistance in mammalian cell lines, *J. Cell Sci.* **17**:381.

Mita, T., and Shibaoka, H., 1983, Changes in microtubules in onion leaf sheath cells during bulb development, *Plant Cell Physiol.* **24**:109.

Mizuno, K., 1985, *In vitro* assembly of microtubules from tubulins of several higher plants, *Cell Biol. Int. Rep.* **9**:13.

Mizuno, K., Koyama, M., and Shibaoka, H., 1981, Isolation of plant tubulin from Azuki bean epicotyls by ethyl *N*-phenylcarbamate-sepharose affinity chromatography, *J. Biochem.* **89**:329.

Mizuno, K., Perkins, J., Sek, F., and Gunning, B., 1985, Some biochemical properties of higher plant tubulins, *Cell Biol. Int. Rep.* **9**:5.

Mohri, H., 1968, Amino-acid composition of "tubulin" constituting microtubules of sperm flagella, *Nature (London)* **217**:1053–1054.

Molè-Bajer, J., and Bajer, A. S., 1983, Action of taxol on mitosis: Modification of microtubule arrangements and function of the mitotic spindle in *Haemanthus* endosperm, *J. Cell Biol.* **96**:527.

Morejohn, L. C., and Fosket, D. E., 1982, Higher plant tubulin identified by self-assembly into microtubules *in vitro*, *Nature (London)* **297**:426.

Morejohn, L. C., and Fosket, D. E., 1984a, Inhibition of plant microtubule polymerization *in vitro* by the phosphoric amide herbicide amiprohos-methyl, *Science* **224**:874.

Morejohn, L. C., and Fosket, D. E., 1984b, Taxol-induced rose microtubule polymerization *in vitro* and its inhibition by colchicine, *J. Cell Biol.* **99**:141.

Morejohn, L. C., Bureau, T. E., Tocchi, L. P., and Fosket, D. E., 1984, Tubulins from different higher plant species are immunologically nonidentical and bind colchicine differentially, *Proc. Natl. Acad. Sci. USA* **81**:1440.

Morejohn, L. C., Bureau, T. E., Cyr, R. J., and Fosket, D. E., 1985, Inhibition of plant proteolytic activities that degrade tubulin *Cell Biol. Int. Rep.* **9**:849.

Morejohn, L. C., Bureau, T. E., Tocchi, L. P., and Fosket, D. E., 1986a, Resistance of plant microtubule polymerization to colchicine results from a low affinity interaction of colchicine and tubulin (submitted for publication).

Morejohn, L. C., Bureau, T. E., Tocchi, L. P., Molè-Bajer, J., Bajer, A. S., and Fosket, D. E., 1986b, Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro* (submitted for publication).

Morgan, J. L., and Seeds, N. W., 1975, Properties of tubulin prepared by affinity chromatography, *Ann. NY Acad. Sci.* **253**:260.

Morris, N. R., Lai, M. H., and Oakley, C. E., 1979, Identification of a gene for α-tubulin in *Aspergillus nidulans*, *Cell* **16**:437.

Mukherjee, J., and Biswas, S., 1982, Tubulin from *Mimosa pudica* and its involvement in leaf movement, *Phytochemistry* **21**:1881.

Murphy, D. B., and Borisy, G. G., 1975, Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly *in vitro*, *Proc. Natl. Acad. Sci. USA* **72**:2696.

Murphy, D. B., Johnson, K. A., and Borisy, G. G., 1977, Role of tubulin-associated proteins in microtubule nucleation and elongation, *J. Mol. Biol.* **117**:33.

Nagle, B. W., and Bryan, J., 1976, Factors affecting nucleation and elongation of microtubules *in vitro*, in: *Cold Spring Harbor Conference on Cell Proliferation*, Book C (R. Goldman, T. Pollard, and J. Rosenbaum eds.), pp. 565, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Naruse, H., and Sakai, H., 1981, Evidence that a polysaccharide from the cortex of sea urchin egg inhibits microtubule assembly through its binding to microtubule-associated proteins, *J. Biochem.* **90**:581.

Nath, J., Flavin, M., and Schiffman, E., 1981, Stimulation of tubulin tyrosinolation in rabbit leukocytes evoked by the chemoattractant formyl-methionyl-leucyl-phenylalanine, *J. Cell Biol.* **91**:232.

Neff, N. F., Thomas, J. H., Grisafi, P., and Botstein, D., 1983, Isolation of the β-tubulin gene from yeast and demonstration of its essential function *in vitro*, *Cell* **33**:211.

Nooden, L. D., 1971, Physiological and developmental effects of colchicine, *Plant Cell Physiol.* **12**:759.

O'Brien, E. T., Jacobs, R. S., and Wilson, L., 1983, Inhibition of bovine brain microtubule assembly *in vitro* by stypoldione, *Mol. Pharmacol.* **24**:493.

O'Farrell, P. H., 1975, High resolution two-dimensional electrophoresis of proteins, *J. Biol. Chem.* **250**:4007.

Okamura, S., 1980, Binding of colchicine to a soluble fraction of carrot cells grown in suspension culture, *Planta* **149**:350–354.

Okamura, S., 1983, Effect of tartrate on the colchicine-binding activity in cultured carrot cell extract, *Protoplasma* **118:**199.

Okamura, S., 1984, Straight and helical filaments formed during microtubule-assembly cycling of cultured carrot cell extract, *J. Electron Micros.* **33:**182.

Okamura, S., Kato, T., and Nishi, A., 1984, Lack of inhibition of carrot colchicine binding activity by podophyllotoxin, *FEBS Lett.* **168:**278.

Olmsted, J. B., and Borisy, G. G., 1973, Characterization of microtubule assembly in porcine brain extracts by viscometry, *Biochemistry* **12:**4282.

Olmsted, J. B., Witman, G. B., Carlson, K., and Rosenbaum, J. L., 1971, Comparison of the microtubule proteins of neuroblastoma cells, brain, and *Chlamydomonas* flagella, *Proc. Natl. Acad. Sci. USA* **68:**2273.

Olson, L. W., 1973, Low molecular weight colchicine binding protein from the aquatic phycomycete *Allomyces neomoniliformis*, *Ark. Mikrobiol.* **91:**281.

Owellen, R. J., Owens, A. H., Jr., and Donigan, D. W., 1972, The binding of vincristine, vinblastine and colchicine to tubulin, *Biochem. Biophys. Res. Commun.* **47:**685.

Palevitz, B. A., and Hepler, P. K., 1974, The control of the plane of division during stomatal differentiation in *Allium* II. Drug studies, *Chromosoma* **46:**327.

Palevitz, B. A., and Hepler, P. K., 1976, Cellulose microfibril orientation and cell shaping in developing guard cells of *Allium:* The role of microtubules and ion accumulation, *Planta* **132:**71.

Parka, S. J., and Soper, O. F., 1977, The physiology and mode of action of the dinitroaniline herbicides, *Weed Sci.* **25:**79.

Parness, J., and Horwitz, S. B., 1981, Taxol binds to polymerized tubulin *in vitro*, *J. Cell Biol.* **91:**479.

Parness, J., Kingston, D. G. I., Powell, R. G., Harracksingh, C., and Horwitz, S. B., 1982, Structure–activity study of cytotoxicity and microtubule assembly *in vitro* by taxol and related taxanes, *Biochem. Biophys. Res. Commun.* **105:**1082.

Parness, J., Asnes, C., F., and Horwitz, S. B., 1983, Taxol binds differentially to flagellar outer doublets and their reassembled microtubules, *Cell Motil.* **4:**123.

Penningroth, S. M., and Kirschner, M. W., 1977, Nucleotide binding and phosphorylation in microtubule assembly *in vitro*, *J. Mol. Biol.* **115:**643.

Pfeffer, S. R., Drubin, D. G., and Kelly, R. B., 1983, Identification of three coated vesicle components as α- and β-tubulin linked to a phosphorylated 50,000 dalton polypeptide, *J. Cell Biol.* **97:**40.

Pfeifer, R. W., and Irons, R. D., 1983, Alteration of lymphocyte function by quinones through a sulfhydryl-dependent disruption of microtubule assembly, *Int. J. Immunopharmacol.* **5:**463.

