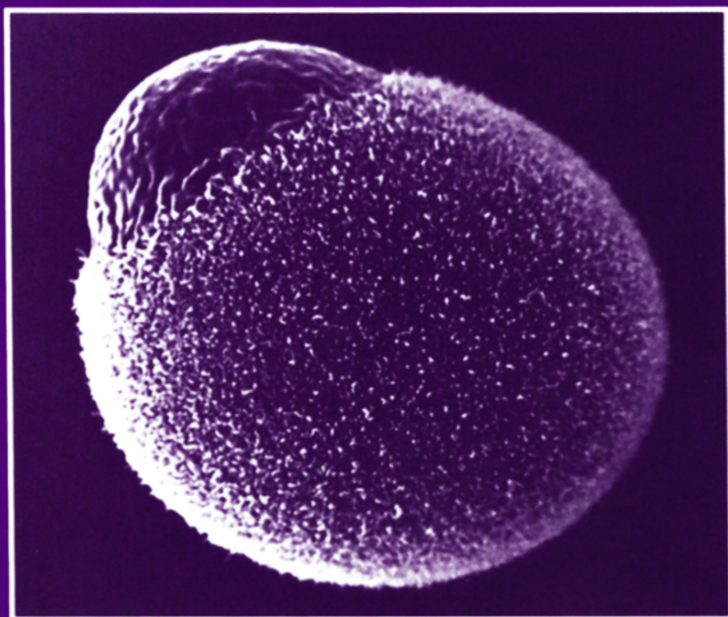
INTERNATIONAL REVIEW OF CYTOLOGY

A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



Volume 177

ACADEMIC PRESS

International Review of
Cytology

A Survey of
Cell Biology

VOLUME 177

SERIES EDITORS

Geoffrey H. Bourne	1949–1988
James F. Danielli	1949–1984
Kwang W. Jeon	1967–
Martin Friedlander	1984–1992
Jonathan Jarvik	1993–1995

EDITORIAL ADVISORY BOARD

Aimee Bakken
Eve Ida Barak
Rosa Beddington
Howard A. Bern
Robert A. Bloodgood
Dean Bok
Stanley Cohen
Rene Couteaux
Marie A. DiBerardino
Charles J. Flickinger
Nicholas Gillham
Elizabeth D. Hay
P. Mark Hogarth
Anthony P. Mahowald
M. Melkonian
Keith E. Mostov
Andreas Oksche
Vladimir R. Pantić
Thomas D. Pollard
L. Evans Roth
Jozef St. Schell
Manfred Schliwa
Hiroh Shibaoka
Wilfred D. Stein
Ralph M. Steinman
M. Tazawa
Yoshio Watanabe
Donald P. Weeks
Robin Wright
Alexander L. Yudin

International Review of Cytology

A Survey of Cell Biology

Edited by

Kwang W. Jeon
Department of Biochemistry
University of Tennessee
Knoxville, Tennessee

VOLUME 177

ACADEMIC PRESS
San Diego London Boston New York Sydney Tokyo Toronto

Front cover photograph: Scanning electron micrograph of an unfertilized mouse egg. (See Ch. 2, Fig. 7A for more details.)

This book is printed on acid-free paper. ♾

Copyright © 1998 by ACADEMIC PRESS

All Rights Reserved.
No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the Publisher.
The appearance of the code at the bottom of the first page of a chapter in this book indicates the Publisher's consent that copies of the chapter may be made for personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per copy fee through the Copyright Clearance Center, Inc. (222 Rosewood Drive, Danvers, Massachusetts 01923), for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. Copy fees for pre-1998 chapters are as shown on the title pages. If no fee code appears on the title page, the copy fee is the same as for current chapters.
0074-7696/98 $25.00

Academic Press
a division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.apnet.com

Academic Press Limited
24-28 Oval Road, London NW1 7DX, UK
http://www.hbuk.co.uk/ap/

International Standard Book Number: 0-12-364581-6

PRINTED IN THE UNITED STATES OF AMERICA
97 98 99 00 01 02 EB 9 8 7 6 5 4 3 2 1

CONTENTS

Contributors ix

The Cellular Basis of Tumor Progression

Gloria H. Heppner and Fred R. Miller

I. Introduction 1
II. Tumor Progression 2
III. Cellular Heterogeneity as the Basis of Tumor Progression 7
IV. Consequences of Tumor Heterogeneity and Progression 32
V. Concluding Remarks 36
References 39

Molecules Involved in Mammalian Sperm–Egg Interaction

S. B. McLeskey, C. Dowds, R. Carballada, R. R. White, and P. M. Saling

I. Introduction 57
II. The Zona Pellucida and Its Interaction with Sperm 62
III. The Egg Plasma Membrane and Its Interaction with Sperm 85
IV. Concluding Remarks 97
References 98

Coordination of Nuclear and Chloroplast Gene Expression in Plant Cells

Michel Goldschmidt-Clermont

I. Introduction 115
II. Classical Genetics of Nuclear–Plastid Interactions 122
III. Plastid Development 128
IV. Nuclear Control of Plastid Gene Expression 135
V. Signaling between the Plastid and the Nucleocytoplasm 160
VI. Concluding Remarks 163
References 165

Signaling in Unicellular Eukaryotes

Søren T. Christensen, Vagn Leick, Leif Rasmussen, and Denys N. Wheatley

I. Introduction 181
II. Signaling Systems in Unicellular Eukaryotes 189
III. Signal Molecules in Cell Survival and Proliferation in *Tetrahymena* 196
IV. Signal Molecules in Chemosensory Behavior in *Tetrahymena* 219
V. Control of Cell Proliferation in *S. cerevisiae* 224
VI. Signaling Systems in Prokaryotes Compared with Those in Eukaryotes 229
VII. Concluding Remarks 231
References 232

Action of SO_2 on Plants and Metabolic Detoxification of SO_2

Ulrich Heber and Katja Hüve

I. Introduction 255
II. Emissions and Immissions 258
III. Deposition 261
IV. Toxicity of SO_2 and Its Hydration Products 265

V. Metabolic Detoxification of SO_2 and Its Hydration Products 271
VI. Effects of SO_2 275
VII. Conclusions 279
References 280

Index 287

This Page Intentionally Left Blank

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions appear.

Rosa Carballada (57), *Institut de Biologie du Developpement de Marseille, 13288 Marseille Cedex 9, France*

Søren T. Christensen (181), *Department of Medical Biochemistry and Genetics, University of Copenhagen, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark*

Carl A. Dowds (57), *Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710*

Michel Goldschmidt-Clermont (115), *Departments of Molecular Biology and Plant Biology, University of Geneva, Sciences II, CH-1211 Geneva 4, Switzerland*

Ulrich Heber (255), *Julius-von-Sachs-Institute of Bioscience, University of Würzburg, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany*

Gloria H. Heppner (1), *Karmanos Cancer Institute, Detroit, Michigan 48201*

Katja Hüve (255), *Julius-von-Sachs-Institute of Bioscience, University of Würzburg, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany*

Vagn Leick (181), *Department of Medical Biochemistry and Genetics, University of Copenhagen, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark*

Susan B. McLeskey (57), *Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710*

Fred R. Miller (1), *Karmanos Cancer Institute, Detroit, Michigan 48201*

Leif Rasmussen (181), *Department of Anatomy and Cell Biology, Institute for Medical Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark*

Patricia M. Saling (57), *Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710*

Denys N. Wheatley (181), *Cell Pathology Unit, Department of Pathology, University Medical School, Aberdeen AB25 2ZD, United Kingdom*

Rebekah R. White (57), *Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710*

The Cellular Basis of Tumor Progression

Gloria H. Heppner and Fred R. Miller
Karmanos Cancer Institute, Detroit, Michigan 48201