Pickett-Heaps, J. D., 1967, The effects of colchicine on the ultrastructure of dividing plant cells, xylem wall differentiation and distribution of cytoplasmic microtubules, *Dev. Biol.* **15:**206.

Pipeleers, D. G., Pipeleers-Marichal, M. A., Sherline, P., and Kipnis, D. M., 1977, A sensitive method for measuring polymerized and depolymerized forms of tubulin in tissues, *J. Cell Biol.* **74:**341.

Piperno, G., and Luck, D. J., 1976, Phosphorylation of axonemal proteins in *Chlamydomonas reinhardtii*, *J. Biol. Chem.* **251:**2161.

Piperno, G., Huang, B., and Luck, D. J. L., 1977, Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*, *Proc. Natl. Acad. Sci. USA* **74:**1600.

Piras, R., and Piras, M. M., 1975, Changes in microtubule phosphorylation during the cell cycle of HeLa cells, *Proc. Natl. Acad. Sci. USA* **72:**1161.

Ponstingl, H., Little, M., Krauhs, E., and Kempf, T., 1979, Carboxy-terminal amino acid sequence of α-tubulin from porcine brain, *Nature (London)* **282:**423.

Ponstingl, H., Krauhs, E., Little, M., and Kempf, K., 1981, Complete amino acid sequence of α-tubulin from porcine brain, *Proc. Natl. Acad. Sci. USA* **78:**2757–2761.

Powell, A. J., Lloyd, C. W., Slabas, A. R., and Cove, D. J., 1980, Demonstration of the microtubular cytoskeleton of the moss, *Physcomitrella patens*, using antibodies against mammalian brain tubulin, *Plant Sci. Lett.* **18:**401.

Preston, S. F., Deanin, G. G., Hanson, R. K., and Gordon, M. W., 1979, The phylogenetic distribution of tubulin:tyrosine ligase, *J. Mol. Evol.* **13**:233.

Quader, H., and Filner, P., 1980, The action of antimitotic herbicides on flagellar regeneration in *Chlamydomonas reinhardtii:* A comparison with the action of colchicine, *Eur. J. Cell Biol.* **21**:301.

Quader, H., Wagenbreth, I., and Robinson, D. G., 1978, Structure, synthesis and orientation of microfibrils V. On the recovery of *Oocystis solitaria* from microtubule inhibitor treatment, *Cytolobiologie* **18**:39.

Quinlan, R. A., Pogson, C. I., and Gull, K., 1980, The influence of the microtubule inhibitor, methyl benzimidazol-2yl carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae, J. Cell Sci.* **46**:341.

Quinlan, R. A., Roobol, A., Pogson, C. I., and Gull, K., 1981, A correlation between *in vitro* and *in vivo* effects of the microtubule inhibitors colchicine, parbendazole and nocodazole on myxamoebae of *Physarum polycephallum, J. Gen. Microbiol.* **122**:1.

Ray, K., Bhattacharyya, B., and Biswas, B. B., 1984, Anion-induced increases in the affinity of colcemid binding to tubulin, *J. Biochem.* **142**:577.

Raybin, D., and Flavin, M., 1975, An enzyme tyrosylating α-tubulin and its role in microtubule assembly, *Biochem. Biophys. Res. Commun.* **65**:1088.

Raybin, D., and Flavin, M., 1977a, Enzyme which specifically adds tyrosine to the α-chain of tubulin, *Biochemistry* **16**:2189.

Raybin, D., and Flavin, M., 1977b, Modification of tubulin by tyrosylation in cells and extracts and its effect on assembly *in vitro, J. Cell Biol* **73**:492.

Richmond, D. V., and Phillips, A., 1975, The effect of benomyl and carbendazim on mitosis in hyphae of *Botrytis cinerea* Pers. ex Fr. and roots of *Allium cepa* L., *Pest. Biochem. Physiol.* **5**:367.

Rikin, A., Atsmon, D., and Gitler, C., 1982, Extraction and immunochemical assays of a tubulin-like factor in cotton seedlings, *Planta* **154**:402.

Rikin, A., Atsmon, D., and Gitler, C., 1983, Quantitation of chill-induced release of a tubulin-like factor and its prevention by abscissic acid in *Gossypium hirsutum* L., *Plant Physiol.* **71**:747.

Ringel, I., and Sternlicht, H., 1984, Carbon-13 nuclear magnetic resonance study of microtubule protein: evidence for a second colchicine site involved in the inhibition of microtubule assembly, *Biochemistry* **23**:5644.

Robinson, D. G., and Herzog, W., 1977, Structure, synthesis and orientation of microfibrils III. A survey of the action of microtubule inhibitors on microtubules and microfibril orientation in *Oocystis solitaria, Cytobiologie* **15**:463.

Robinson, D. G., Grimm, I., and Sachs, H., 1976, Colchicine and microfibril orientation, *Protoplasma* **89**:375.

Rodriguez, J. A., and Borisy, G. G., 1978a, Modification of the C-terminus of brain tubulin during development, *Biochem. Biophys. Res. Commun.* **89**:893.

Roobol, A., and Gull, K., 1980, Microtubules and microtubule proteins of *Physarum polycephalum,* in: *Microtubules and Microtubule Inhibitors* (M. Borgers and M. DeBrabander, eds.), pp. 523–532, North Holland, Amsterdam.

Roobol, A., and Gull, K., 1982, Purification of microtubule proteins from *Physarum polycephalum* by *in vitro* microtubule assembly, in: *Microtubules in Microorganisms* (P. Cappuccinelli and N. R. Morris, eds.), pp. 227–233, Marcel Dekker, New York.

Roobol, A., Pogson, C. I., and Gull, K., 1980a, *In vitro* assembly of microtubule proteins from myxamoebae of *Physarum polycephalum, Exp. Cell Res.* **130**:203.

Roobol, A., Pogson, C. I., and Gull, K., 1980b, Identification and characterization of microtubule proteins from myxamoebae of *Physarum polycephalum, Biochem. J.* **189**:305.

Rosenbaum, J. L., and Child, F. M., 1967, Flagellar regeneration in protozoan flagellates, *J. Cell Biol.* **34**:345.

Rousset, B., Bernier-Valentin, F., Wolff, J., and Roux, B., 1983, Alterations in tubulin immunoreactivity; relation to secondary structure, *Eur. J. Biochem.* **131**:31.

Rubin, R. W., and Cousins, E. E., 1976, Isolation of a tubulin-like protein from *Phaseolus, Phytochemistry* **15**:1837.

Rubin, R. W., Quillen, M., Cocoran, J. J., Ganapathi, R., and Krishan, R., 1982, Tubulin as a major cell surface protein in human lymphoid cells of leukemic origin, *Cancer Res.* **42**:1384.

Ruderman, J. V., and Alexandraki, D., 1983, Organization and expression of the tubulin gene families in the sea urchin, *J. Submicrosc. Cytol.* **15**:349.

Russell, D. G., and Gull, K., 1984, Flagellar regeneration of the trypanosome *Crithidia fasciculata* involves posttranslational modification of cytoplasmic alpha tubulin, *Mol. Cell. Biol.* **4**:1182.

Russell, D. G., Miller, D., and Gull, K., 1984, Tubulin heterogeneity in the trypanosome *Crithidia fasciculata, Mol. Cell. Biol.* **4**:779.

Sabnis, D. D., 1981, Lumicolchicine as a tool in the study of plant microtubules: Some biological effects of sequential products formed during phototransformations of colchicine, *J. Exp. Bot.* **32**:271.

Sabnis, D. D., and Hart, J. W., 1982, Microtubule proeins and p-proteins, in: *Encyclopedia of Plant Physiology New Series*, Vol. 14A. *Nucleic Acids and Proteins in Plants. I. Structure, Biochemistry and Physiology of Proteins* (D. Boulter and B. Parthier, eds.), pp. 401–437, Springer-Verlag, Berlin.

Salmon, E. D., and Wolniak, S., 1983, Taxol stabilization of mitotic spindle microtubules: Analysis using calcium-induced depolymerization, *Cell Motil.* **4**:155.