Variability in disease presentation and course is a hallmark of cancer. Variability is seen among similarly diagnosed cancers in different patients or animal hosts and in the same cancer at different periods of time. This latter type of variability, termed "tumor progression," was defined by Foulds in a series of six rules that describe the independent behavior of individual cancers and the independent evolution of different cancer characteristics. Tumor progression is believed to result from variability among subpopulations of tumor cells within individual cancers and from selection of these subpopulations by conditions within the cancer environment, such that different subpopulations come to prominence over the course of cancer development and growth. Interactions among subpopulations, however, modulate tumor behavior as well as tumor evolution. The leading hypothesis for the origin of tumor subpopulations is the genetic instability of cancer cells. There are a number of possible mechanisms of genetic instability, some internal to cancer cells (mutation, amplification, mutator phenotypes, DNA repair deficiencies) and some present in the tumor microenvironment (endogenous mutagens). There are also potential epigenetic mechanisms of variability, including alterations in gene regulation, differentiation, adaptation, and cell fusion. Regardless of mechanism, the heterogeneity of tumor subpopulations poses a number of challenges to the practice of cancer research, including the design of reproducible and meaningful experiments. Tumor heterogeneity also has significant consequences for the clinical assessment of tumor prognosis and the development of effective treatment regimens.

I. Introduction

Cancer researchers are frequently asked the questions "With so much money being poured into cancer research, why hasn't there been more

0074-7696/98 $25.00
Copyright © 1998 by Academic Press.
All rights of reproduction in any form reserved.

progress; why hasn't cancer been cured?" Our usual response is to point to the successes to date—childhood leukemia and Hodgkin's Disease, a very short list—and to mumble something about the complexity of the problem and the probable lack of a "magic bullet." No wonder the public is skeptical! Just what is it that makes cancer so complex that the combined efforts of thousands of scientists and many millions of dollars cannot solve its mysteries? The answer to this question is not obvious to the general public and probably not all that clear to the professional cell and molecular biologists who make up the readership of the *International Review of Cytology*. But the answer is not hidden or unique to cancer. It involves two of the most fundamental properties of life, namely, the proclivity of organisms, including cells, to undergo change and the capacity of the environment to utilize that change to mold a new, evolving population. In short, cancer exhibits in a dramatic and immediate way, the same hallmarks as does life in general, variation and selection and the interplay between the two that leads to overall survival. The fact that in the case of cancer the forces that lead to the survival of the neoplastic population, if unchecked, ultimately destroy its environment, its host, should not be surprising, given the many similar examples in the evolution of free-living organisms. However, the public, as well as researchers and oncologists, generally confronts cancer on a less cosmic scale, in particular experimental systems and individual patients. At this level the forces of evolution are manifested as unwanted, and oftentimes unexpected, variability—inconsistency in experimental results and patient outcome. The variability in the "trees" makes up the complexity of the "forest." The purposes of this review are to describe the types and mechanisms of variability in cancer, to show how they result in the phenomenon known as "tumor progression," and hopefully to enhance the reader's appreciation of the complexity of the neoplastic process.

II. Tumor Progression

A. Variability in Tumor Behavior

1. Among Patients

One of the most fascinating experiences for a cancer experimentalist is to attend a "multidisciplinary" clinical conference in a large-practice cancer center and to hear the presentation of the week's "cases" and the discussion by the various physicians—surgeons, pathologists, diagnostic radiologists, and radiation and medical oncologists—on the best course of action for treatment. What soon becomes apparent is that individual cases which

nominally carry the same diagnosis, for example, infiltrating ductal carcinoma of the breast, are anything but routine. The physicians will weigh every bit of information they have on each patient—age, family history, previous and current medical history, extent of cancer spread (local, to lymph nodes, in distant organs), and pathological features, the expression of tumor "markers", etc.—and then arrive at a consensus decision couched in statistical predictions—recurrence occurs 10% of the time in this type of patient, or 5-year survival is 45%, and so on. What eventually becomes clear is that no two patients are alike and that disease outcome cannot be predicted with certainty. The apparently most hopeless case may achieve a complete cure; the less problematic one may turn out to be catastrophic. This extensive interpatient variability is at the heart of medical decision-making and is the major reason why clinical trials of new treatments require such large numbers of patients and such long times to complete. Indeed, even in large cancer centers there are seldom enough of a particular type of patient to carry out a definitive study, necessitating the organization of multiinstitutional cooperative trials to make even a small advance. Thus, the first "type" of variability that must be appreciated is among individual cancer patients and includes both patient variables and differences among their neoplasms.

2. Over Time

The second type of variability likewise contributes to the difficulty in making treatment decisions because it deals with the possibility of change over time. A patient's breast cancer may present with the molecular markers, estrogen and progesterone receptors (ER, PR), that predict for sensitivity to hormone-based therapy, nowadays most likely with tamoxifen. Following surgery to remove the primary cancer, the patient may be placed on a regimen of tamoxifen. Fortunately, at least half of such patients will respond to the treatment and many will have no further difficulties. However, for some patients the cancer may eventually recur and it will be found to no longer express ER or PR and no longer respond to tamoxifen. Over time, a hormone-responsive cancer has evolved into a nonresponsive one. Similarly, a cancer that was initially responsive to a particular set of chemotherapeutic drugs may evolve into a resistant cancer. A cancer that on primary diagnosis appeared to be relatively well-differentiated and contained may reappear as an aneuploid, highly aggressive malignancy. In short, the basic characteristics of an individual cancer may become fundamentally different over a period of time such that the original treatment plan may no longer be appropriate and the patient's prognosis has become drastically changed. This ability of individual neoplasms to change over time is called "tumor progression."

B. The Concept of Tumor Progression

1. Foulds' Definition

The formulation of the concept of tumor progression was the seminal contribution of Leslie Foulds whose encyclopedic knowledge of the behavior of both experimental and clinical cancers allowed him to perceive the underlying generalizations masked by the otherwise overwhelming variation, to define the forest as distinct from the trees. Foulds' concepts and their documentation are contained in a brilliant two-volume work entitled *Neoplastic Development* (Foulds, 1969, 1975). These volumes, no longer in print, represent one of the most profound syntheses in cancer research. Foulds defined progression as the "acquisition of permanent, irreversible qualitative changes of one or more characteristics in a neoplasm." It is important to emphasize that progression deals with qualitative changes in fundamental characteristics, not just the extension of a cancer in time or space, for which the term progression is often misapplied. The concept of progression also deals with changes in a cancer, not with changes in cancer cells—it is a tissue, not a cellular phenomenon. Although Foulds included the word "irreversible" in the definition, this may not be literally true since sometimes cancers may undergo spontaneous regression (which is a special, unfortunately infrequent case of progression) or may be induced to regress by, for example, noncytotoxic, differentiating agents or embryonic fields (Pierce *et al.,* 1984). These exceptions, however, do not disprove the rule. Finally, the term progression can be applied over the entire course of neoplastic change, from seemingly normal, but "initiated" cells to advanced, metastatic disease.

2. Foulds' Rules

The intellectual strength of Foulds' concept of tumor progression lies in a series of six "rules" which he extracted from his detailed reading of the clinical and experimental literature. Although a great deal has been learned about cancer, particularly at the molecular level, since Foulds' time, most, if not all, of that knowledge has provided more examples of its truth, without shaking the foundations of his concept. The rules are as useful as when they were first formulated.

Rule 1 states that "progression occurs independently in different tumors in the same host." Multiple tumors are quite common in animal cancer models, particularly those of viral or germline origin. Multiple simultaneous neoplasms, for example, carcinoma *in situ* and invasive carcinoma in the breast (Teixera *et al.,* 1994, 1996; Fujii *et al.,* 1996), multiple polyps and cancer in the colon (Fucci *et al,* 1994; Fante *et al.,* 1996), or second or even

more cancers of the same or different histotype as these or other cancers (Czarneki *et al.,* 1994; Azizi *et al.,* 1995; Yokoyama *et al.,* 1996), are also frequent in humans. Rule 1 addresses the independence of the natural history of these lesions. Thus, although multiple independent foci of carcinoma *in situ* may occur in the same patient, in the same or different breasts, they do not all have the same probability of progression to invasive cancers, nor do the cancers that may occur necessarily share similar sensitivities to treatment or proclivities for metastatic spread.

Rule 2 is "progression occurs independently in different characters in the same tumor." A great deal of effort has gone, and is still going, into the identification of "prognostic indicators," pathological, cellular, and molecular markers that will allow a physician to predict whether an individual cancer will follow a relatively benign course and be controlled by moderate therapy or whether it will prove to be highly malignant and require correspondingly aggressive treatment. Much experimental ingenuity and statistical analysis has been used to specify independent markers of risk or to construct multivariate indices of prognosis. Without denying the usefulness of these approaches as general guidelines, it is clear that there are no absolute indicators. Cancer development and progression are multifactorial processes. The expression of any one characteristic is not a guarantee of the eventual expression of any other.