Sánchez, F., Natzle, J. E., Cleveland, D. W., Kirschner, M. W., and McCarthy, B. J., 1980, A dispersed miltigene family encoding tubulin in *Drosophila melanogaster, Cell,* **22**:845.

Sandoval, I. V., and Cuatrecasas, P., 1976, Proteins associated with tubulin, *Biochem. Biophys. Res. Commun.* **68**:169.

Schatten, G., Schatten, H., Bestor, T. H., and Balczon, R., 1982, Taxol inhibits the nuclear movements during fertilization and induces asters in unfertilized sea urcin eggs, *J. Cell Biol.* **94**:455.

Schedl, T., Burland, T. G., Gull, K., and Dove, W. F., 1984a, Cell cycle regulation of tubulin RNA level, tubulin protein synthesis, and assembly of microtubules in *Physarum, J. Cell Biol.* **99**:155.

Schedl, T., Owens, J., Dove, W. F., and Burland, T. G., 1984b, Genetics of the tubulin gene families of *Physarum, Genetics* **108**:143.

Schiff, P. B., and Horwitz, S. B., 1980, Taxol stabilized microtubules in mouse fibroblast cells, *Proc. Natl. Acad. Sci. USA* **77**:1561.

Schiff, P. B., and Horwitz, S. B., 1981, Taxol assembles tubulin in the absence of exogenous guanosine 5′-triphosphate or microtubule-associated proteins, *Biochemistry* **20**:3247.

Schiff, P. B., Fant, J., and Horwitz, S. B., 1979, Promotion of microtubule assembly *in vitro* by taxol, *Nature (London)* **277**:665.

Schmit, A-C., Vantard, M., DeMey, J., and Lambert, A.-M., 1983, Aster-like microtubule centers establish spindle polarity during interphase-mitosis transition in higher plant cells, *Plant Cell Rep.* **2**:285.

Schmitt, H., and Atlas, D., 1976, Specific affinity labeling of tubulin with bromocolchicine, *J. Mol. Biol.* **102**:743.

Schroder, H. C., Wehland, J., and Weber, K., 1985, Purification of brain tubulin-tyrosine ligase by biochemical and immunological methods, *J. Cell Biol.* **100**:276.

Seebeck, T., Whittaker, P. A., Imboden, M. A., Hardman, N., and Braun, R., 1983, Tubulin genes of *Trypanosoma brucei:* A tightly clustered family of alternating genes, *Proc. Natl. Acad. Sci. USA* **80**:4634.

Seiler, J. P., 1975, Toxicology and genetic effects of benzimidazole compounds, *Mutat. Res.* **32**:151.

Sheir-Neiss, G., Nardi, R. B., Gealt, M. A., and Morris, N. R., 1976, Tubulin-like protein from *Aspergillus nidulans, Biochem. Biophys. Res. Commun.* **69**:285.

Sheir-Neiss, G., Lai, M. H., and Morris, N. R., 1978, Identification of a gene for β-tubulins in *Aspergillus nidulans, Cell* **15**:639.

Shelanski, M. L., and Taylor, E. W., 1967, Isolation of a protein subunit from microtubules, *J. Cell Biol.* **34**:549.

Shelanski, M. L., Gaskin, F. and Cantor, C. R., 1973, Microtubule assembly in the absence of added nucleotides, *Proc. Natl. Acad. Sci. USA* **70**:765.

Sherline, P., and Schiavone, K., 1977, Immunofluorescence localization of proteins of high molecular weight along intracellular microtubules, *Science* **198**:1038–1040.

Sherline, P., Leung, J. T., and Kipnis, D., 1975, Binding of colchicine to purified microubule protein, *J. Biol. Chem.* **250**:5481.

Sherline, P., Schiavone, K., and Brocato, S., 1979, Endogenous inhibitor of colchicine-tubulin binding in rat brain, *Science* **205**:593–595.

Sheterline, P., 1978, Localization of the major high molecular weight protein on microtubules *in vitro* and in cultured cells, *Exp. Cell Res.* **115**:460.

Shields, R., Robinson, S. J., and Anslow, P. A., 1984, Use of fungicides in plant tissue culture, *Plant Cell Rep.* **3**:33.

Silflow, C. D., and Rosenbaum, J. L., 1981, Multiple α- and β-tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation, *Cell* **24**:81.

Silflow, C. D., Chisholm, R. L., Conner, T. W., and Ranum, L. P. W., 1985, The two alpha tubulin genes of *Chlamydomonas reinhardtii* code for slightly different proteins, *Mol. Cell. Biol.* **5**:2389.

Slabas, A. R., MacDonald, G., and Lloyd, C. W., 1980, Selective purification of plant proteins which co-polymerise with mammalian microtubules, *FEBS Lett.* **110**:77.

Slifkin, M. K., 1967, Nuclear division in *Saprolegnia* as revealed by colchicine, *Mycologia* **59**:431.

Sloan, M. E., and Camper, N. D., 1981, Effects of colchicine on carrot callus-growth and energy status, *Plant Cell Tiss. Organ Cult.* **1**:69.

Sloboda, R. D., Dentler, W. L., and Rosenbaum, J. L., 1975, Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*, *Biochemistry* **15**:4497.

Soifer, D., Laszlo, A., Mack, K., Scotto, J., and Siconolfi, L., 1975, The association of a cyclic AMP-dependent protein kinase activity with microtubule protein, *Ann. NY Acad. Sci.* **253**:598.

Solomon, F., Monard, D., and Rentsch, M. 1973, Stabilization of colchicine-binding activity of neuroblastoma, *J. Mol. Biol.* **78**:569.

Stadler, J., and Franke, W. W., 1974, Characterization of the colchicine binding of membrane fractions from rat and mouse liver, *J. Cell Biol.* **60**:297.

Stearns, M. E., and Brown, D. L., 1979. Purification of cytoplasmic tubulin and microtubule organizing center proteins functioning in microtubule initiation from the alga *Polytomella*, *Proc. Natl. Acad. Sci. USA* **76**:5745.

Stephens, R. E., 1970, Thermal fractionation of outer doublet microtubules into A and B-subfiber component: A-and B-tubulin, *J. Mol. Biol.* **47**:353.

Stephens, R. E., 1975, Structural chemistry of the axoneme: Evidence for chemically and functionally unique tubulin dimers in outer fibers, in: *Molecules in Cell Movement* (S. Inoue and R. E. Stephens, eds.), pp. 181, Raven Press, New York.

Stephens, R. E., 1977, Major membrane protein differences in cilia and flagella: Evidence for a membrane-associated tubulin, *Biochemistry* **16**:2047.

Stephens, R. E., 1978, Primary structural differences among tubulin subunits from flagella, cilia and the cytoplasm, *Biochemistry* **17**:2882.

Stephens, R. E., 1981, Chemical differences distinguish ciliary membrane and axonemal tubulins, *Biochemistry* **20**:4716–4723.

Stephens, R. E., 1983, Reconstitution of ciliary membranes containing tubulin, *J. Cell Biol.* **96**:68.

Sternlicht, H., and Ringel, I., 1979, Colchicine inhibition of microtubule assembly via copolymer formation, *J. Biol. Chem.* **254**:10540.

Stieger, J., Wyler, T., and Seebeck, T., 1984, Partial purification and characterization of microtubular protein from *Trypanosoma brucei*, *J. Biol. Chem.* **259**:4596.

Strachan, S. D., and Hess, F. D., 1983, The biochemical mechanism of action of the dinitroaniline herbicide oryzalin, *Pest. Biochem. Physiol.* **20**:14.

Stubblefield, E., and Klevecz, R., 1965, Synchronization of Chinese hamster cells by reversal of colcemid inhibition, *Exp. Cell Res.* **40**:660.

Studier, F. W., 1973, Analysis of bacteriophage T7 early RNAs and proteins on slab gels, *J. Mol. Biol.* **79**:237.

Styles, J. A., and Garner, R., 1974, Benzimidazole carbamate methyl ester—Evaluation of its effects *in vivo* and *in vitro*, *Mutation Res.* **26**:177.

Sullivan, K. F., and Cleveland D. W., 1984, Sequence of highly diverent β-tubulin gene reveals regional heterogeneity in the β-tubulin polypeptide, *J. Cell Biol.* **99**:1754.

Suprenant, K. A., and Rebhun, L. I., 1983, Assembly of unfertilized sea urchin egg tubulin at physiological temperatures, *J. Biol. Chem.* **258**:4518.

Taylor, E. W., 1965, The mechanism of colchicine inhibition of mitosis I. Kinetics of inhibition and the binding of H^3-colchicine, *J. Cell Biol.* **25:**145.

Thomashow, L. S. Wilhausen, M., Rutter, W. J., and Agabian, N., 1983, Tubulin genes are tandemly linked and clustered in the genome of *Trypanosoma brucei, Cell* **32:**35.

Thompson, W. C., 1977, Post-translational addition of tyrosine to alpha tubulin *in vitro* in intact brain and in myogenic cells in culture, *FEBS Lett.* **80:**9.

Thompson, W. C., 1982, The cyclic tyrosination/detyrosination of alpha tubulin, in: *Methods in Cell Biology* (L. Wilson, ed.), pp. 235–255, Academic Press, New York.

Thompson, W. C., Deanin, G. G., and Gordon, M. W., 1979, Intact microtubules are required for rapid turnover of carboxyl-terminal tyrosine of α-tubulin in cell cultures, *Proc. Natl. Acad. Sci. USA* **76:**1318.

Thompson, W. C., Wilson, L., and Purich, D. L., 1981, Taxol induces microtubule assembly at low temperature, *Cell Motil.* **1:**445.

Thompson, W. C., Asai, D. J., and Carney, D. H., 1984, Heterogeneity among microtubules of the cytoplasmic microtubule complex detected by a monoclonal antibody to α-tubulin, *J. Cell Biol.* **98:**1017.

Tilney, L. G., 1968, Studies on the microtubules in Heliozoa IV. The effect of colchicine on the formation and maintenance of the axopodia and the redevelopment of pattern in *Actinosphaerium nucleofilum* (Barrett), *J. Cell Sci.* **3:**549.

Tilney, L. G., and Gibbins, J. R., 1968, Differential effects of antimitotic agents on the stability and behavior of cytoplasmic and ciliary microtubules, *Protoplasma* **65:**167.

Toda, T., Adachi, Y., Hiraoka, Y., and Yanagida, M., 1984, Identification of the pleiotropic cell division cycle gene *NDA2* as one of two different α-tubulin genes in *Schizosaccharomyces pombe, Cell* **37:**233.

Torian, B. E., Barnes, R. C., Stephens, R. S., and Stibbs, H. H., 1984, Tubulin and high-molecular weight polypeptides as *Giardia lamblia* antigens, *Infect. Immun.* **46:**152.

Turner, F. R., 1970, The effects of colchicine on spermatogenesis in *Nitella, J. Cell. Biol.* **46:** 220.

Umesono, K., Toda, T., Hayashi, S., and Yanagida, M. 1983, Two cell division cycle genes *NDA2* and *NDA3* of the fission yeast *Schizosaccharomces pombe* control microubular organization and sensitivity to anti-mitotic benzimidazole compounds, *J. Mol. Biol.* **168:**271.

Upadhyaya, M. K., and Nooden, L. D., 1980, Mode of dinitroaniline herbicide action II. Characterization of ^{14}C-oryzalin uptake and binding, *Plant Physiol.* **66:**1048.

Valenzuela, P., Quiroga, M., Valdivar, J., Rutter, W. J., Kirschner, M. W., and Cleveland, D. W., 1981, Nucleotide and corresponding amino acid sequences encoded by α- and β-tubulin mRNAs, *Nature (London)* **289:**650.

Vallee, R. B., 1982, A taxol-dependent procedure for the isolation of microtubule-associated proteins (MAPs), *J. Cell Biol.* **92:**435.

van der Valk, P., Rennie, P. J., Connolly, J. A., and Fowke, L. C., 1981, Distribution of cortical microtubules in tobacco protoplasts. An immunofluorescence microscopic and ultrastructural study, *Protoplasma* **106:**193.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., and McPhail, A. T., 1971, Plant antitumor agents, VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia, J. Am. Chem. Soc.* **93:**2325.

Wanka, F., 1968, Ultrastructural changes during normal and colchicine-inhibited cell division in *Chlorella, Protoplasma* **66:**105.

Water, R. D., and Kleinsmith, J. L., 1976, Identification of α- and β-tubulin in yeast, *Biochem. Biophys. Res. Commun.* **70:**704.

Weatherbee, J. A., and Morris, N. R., 1984, *Aspergillus* contains multiple tubulin genes, *J. Biol. Chem.* **259:**1542.

Weber, K., and Osborn, M., 1969, The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis, *J. Biol. Chem.* **244:**4406.

Weeks, D. D., and Collis, P. S., 1976, Induction of microtubule protein synthesis in *Chlamydomonas reinhardi* during flagellar regeneration, *Cell* **9:**15.

Wehland, J., Schroeder, M., and Weber, K., 1984, Organization of microtubules in stabilized

meristematic plant cells revealed by a rat monoclonal antibody reacting only with the tyrosinated form of α-tubulin, *Cell Biol. Int. Rep.* **8**:147.

Weinert, T. A., and Cappuccinelli, P., 1982, A novel tubulin inactivating protein from *Dictyostelium discoideum*, in: *Microtubules in Microorganisms* (P. Cappuccinelli and N R. Morris, eds.), pp. 129, Marcel Dekker, New York.

Weinert, T., Cappuccinelli, P., and Wiche, G., 1982, Potent microtubule inhibitor protein from *Dictyostelium discoideum*, *Biochemistry* **21**:782.

Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., and Kirschner, M. W., 1975, A protein factor essential for microtubule assembly, *Proc. Natl. Acad. Sci. USA* **72**:1858.

Weisenberg, R. C., 1972, Microtubule formation *in vitro* in solutions containing low calcium concentrations, *Science* **177**:1104.

Weisenberg, R. C., Borisy, G. G., and Taylor, E. W., 1968, The colchicine-binding protein of mammalian brain and its relation to microtubules, *Biochemistry* **7**:4466.

Welker, D. L., and Williams, K. L., 1980, Mitotic arrest and chromosome doubling using thiabendazole, cambendazole, nocodazole and ben late in the slime mould *Dictyostelium discoideum*, *J. Gen. Microbiol.* **116**:397.

White, E., Scandella, D., and Katz, E. R., 1981, Inhibition by CIPC of mitosis and development in *Dictyostelium discoideum* and the isolation of CIPC-resistant mutants, *Dev. Genet.* **2**:99–111.

White, E., Tolbert, E. M., and Katz, E. R., 1983, Identification of tubulin in *Dictyostelium discoideum:* Characterization of some unique properties, *J. Cell Biol.* **97**:1011.

Wick, S. M., and Duniec, J., 1983, Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. I. Preprophase band development and concomitant appearance of nuclear envelope-associated tubulin, *J. Cell Biol.* **97**:235.

Wick, S. M, Seagull, R. W., Osborn, M., Weber, K., and Gunning, B. E. S., 1981, Immunofluorescence microscopy of organized micotubule arrays in structurally stabilized meristematic plant cells, *J. Cell Biol.* **89**:685.

Wilde, C. D., Chow, L. T., Wefald, F. C., and Cowan, N. J., 1982, Structure of two human α-tubulin genes, *Proc. Natl. Acad. Sci. USA* **79**:96.

Williams, K. L., 1980, Examination of the chromosomes of *Polyphondylium pallidium* following metaphase arrest by benzimidazole derivatives and colchicine, *J. Gen Microbiol.* **116**:409.

Williams, N. E., Vaudaux, P. E., and Skrives, L., 1979, Cytoskeletal proteins of the cell surface in *Tetrahymena* I. Identification and localization of major proteins, *Exp. Cell Res.* **123**:311.

Williams, R. F., Aivaliotis, M. J., Barnes, L. D., and Robinson, A. K., 1983, High performance liquid chromatographic application of the Hummel and Dryer method for the determination of colchicine-tubulin binding parameters, *J. Chromatogr.* **266**:141.