Rule 3, "progression is independent of growth," with its corollaries "at its first clinical manifestation a tumor may be at any stage of progression" and "progression is independent of the size or clinical duration of a tumor," appears to be counterintuitive: after all, isn't the basis of cancer "uncontrolled growth?" Discussion of this assumption, one of the most common misconceptions in cancer research, is beyond the scope of this review. However, for an illustration of the validity of Rule 3, it is only necessary to consider "cancers of unknown origin," a not uncommon diagnosis in which the cancer presents as widely disseminated disease without an obvious primary, even at extensive autopsy (Mackay and Ordonez, 1993; Abbruzzese *et al.,* 1994).

Rule 4, "progression is continuous or discontinuous, by gradual change or by abrupt steps," refers to the sometimes startling rapidity with which a cancer may appear to "take off" (although it may also be a consequence of how frequently the neoplasm is being monitored).

Rule 5, "progression follows one of alternate paths of development," is related to Rule 1 and is most meaningful when applied to the relationship between preneoplastic lesions, whether histological or molecular, and the eventual development of overt cancer. Progression *can* follow a particular sequence of events, but it does not have to.

Rule 6 particularly applies to experimental work: "progression does not always reach an end point within the lifetime of a host." Cancers that have

been propagated for long times, either in experimental animals or in cell culture, are still subject to change and the rules of progression.

C. Examples of Progression and Foulds' Rules

As mentioned above, tumor progression can be seen in a wide range of biologically and clinically significant cancer characteristics: growth rate, sensitivity to endogenous (hormones, growth factors) and exogenous growth regulators or cytotoxic agents (antihormone drugs, chemo- and radiation therapy), as well as in the development of metastases. The literature on this topic is extensive and only a few illustrative examples can be presented here.

Foulds (1969) carried out an extensive analysis of the progression of pregnancy-dependent mouse mammary tumors. These tumors arise during pregnancy and then regress after weaning. Foulds followed a number of such tumors that had arisen in different mammary glands of a single mouse. Over the course of several pregnancies the tumors grew and regressed until one of them did not regress but continued to grow in the absence of hormone stimulation (see Rule 1).

Some years ago our laboratory (Hager *et al.*, 1978) measured a number of behavioral characteristics (latency period, growth rate, invasiveness, and metastasis) in seven, independently arising mouse mammary tumors serially passaged for up to 10 generations. In general the data illustrated progression of these characteristics over time and, in addition, provided further examples of the validity of Foulds' rules, in particular the independent evolution of the characteristics measured, including enhanced growth rate versus ability to metastasize (Rule 2). However, our tumors also demonstrated considerable fluctuation in progression from 1 serial passage generation to the next. For example, one tumor became metastatic, lost the capacity, regained it, and lost it again over successive passages, suggesting that the course of tumor evolution need not be inexorable but may be subject to control mechanisms, a theme which will be discussed more fully below.

Rubin (1994b) utilized a cell culture system to address the validity of Rules 3, 4, and 5. Rubin's system consists of a subline of NIH 3T3 cells that undergo progression, manifested as foci of increasing population density, when subjected to sequential rounds of growth to and maintenance of confluence. Interestingly, progression was favored by constraint of proliferation (see Rule 3). Furthermore, although the appearance of dense foci was usually gradual, they sometimes appeared very quickly, at a rate consonant with one-step transformation (Rule 4). The system also gave evidence, by the unique morphology of the dense foci from independent lineages, of

the validity of Rule 5, that progression follows alternate paths of development.

A very active area of current research is the identification of molecular genetic and cytogenetic correlates of tumor progression and the relationship of these to histopathological lesions that cover the progression spectrum of normal to dysplastic to preneoplastic to *in situ* and finally invasive cancers. The underlying premise is that the cause of progression is genetic (see below) and in particular involves alterations in key oncogenes and suppressor genes. This scheme of genetic progression, originally articulated for colon cancer by Fearon and Vogelstein (1990), also illustrates some of Foulds' rules: for example, although it is possible to discern an order of genetic alterations in the progression to malignancy, the order is not necessarily the same in each tumor (Rules 1, 2, and 4). For a recent, thoughtful application of the Fearon and Vogelstein scheme to head and neck cancer, the reader is referred to Califano *et al.* (1996).

In addition to progression occurring within sites of primary tumor development, Foulds' Rule 6 addresses the issue of progression beyond the life of the host. This rule has long been appreciated by experimentalists who have used serial transplantation as a method to develop animal models of aggressive and metastatic tumor growth (Hauschka, 1953). A corollary to this rule is that progression does not end within primary cancers but can also be seen within individual metastatic lesions from the same primary cancer, which may continue to evolve new properties, including the tendency to metastasize to new sites (Poste *et al.,* 1982) and different levels of sensitivity and resistance to chemotherapeutic drugs (Talmadge *et al.,* 1984).

III. Cellular Heterogeneity as the Basis of Tumor Progression

A. Nowell Hypothesis

The underlying mechanisms of tumor progression had been a subject of conjecture and debate before Foulds so elegantly defined the phenomenon and enunciated his rules. Parallels were noted with the emergence of drug and phage resistance in bacteria and similar debate occurred on whether what appeared to be a wholesale change in population phenotype was due to induction of new behavior by an environmental factor or was evidence of the emergence of variants, preexisting within the original population, which had been selected to dominance through changing environmental conditions (Hauschka, 1953). Indeed, Law (1952) adapted the Luria and Delbrück (1943) fluctuation test to demonstrate that emergence of A-

methopterin resistance in leukemia cells was due to selection of preexisting, resistant mutants.

In 1976 a very influential paper appeared in *Science* that clearly and succinctly focused on the cellular basis of tumor progression (Nowell, 1976). In this paper Nowell hypothesized that "tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines." A particular feature of Nowell's concept was the contention that a fundamental property of cancer cells, as opposed to normal cells, was heightened genetic instability and that instability may become more pronounced as neoplasms evolve. Genetic instability, which could be due to a variety of mechanisms, was postulated to result in the production of clonal variants, some of which have a selective advantage in their environmental milieu and hence come to be dominant forces in the growing cancer such that their behavioral characteristics determine the behavior of the entire neoplasm.

In order for the Nowell hypothesis to have any validity, cancers must be heterogenous at the cellular level. At some time during the evolution of a single neoplasm, it must be possible to demonstrate coexisting tumor cell subpopulations that differ in the type of behavioral properties known to change during tumor progression. If the basis for the cellular heterogeneity is genetic, these subpopulations should be clonal, that is, they should be able to reproduce themselves or "breed-true," at least until further genetic variation occurs. It should also be possible to demonstrate the emergence and diminution of individual subpopulations (clones) within a single neoplasm, over time, as they are affected by selection pressure.

Although an attractive part of Nowell's hypothesis, tumor cells need not be especially unstable genetically or become more so during neoplastic development for progression to occur. Tomlinson *et al.* (1996) have used mathematical models of tumorigenesis (based on data from colorectal cancer) to show that selection is more important in driving tumorigenesis than is an increased mutation rate. An enhanced mutation rate may result in more rapid progression but is not required for it. Furthermore, as will be discussed, not all cellular heterogeneity in neoplasms is necessarily due to primary genetic alterations, nor does the ability of a cell to pass on a trait to its daughter cells necessarily mean that the trait is inherited.

Subsequent research has also shown that tumor progression need not be simply the reflection of the "survival of the fittest." As mentioned above, there can be fluctuations in progression and, as will also be discussed, modifying intersubpopulation interactions that influence the direction and extent of evolution.