Wilson, L., 1975, Microtubules as drug receptors: Pharmacological properties of microtubule protein, *Ann. NY Acad. Sci.* **253**:213.

Wilson, L., and Bryan, J., 1974, Biochemical and pharmacological properties of microtubules, *Adv. Cell and Mol. Biol* **3**:22.

Wilson, L., and Friedkin, M., 1967, The biochemical events of mitosis II. The *in vivo* and *in vitro* binding of colchicine in grasshopper embryos and its possible relation to inhibition of mitosis, *Biochemistry* **6**:3126.

Wilson, L., and Meza, I., 1973, The mechanism of action of colchicine. Colchicine binding properties of sea urchin sperm tail outer doublet tubulin, *J. Cell. Biol.* **58**:709.

Wilson, L., Bryan, J., Ruby, A., and Mazia, D., 1970, Precipitation of proteins by vinblastine and calcium ions, *Proc. Natl. Acad. Sci. USA* **66**:807.

Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., and Creswell, K. M., 1974, Interaction of drugs with microtubule proteins, *Fed. Proc.* **33**:158.

Witman, G. B., Carlson, K., and Rosenbaum, J. L., 1972, *Chlamydomonas* flagella. 11. The distribution of tubulins 1 and 2 in outer doublet microtubules, *J. Cell Biol.* **54**:540.

Wright, M., and Oustrin, M. L., 1983, Taxol, a mitotic inhibitor for chromosomal studies in Myxomycetes, *FEBS Microbiol. Lett.* **18**:89.

Wright, M., Moisand, A., and Oustrin, M. L., 1982, Stabilization of monoasters by taxol in the amoebae of *Physarum polycephalum* (Myxomycetes), *Protoplasma* **113**:44.

Wunderlich, F., and Speth, V., 1970, Antimitotic agents and macronuclear division of ciliates IV. Reassembly of microtubules in macronuclei of *Tetrahymena* adapting to colchicine, *Protoplasma* **70:**139.

Yadav, N. S., and Filner, P., 1983, Tubulin from cultured tobacco cells: Isolation and identification based on similarities to brain tubulin, *Planta* **157:**46.

Yang, S., and Criddle, R. S., 1970, *In vitro* biosynthesis of membrane proteins in isolated mitochondria from *Saccharomyces carlsbergensis*, *Biochemistry* **9:**3063.

Youngblom, J., Schloss, J. A., and Silflow, C. D., 1984, The two β-tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins, *Mol. Cell. Biol.* **4:**2686.

Zisapel, N., Levi, M., and Gozes, I., 1980, Tubulin: An integral protein of mammalian synaptic vesicle membranes, *J. Neurochem.* **34:**26.

Index

Acetylation, of tubulin, 289–290
Actin
  diameter, 109
  microinjection studies, 9–13
  nuclear, 12–13, 144–145
    antiactin effects, 21
  polarity, 179
    marker, 179
    positive end, 14, 15
  mRNA, 135–136, 137, 138, 141
  stress fibers, 14–19
  synthesis, 135–136, 140
  tropomyosin and, 18
Actin-associated protein
  antibodies, 20–26
  microinjection studies, 13–19
Actin-capping proteins, 14
Actin depolymerizing factor, 14–17
Actin gene expression
  in *Caenorhabditis elegans*, 151–178
    amino acid sequences, 155–157, 158
    cell-specific, 154
    cytoplasmic, 163
    embryonic, 161
    evolution, 157–158
    gene-specific probes, 158, 159, 161, 162
    genomic organization, 152–153
  hybridization, 158, 160–161, 162
  introns, 152, 154, 157
  multigene families, 161
  mutants, 151–152, 165–168
  mutants, revertant, 167–175
  polymorphism, 152
  sequence analysis, 153–158
  transcription, 158–163, 169–176
Actin gene expression (*cont.*)
  translational products, 163–165
  ultrastructural defects, 151–152
  in cell growth/differentiation, 131–149
  cytoskeleton organization, 133–135
  growth factors and, 136–138, 139, 143–144
  as protooncogene, 141–143
  mRNA, 135–136, 137, 138–141
  protein transcription, 21
Actin-severing protein, 14
β-Actin
  gene expression, 141
  in neoplastic transformation, 142–143
Actomyosin, contraction, 10
Adenosine 3′:5′-cyclic phosphate-inducing agents, 86, 100
Adenosine triphosphatase, in tubulin, 259
Adhesion plaques, 134
Affinity chromatography, of tubulin, 262
Algae
  cytoskeleton isolation, 267
  tubulin isolation, 261
Ameba, cytoskeleton isolation, 267
Amino acid sequences
  actin, 155–157, 158
  intermediate filament proteins, 113, 114
  keratin, 69, 71–72, 73, 86–88, 90, 91, 93
  tubulin, 271–274
Anchorage dependence, 131
Anchorage-dependent cells, cell configuration changes, 131–132
Anthelmintics, 304–306
Antimicrotubule drugs
  benzimidazole, 304–306

Antimicrotubule drugs (*cont.*)
caffeine, 309
griseofulvin, 309
herbicides, 293, 300–304
melatonin, 309
podophyllotoxin, 309
taxol, 306–309
tubulin effects, 204–207, 269–270, 293–309
vinblastine, 309
Antisera, keratin specific, 71, 73, 89
Antitubulin, 6–9, 260
microinjection, 6–9
Assays
microtubule polarity, 182–187
tubulin, 259–260
Aster microtubules, 187, 191
Axon transport
microtubule polarity and, 195, 196
tubulin, 232
Axoneme
dynein, 182, 184–185
microtubule polarity, 182
tubulin, 265, 267
Axopodia transport, microtubule polarity and, 195

Benomyl, 304, 305
Benzimidazole derivatives, 304–306
Brain, tubulin gene expression, 227–256
cytoplasmic versus membrane-bound, 230–231
developmental regulation, 232–234
genes, 234–235
hybridization analysis, 235–237, 240–245
isoform(s), 228–234
isoform localization, 229–234
isoform microheterogeneity, 228–229, 231, 233
myelin-associated, 230
posttranslational modification, 231–232
mRNA, 235–245
subcellular distribution, 229–230
translational products, 235, 237–240, 241
Browning reaction, in tubulin isolation, 270

Caenorhabditis elegans, actin gene expression in, 151–178
amino acid sequences, 155–157, 158
cell-specific, 154
cytoplasmic, 163
embryonic, 161
evolution, 157–158
gene-specific probes, 158, 159, 161, 162
genomic organization, 152–153

Caenorhabditis elegans, actin gene expression in (*cont.*)
amino acid sequences (*cont.*)
hybridization analysis, 158, 160–161, 162
introns, 152, 154, 157
multigene family, 161
mutants, 151–152, 165–168
mutants, revertant, 167–175
polymorphism, 152
sequence analysis, 153–158
transcription, 158–163, 169–176
translational products, 163–165
ultrastructural defects, 151–152
Caffeine, as antimicrotubule drug, 309
Calcium
actomyosin contraction and, 10
epithelial cell growth and, 100
microtubule polymerization and, 3
Capping proteins, 14–19
Carbamate herbicides, 300, 301, 303–304
Cell cycle, tubulin synthesis during, 220–222
in *Chlamydomonas*, 221–222
in *Physarum*, 220–221
Cell morphology
actin synthesis and, 140
anchorage-dependence and, 131–133
cell proliferation and, 132
cytoskeleton organization and, 133–134
differentiation and, 132–133
Cell proliferation, cell morphology and, 132
Cellular retinoic acid binding protein, 100–101
Cerebellum, β-tubulin, 238–239, 242–243, 244–245
c-fos oncogene, 142
Chlamydomonas
axoneme, 183, 184
tubulin synthesis, 214–218
cell cyle regulation, 221–222
DNA sequences, 218
Chromatography, for tubulin isolation, 260–263
Cilia
microtubule polarity, 182
tubulin isolation, 265, 267
Clathrin, 29–30
c-myc oncogene, 142
Coated pit, 30, 31
Coated vesicle, 1
clathrin, 29–30
functions, 29–30
microfilament contact, 30, 31
Colcemid, tubulin-binding, 297
Colchicine, antimicrotubule effects, 293, 294–300
in vitro, 296–300

Colchicine, antimicrotubule effects (*cont.*)
in vivo, 294–296
intermediate filament effects, 109
tubulin effects, 204–206, 213
in affinity chromatography, 262
Colchicine-binding assay, 259–260
Colchicine-binding protein, 292–293
Copolymerization, in tubulin isolation, 265
Cortex, cerebral, β-tubulin, 240, 243
Cytokeratin, *see also* Keratin
filaments
microinjection studies, 27–29
protofilaments, 53
reorganization, 27
mRNA, 29
Cytoplast, tubulin synthesis regulations, 135–145
Cytoskeleton
in cell growth/differentiation regulation, 135–145
components, 1
desmin, 119–120, 121, 122
isolation, 267
protein synthesis, 1

Deoxyribonuclease I, endoplasmic streaming effects, 18–19
Deoxyribonucleic acid
synthesis, microtubule depolymerization and, 134
tubulin genes
transfection, 211–213
sequences, 218
Deoxyribonucleic acid-binding protein, 60
cDeoxyribonucleic acid (cDNA) clone probe
desmin genes, 115
intermediate filament genes, 114–115
keratin genes, 70–71, 94, 96, 97, 114–115
Desmin
aggregation, 121, 122
characteristics, 112
domain organization, 42, 43
filaments, 55–56
headpieces, 58–60
heterogenous cytoskeleton, 119–120, 121, 122
rod domain, 51, 52, 53
protofilament, 53–58
sequence organization, 49, 50–51
structural homology, 111, 114
subunits, 72
T desmin, 60
tailpieces, 60–61
carboxyterminal appendages, 62–63
vimentin colocalization, 121, 123, 124

Desmin gene(s)
chromosomal localization, 125–127
cloned, 118–124
cDNA clone probe, 115
single-copy, 115
structure, 115, 116, 117, 118
Desmin gene expression, 118, 120–124
HeLa cells, 121, 123–124
heterogenous cytoskeleton, 119–120, 121, 122
lens cells, 118, 120–121, 122
species specificity, 121, 123–124
Dictyostelium
actin genes, 152, 153, 161
cytoskeleton isolation, 267
Differentiation, cellular
actin regulation
actin gene expression, 135–141
cytoskeleton organization, 133–135
growth factors, 136–138, 139, 143–144
mechanisms, 143–145
protooncogene, 141–143
mRNA, 135–136, 137, 138, 141
cell configuration and, 132–133
during embryogenesis, 132–133
extracellular matrix, 132–133, 134–135
keratin gene expression, 70–73, 87
vitamin A and, 100–103
Dinitroaniline herbicides, 300–303, 304
Drosophila, actin genes, 153, 155, 158, 161
Drug affinity chromatography, for tubulin isolation, 262–263
Dynein
axonemal, 182, 184–185
as microtubule polarity indicator, 182, 185, 187

Electrophoretic separation, of tubulin subunits, 275–279
Embryo, actin gene expression, 161
Embryogenesis, differentiation during, 132–133
End domains, of intermediate filaments, 78–81
Endoplasmic streaming, DNAase I effects, 18–19
Epidermis, *see also* Keratin
culture, 86
definition, 85
proliferation, 85–86
Epithelium, *see also* Keratin
differentiation, 100
Exchange chromatography, for tubulin isolation, 260–262

Exons
intermediate filament genes, 74, 75, 115, 116, 118
keratin genes, 97, 98, 99
Extracellular matrix, in differentiation, 132–133, 134–135

Fenbendazole, 304, 305, 306
Fibronectin, 133–135
Fillagrin, 85, 95
Flagella
axoneme, microtubule polarity, 182
tubulin, 265, 267
tubulin gene expression, 213–220
in *Chlamydomonas*, 214–218, 221–222
DNA sequences, 218
in *Leishmania*, 219–220
in *Naegleria*, 219
Fungi, tubulin
antimicrotubule drug effects, 293–309
dimer structure, 271–293
identification/isolation, 257, 258–271
Fungicides, antimicrotubule, 262, 304–306

Gene(s)
clustering, 153
desmin
chromosomal localization, 125–127
cloned, 118–124
cDNA clone probe, 115
single-copy, 115
structure, 115, 116, 117, 118
glial fibrillary acidic protein, 115
intermediate filament, 114–127
evolution, 75–81, 115, 118
structure, 73–75
keratin
enhancer sequences, 101, 102
exon(s), 97, 98, 99
exon/intron pattern, 118
homology, 97–99
intermediate filament gene relationship, 99–100
introns, 97, 98, 99–100, 118
multifamily, 115
mutagenesis, site-directed, 101, 102–103
number, 94
structure, 74, 97–100
subfamily, 94, 96–97
keratin type I
introns, 77–78
structure, 74, 97–99
keratin type II
introns, 78
Gene(s) (*cont.*)
keratin type II (*cont.*)
linkage, 78
structure, 74, 97–99
keratin type III, 99
tubulin
in higher eukaryotes, 234–235
multiple, 290–292
transfected, 211–213
vimentin
chromosomal localization, 125, 126
single-copy, 115
structure, 73–74, 115, 116, 117, 118, 119
Gene conversion, 157
Gene expression
actin
amino acid sequences, 155–157, 158
in *Caenorhabditis elegans*, 151–178
in cell growth/differentiation, 135–141
cell-specific, 154
cytoplasmic, 163
cytoskeleton organization, 133–135
embryonic, 161
evolution, 157–158
gene-specific probes, 158, 159, 161, 162
genomic organization, 152–153
growth factors, 143–145
hybridization analysis, 158, 160–161, 162
introns, 152, 154, 157
mechanisms, 143–145
multigene family, 161
mutants, 151–152, 165–168
mutants, revertant, 167–175
polymorphism, 152
protein transcription, 21
protooncogene, 141–143
regulation, 135–141
mRNA, 135–136, 137, 138, 141
sequence analysis, 153–158
transcription, 158–163, 169–176
translational products, 118, 120–124
ultrastructural defects, 151–152
β-actin, 141
desmin, 118, 120–124
HeLa cells, 121, 123–124
heterogenous cytoskeleton, 119–120, 121, 122
lens cells, 118, 120–121, 122
species specificity, 121, 123, 124
intermediate filament, 118
keratin, 85–107
differentiation and, 70–73, 87, 85–107
vitamin A and, 100–103
tubulin, 203–225, 227–256
autoregulatory control, 204–213

Gene expression (*cont.*)
tubulin (*cont.*)
in brain, 227–256
during cell cycle, 220–222
in *Chlamydomonas*, 214–218, 221–222
in cloned transfected tubulin genes, 211–213
colchicine effects, 204–206, 213
cytoplasmic events, 210–211
DNA sequences, 218
during flagellar growth, 213–220
interferon induction, 213
isoform(s), 228–234
isoform localization, 229–231
in *Leishmania*, 219–220
molecular levels, 207–213
in *Naegleria*, 219
nocodazole effects, 206
RNA sequestration/modification, 208–209, 220
mRNA, 208–209, 213, 214–218, 220–222
transcriptional control, 209–210, 214–217, 219
unpolymerized subunits, 204–207, 211
Gene families
evolution, 157
multigene
actin, 161
keratin, 115
tubulin, 290–292
Genetic drift, 157–158
Glial fibrillary acidic protein
characteristics, 111, 112
headpieces, 58, 60
single-copy, 115
structure, 43, 44, 46, 49, 50–51, 58, 60, 62, 63
homology, 111, 114
subunits, 72
tailpieces, 60–61
carboxyterminal appendages, 62–63
Glycosylation, of tubulin, 207
Griseofulvin, 309
Growth, cellular, actin regulation, 131–149
cytoskeleton organization, 133–135
growth factors and, 136–138, 139, 143–144
protooncogene, 141–143
mRNA, 135–136, 137, 138, 141
Growth factors
in actin cell growth/differentiation regulation, 136–138, 139, 143–144
binding, 143–144
epithelial, 86, 100
Guanosine triphosphatase, in tubulin, 259, 268