A stumbling block for some to accepting the concept of intratumor cellular heterogeneity is the well-documented evidence for the monoclonal origin of the vast majority of cancers (Fialkow, 1979; Tanooka and Tanaka,

1982; Vogelstein *et al.*, 1985; Tanooka, 1988; Noguchi *et al.*, 1992). Further reflection, however, reveals no inherent inconsistency. Clonality does not signify the absence of change. A cancer, just as a whole, multicellular organism, may originate with a single "stem" cell which nevertheless may change, by either normal differentiative processes or actual genetic alterations, during the course of development. Some markers of clonal identity, such as X-inactivation, may be less subject to change, than, for example, resistance to a particular chemotherapeutic drug: one can surely have subclones, derived from an original, parental clone, that continue to express common features, as well as new ones. Similarly, individual metastases from a single cancer may be clonal in origin (Talmadge *et al.*, 1982) and yet develop subclonal heterogeneity over time (Poste *et al.*, 1982). Furthermore, there is considerable evidence for clonal selection during the early phases of cancer development (Mitelman, 1971), even within preneoplastic or preinvasive lesions (Hsu *et al.*, 1983; Shibeta *et al.*, 1993; Fujii *et al.*, 1996), so that the appearance of clonality within an established tumor may mask genetic diversity at the true point of origin (assuming that could be defined) (Ogawa *et al.*, 1980). In any event, there is a great deal of evidence that individual cancers can, in fact, contain multiple cellular subpopulations that behave in a clonal manner.

B. Cellular Heterogeneity of Cancer

1. Animal Tumors

The experimental study of cancer heterogeneity received major impetus with the publication in the late 1970s by Fidler and Kripke (1977) of a paper demonstrating the preexistence (prior to assay) within the B16 melanoma cell line of clones of cells that differed markedly in their ability to form lung colonies upon intravenous injection into syngeneic mice (this procedure is known as the experimental metastasis assay). Fidler and Kripke, as had Law before (1952), used an adaptation of the fluctuation test to show that a cancer was heterogenous at the cellular level. Soon thereafter our own laboratory (Dexter *et al.*, 1978) reported the isolation, based on morphological criteria, of four tumor-producing subpopulations from a single, primary mammary carcinoma that had arisen in a strain Balb/cfC_3H mouse. A fifth subpopulation was later isolated from a spontaneous metastasis in a mouse bearing a serial transplant from that same primary (Heppner *et al.*, 1978). These five subpopulations were shown to differ in basic behavioral properties, including growth rate (Dexter *et al.*, 1978), intrinsic sensitivity to chemotherapeutic drugs (Heppner *et al.*, 1978), immunogenicity in syngeneic hosts (Miller and Heppner, 1979), and ability to

metastasize (Miller *et al.,* 1983). The populations bred true, that is, their basic characteristics were stable upon repeated propagation.

Subsequently, there have been repeated demonstrations of similar cellular heterogeneity in experimental tumors of recent origin as well as in well-established tumor models. This review can only cite a fraction of the published literature. Virtually every type of cancer has been used: as mentioned, melanoma, lymphoma–leukemia (Olsson and Ebbesen, 1979), sarcoma (Nicolson *et al.,* 1978; Varani *et al.,* 1978; Kripke *et al.,* 1978; Mantonvani *et al.,* 1981; Boone *et al.,* 1979; Wang *et al.,* 1982), and carcinoma (Martin *et al.,* 1983; Welch *et al.,* 1984a,b; Brodt *et al.,* 1985). Clonal heterogeneity has been demonstrated in tumors induced by chemicals (Wang *et al.,* 1982; Martin *et al.,* 1983), by physical agents (Kripke *et al.,* 1978; Boone *et al.,* 1979), by steroids (Dominguez and Huseby, 1968), or by viruses (Dexter *et al.,* 1978; Brodt *et al.,* 1985; Olsson and Ebbesen, 1979), as well as in spontaneously transformed clones *in vitro* (Rubin, 1988). Clonal tumor heterogeneity has been noted in a wide range of properties: karyotype (Dexter *et al.,* 1978; Boone *et al.,* 1979; Wang *et al.,* 1982), histopathology (Dexter *et al.,* 1978), metastatic and invasive behavior (Fidler and Kripke, 1977; Boone *et al.,* 1979; Mantovani *et al.,* 1981; Wang *et al.,* 1982; Miller *et al.,* 1983; Welch *et al.,* 1984a), antigenicity, and immunogenicity (Miller and Heppner, 1979; Brodt *et al.,* 1985; Hostetler and Kripke, 1988), and sensitivity to chemotherapy (Law, 1952; Heppner *et al.,* 1978; Tsuro and Fidler, 1981; Welch and Nicolson, 1983), to X-irradiation (Hill *et al.,* 1979; Leith *et al.,* 1981; Welch *et al.,* 1984b; Wallen *et al.,* 1985; Jamasbi and Perkins, 1990), to γ-radiation (Welch *et al.,* 1983), and to hyperthermia (Tomasovic *et al.,* 1984; Rhee *et al.,* 1987). Biological agents, such as hormones (Geradts *et al.,* 1986) and inducers of differentiation (Gerharz *et al.,* 1989), likewise elicit differing responses among clones isolated from the same animal tumor.