Hair, keratin, 80–81, 93–94
HeLa cells, desmin gene expression, 121, 123–124
Herbicides, antimicrotubule, 262, 293, 300–304
Hybridization analysis
actin genes, 158, 160–161, 162
tubulin mRNA, 235–237, 240–245
Hydrocortisone, 100

Immunoblotting, tubulin, 269
Immunoglobulin assay, tubulin, 260
Insulin, epithelial cell growth and, 100
Intermediate filament(s)
biochemical properties, 41–42, 109–110
classes, 27
colchicine effects, 109
copolymerization, 28
definition, 69
diameter, 109
discovery, 109–110
hydrophobic residues, 89
isolation, 110
microinjection studies, 27–30, 31
antibodies, 27–29
coated vesicles, 29–30
mRNA, 29
polymerization, 110, 114
subclasses, 69
subunits
characteristics, 111, 112
diversity, 110–114
model, 72–73
secondary structure, 69–70
sequence types, 72, 73
Intermediate filament gene(s), 114–127
evolution, 75–81, 115, 118
ancestral gene, 76–77
coiled-coil domain, 75–76
end domain, 78–81
primordial gene, 75–76
rod domain, 75–78
type-specific progenitor, 76, 77–78
structure, 73–75, 115–118
exons, 74–75
introns, 74, 75–76, 77–78, 113, 115
Intermediate filament gene expression, 118
Intermediate filament proteins, 41–68
amino acid sequences, 113, 114
coiled-coils, 42–43, 44, 45–46, 48–49
as DNA-binding protein, 60
domains, 42–46, 111, 114

Intermediate filament proteins (*cont.*)
homology, 114
invertebrate, 51
model, 111, 113, 114
neurofilaments, 44, 46–48
sequence organization, 49–51
protofilaments, 53–58
purification, 110–111
rod domain, 42–44, 48–53
nonepithelial protein comparison, 49–51
sequence organization, 48–49
structural characteristics, 51–53
subclasses, 41
terminal domains, 58–63
headpieces, 58–60
tailpieces, 60–63
tissue specificity, 111
Interferon, 213
Introns
actin genes, 152, 154, 157
intermediate filament genes, 74, 75–76, 77–78, 113, 115
keratin genes, 97, 98, 99–100, 118
keratin type I genes, 77–78
keratin type II genes, 78
Invertebrates, intermediate filament proteins, 51

Keratin
amino acid sequences, 69, 71–72, 73, 86–88, 90, 91, 93
antibodies, 27–29
antisera, 71, 73, 89
characteristics, 112
domain organization, 44–45, 46
filament assembly, 92–93
headpieces, 60
heteropolymer, 114
homology, 111
hydrophobic residues, 89, 91
macrofibrils, 93–94, 95
nonepidermal, 80–81, 93–94
number, 41
properties, 93–94
protofilaments, 53–58, 92
prototypes, 49
mRNA, 86
rod domain, 52
of stratum corneum, 86
structure, 86–94
DNA sequences, 60
model, 92–93
nonhelical ends, 91, 92, 93
subclasses, 114–115
subdomains, 73, 78–80, 81
Keratin (*cont.*)
subunits, 72, 73
differential expression, 71–73
primary sequences, 69
tailpieces, 63
Keratin gene(s)
cDNA clone probes, 70–71, 94, 96, 97, 114–115
enhancer sequences, 101, 102
exon(s), 97, 98, 99
exon/intron patterns, 118
homology, 97–99
intermediate filament gene relationship, 99–100
introns, 97, 98, 99–100, 118
multifamily, 115
mutagenesis, site-directed, 101, 102–103
number, 94
structure, 74, 97–100
subfamilies, 94, 96–97
Keratin gene expression, 85–107
differential, 70–73, 85–107
encoding genes, 94–97
epidermal keratin structure, 86–94
gene structure relationships, 97–100
vitamin A and, 100–103
Keratin type I
characteristics, 86
end domain, 78–81
helical domain, 88–93
homology, 111
as keratin prototype, 49
structure, 72, 73
Keratin type I gene(s)
introns, 77–78
structure, 74, 97–99
Keratin type I gene expression, 100–102
Keratin type II
characteristics, 86–87
end domains, 78–81
helical domain, 88–93
homology, 111
subdomains, 73
Keratin type II gene(s)
introns, 78
linkage, 78
structure, 74, 97–99
Keratin type II gene expression, 100–102
Keratin type III, 73
Keratin type III gene, 99
Kinetochore, polarity, 187, 188, 189, 190–191, 193

Lampbrush chromosomes, actin, 21, 22, 145
Leishmania, tubulin synthesis, 219–220

Lens cells, desmin gene expression, 118, 120–121, 122
Lumicolchicine, tubulin-binding, 297, 299

MAP, *see* Microtubule-associated proteins
Mebendazole, 304, 305–306
Meiotic recombination, 157
Melanophore, microtubule polarity, 194–195
Melatonin, 309
Membrane-associated tubulin, 292–293
Methyl benzimidazol-2-yl carbamate, 304, 305, 306
Microfilament(s)
  microinjection studies, 9–26
    actin-associated protein antibodies, 20–26
    actin-associated protein dynamics, 13–19
    actin dynamics, 9–13
    microfilament reorganization, 14–19
    phalloidin, 19–20
Microinjection, of cytoskeletal elements, 1–40
  intermediate filaments, 27–30, 31
    antibodies, 27–29
    coated vesicles, 29–30
    mRNA, 29
  microfilaments, 9–26
    actin-associated protein antibodies, 20–26
    actin-associated protein dynamics, 13–19
    actin dynamics, 9–13
    microfilament reorganization, 14–19
    phalloidin, 19–20
  microtubules, 2–9
    antitubulins, 6–9
    microtubule dynamics, 3–5
    microtubule-bound organelle transport, 5–6
    tubulin/microtubule equilibrium, 2–3
  microtubule-associated proteins, 3–5
  tubulin, 2–7, 208
Microtubule(s)
  assembly
    into axonemes, 183
    in vivo, 2
  classes, 187
  components, 258
  definition, 258
  depolymerization
    DNA synthesis and, 134
    tubulin synthesis and, 2–3
  diameter, 109, 179
  dimer organization, 180–181
  functions, 203, 258
  microinjection studies, 2–9
    antibodies, 6–9
Microtubule(s) (*cont.*)
  microinjection studies (*cont.*)
    microtubular dynamics, 3–5
    microtubule-bound organelle transport, 5–6
    tubulin/microtubule equilibrium, 2–3
  polarity, 179–202
    determination methods, 179–187
    functions, 180, 182
    intracellular motility and, 193–196
    kinetochores, 187, 188, 189, 190–191, 193
    microtubule-organizing centers and, 196
    in mitosis, 187–193
    nonkinetochore, 187, 191–193
    spindle function and, 187–193
  polymerization, calcium and, 3
  polymerization-depolymerization, in tubulin isolation, 263–265
  in prokaryotes, 258
  structure, 179–181
  subunits, 179–180
Microtubule-associated proteins
  microinjection, 3–5
  microtubule assembly and, 2
  in tubulin isolation, 264
Microtubule-organizing centers, 196
Mitosis
  colchicine effects, 294–295
  microtubule polarity and, 187–193
Multigene families
  actin, 161
  keratin, 115
  tubulin, 290–292
Mutation
  actin genes, 151–152, 165–168
    revertants, 167–175
  keratin genes, 101, 102–103
Myelin, tubulin-associated, 230
Myosin
  antiactin effects, 22–24
  diameter, 109
myo-3 gene, 153

Naegleria, tubulin gene expression in, 219
Neuroblastoma cell
  tubulin phosphorylation, 231–232
  β-tubulin mRNA, 237–238
Neurofilament(s)
  characteristics, 112
  components, 46
Neurofilament proteins
  domains, 44
  headpieces, 58
  M, 47, 48, 49–50

Neurofilament proteins (*cont.*)
molecular weight estimation, 62
sequence organization, 49–51
structural models, 46–48
tailpieces, 61–62
carboxyterminal appendages, 63
Nocodazole, 206, 304, 305, 306
Nuclear matrix, 145
Nuclease protection assay, of tubulin transfected genes, 211, 212
Nucleus, actin, 12–13, 144–145
antiactin effects, 21

Oncogenes, 142
proto-, 141–143
Organelle(s), *see also* specific organelles
tubulin-rich, 265, 267–268
Organelle transport
microtubule-bound, 5–6
microtubule polarity and, 193–196

Parbendazole, 304, 305, 306
Paramyosin, 152
Pellicular membrane, tubulin, 267–268
Peptide mapping, tubulin, 279–282, 286
Phalloidin, 19–20
Phosphoric amide herbicides, 300–301, 303, 304
Phosphorylation, tubulin, 231–232, 288–289
Phragmoplast, 187
Physarum, tubulin synthesis, 220–221
Pigment transport, microtubule polarity and, 194–195
Plant(s), tubulin
antimicrotubule drug effects, 293–309
dimer structure, 271–293
genes, 291–292
identification/isolation, 257, 258–271
Plant alkaloids, antimicrotubule, 293
Plasma membrane, spectrin/vimentin association, 25
Podophyllotoxin
antimicrotubule, 309
tubulin-binding, 297, 298, 299–300
Polarity
actin, 179
microtubules, 179–202
determination methods, 179–187
intracellular motility and, 193–196
kinetochores, 187, 188, 189, 190–191, 193
microtubule-organizing centers and, 196
in mitosis, 187–193
nonkinetochore, 187, 191–193
spindle function and, 187–193
Polymorphism, actin genes, 152
Prokaryote
macrotubules, 258
tubulinlike proteins, 258
Protein(s), *see also* specific proteins
synthesis, cytoskeletal, 1
transcription, actin in, 21
Protista, tubulin
antimicrotubule drug effects, 293–309
dimer structure, 271–293
identification/isolation, 257, 258–271
Protofilament, 53–58, 92
Protooncogene, actin as, 141–143
Pseudopod, actomyosin contraction, 10

Ribonucleic acid, tubulin, 209–210, 214–217
mRNA
actin, 135–136, 137, 138, 141
intermediate filaments, 118
microinjection, 29
keratin, 70–71, 86
tubulin, 138–139, 141
expression in brain, 235–245, 246–250
interferon effects, 213
synthesis regulation, 208–209, 214–218, 220–222
Rod domains
intermediate filaments, 42–44, 48–53, 75–78
keratin, 52
vimentin, 52

Saltatory particle movement, microtubule polarity and, 193–196
Sea urchin
actin genes, 153, 155
tubulin synthesis, 214
Secondary products, in tubulin isolation, 269–270
Serum factors, epithelial cell growth and, 100
Spectrin, 24–25
Spindle
diagram, 187
immunogold staining, 284
microtubule polarity and, 187–193
st 15 gene, 166
st 119 gene, 166
st 120 gene, 166
Stratum corneum, keratin, 86
Stress fibers
actin and, 14–19
depolymerizing factor effects, 14–17
assembly, 12
sup-3 locus, 153
Synaptosome, tubulin, 230–231

Taxol, 306–309
Thiabendazole, 304
Thyroid hormone, 233–234
Transcription
  actin gene, 158–163
    in mutant revertants, 169–175
  tubulin genes, 209–210, 214–217, 219
Transfection, tubulin genes, 211–213
Transformation, neoplastic, 142–143
Tropomyosin, 18
Tubulin
  acetylation, 289–290
  antibodies, 6–9, 260
    proteolytic inhibition and, 269
  antimicrotubule drug effects, 293–309
    benzimidazole, 304–306
    caffeine, 309
    colchicine, 293, 294–300
    griseofulvin, 309
    herbicides, 300–304
    melatonin, 309
    podophyllotoxin, 309
    taxol, 306–309
    vinblastine, 309
  axon transport, 232
  cytoplasmic, 264–265
  dimer structure, 179–180, 258, 271–293
    amino acid sequences, 271–272
    immunology, 282–284, 285, 286
    membrane-associated, 292–293
    multiple tubulin genes, 290–292
    peptide mapping, 279–282, 286
    posttranslational modifications, 287–290
    subunit electrophoretic resolution, 275–279
    subunit evolution, 272–274
    subunit microheterogeneity, 284–287
  enzymatic activity, 259
  glycosylation, 287
  identification/isolation, 258–271
    antimicrotubule drugs and, 269–270
    assays, 259–260
    browning reaction, 270
    copolymerization, 265
    criteria, 260
    drug affinity methods, 262–263
    exchange chromatography, 260–263
    immunoblotting, 269
    polymerization-depolymerization, 263–265
    secondary products and, 269–270
    starting material, 270–271
    technical problems, 268–271
    from tubulin-rich organelles, 265, 267–268
Tubulin (*cont.*)
  microtubule depolymerization and, 2–3
  microtubule equilibrium and, 2–3
  as microtubule polarity indicator, 182, 185–186, 187
  multitubulin hypothesis, 286
  nonanimal, 257–329
    future research areas, 310–311
  phosphorylation, 288–289
  RNA transcription, 209–210, 214–217
  mRNA, 138–139, 141
    interferon effects, 213
    synthesis regulation, 208–209, 214–218, 220–222
  structural divergence, 257
  tyrosinolation, 231, 287–288
Tubulin gene(s)
  in higher eukaryotes, 234–235
  multiple, 290–292
  transfected, 211–213
Tubulin gene expression, 203–225, 227–256
  autoregulatory control, 204–213
  in brain, 227–256
    cytoplasmic versus membrane-bound, 230–231
    developmental regulation, 232–234
    genes, 234–235
    hybridization analysis, 235–237, 240–245
    isoform(s), 228–234
    isoform localization, 229–234
    isoform microheterogeneity, 228–229, 231, 233
    myelin-associated, 230
    posttranslational modifications, 231–232
    mRNA, 235–245
    subcellular distribution, 229–230
    translational products, 235, 237–240, 241
  during cell cycle, 220–222
  in *Chlamydomonas*, 214–218
  in cloned transfected genes, 211–213
  colchicine effects, 204–206, 213
  cytoplasmic events, 210–211
  DNA sequences, 218
  during flagellar growth, 213–220
  interferon induction, 213
  isoform(s), 228–231
  isoform localization, 229–231
  in *Leishmania*, 219–220
  molecular levels, 207–213
  in *Naegleria*, 219
  nocodazole effects, 206
  RNA sequestration/modification, 208–209, 220
  mRNA, 208–209, 213, 214–218, 220–222

Tubulin gene expression (*cont.*)
transcriptional control, 209–210, 214–217, 219
unpolymerized subunits, 204–207, 211
Tubulinlike protein, 258
Tubulin-tyrosine ligase, 287, 288
α-Tubulin
in brain, 229, 230–231, 212–233, 234, 235–237, 245
differential localization, 229, 230–231, 232–233
genes, 234
isoforms, 228, 230–231, 232–233
mRNA, 235–237
β-Tubulin
in brain, 228, 229, 230, 231, 232–233, 234–235, 237–245, 249–250
differential localization, 229, 230, 231, 232, 233
genes, 234–235
isoforms, 228, 229, 230, 231, 232–233
mRNA, 237–245, 249–250
hybridization analysis, 240–245
translation products, 237–240, 241
Tyrosinolation, of tubulin, 231, 287–288

unc-15 I gene, 152
unc-54 I gene, 152, 153

v-fgr oncogene, 142
Villin, microinjection, 17
Vimentin
aggregation, 121, 123
antibody, 27
desmin colocalization, 121, 123, 124
cDNA probe, 115
headpieces, 58, 59
intermediate filament copolymerization, 28
rod domain, 52
sequence organization, 49, 50, 51
spectrin/plasma membrane association, 25
structural homology, 111, 114
structural model, 113
subunits, 72
tailpieces, 60–61
carboxyterminal appendages, 62–63
Vimentin genes
chromosomal localization, 125, 126
single-copy, 115
structure, 73–74, 115, 116, 117, 118, 119
Vinblastine
antimicrotubule, 206, 309
tubulin precipitation with, 262–263
tubulin synthesis effects, 206
Vinculin, 24, 25, 26
Vitamin A
epithelial cell differentiation and, 86, 100
keratin gene expression and, 100–103

Wool, keratin, 93–94