In general, the clones isolated from all these cancers remain stable, at least for some time, in regard to their specific traits, upon repeated propagation. Also, in general, the specific traits exhibited by the varying clones are randomly distributed among them, that is, metastasizing ability does not sort with drug sensitivity, and so on (see Fould's Rule 2).

2. Human Cancers

Investigation of the cellular heterogeneity of human cancer is logistically more difficult than it is for animal cancer and the proof of clonality is oftentimes indirect, due to difficulties in successfully propagating many human cancers either *in vitro* or as xenografts. Nevertheless, there is an impressive number of publications in which investigators have shown by direct isolation the existence of multiple subpopulations (clones) in human

cancers, either in primary cancers or in different metastatic foci from the same patient, or in human cancer cell lines. As with animal tumors, the types of cancers for which this has been achieved are all-inclusive: melanoma (Albino *et al.*, 1981; Natali *et al.*, 1983; Rofstad, 1992), colon carcinoma (Brattain *et al.*, 1981, 1983; Leith *et al.*, 1982a,b, 1983, 1984, 1985; Zhou *et al.*, 1995), glioma (Yung *et al.*, 1982; Wikstrand *et al.*, 1983; Allalunis-Turner *et al.*, 1992; Coons *et al.*, 1995), lung cancer (de Vries *et al.*, 1989; Linsley *et al.*, 1986), breast cancer (Brennan *et al.*, 1979; Teixeira *et al.*, 1995), bladder cancer (Brown *et al.*, 1990), salivary gland cancer (Shirasuna *et al.*, 1986), and thyroid cancer (Eng *et al.*, 1996) among others (Slocum *et al.*, 1985). Also, as with animal tumors, the heterogeneity is seen for a wide range of characteristics, including *in vitro* growth properties (Albino *et al.*, 1981; Brattain *et al.*, 1983), karyotype (Leith *et al.*, 1982b; Coons *et al.*, 1995; Teixeira *et al.*, 1995), sensitivity to growth regulators or expression of growth factor or hormone receptors (Brennan *et al.*, 1979; Brattain *et al.*, 1983; Zhou *et al.*, 1995), molecular genetic markers (Eng *et al.*, 1996), antigen expression (Albino *et al.*, 1981; Natali *et al.*, 1983; Wikstrand *et al.*, 1983; Linsley *et al.*, 1986), ability to grow as xenografts (Brown *et al.*, 1990; Shirasuna *et al.*, 1986), and sensitivity to chemotherapeutic drugs (Yung *et al.*, 1982; de Vries *et al.*, 1989; Allalunis-Turner *et al.*, 1992), various types of radiation (Leith *et al.*, 1982a,b, 1984; Rofstad, 1992; Allalunis-Turner *et al.*, 1992), hyperthermia (Leith *et al.*, 1983, 1985), and biological therapy (Leith *et al.*, 1982a; Allalunis-Turner *et al.*, 1992). (Again, the references cited are only a fraction of the published literature.)

A particularly elegant study on the extent and distribution of distinct clones (detected by flow cytometry and cytogenetic analysis of first division cells) was recently published for a single, human low-grade oligoastrocytoma (Coons *et al.*, 1995). The authors sampled 38 spacially mapped areas in the tumor and found extensive variation in karyotype and DNA content among them. One area in particular, in addition to containing a distinct, clonal stem line, also contained a variety of nonclonal abnormalities, suggesting that this area was a hot spot of genetic instability. This study is a fine illustration of regional heterogeneity, that is, heterogeneity in the distribution of tumor cell clones within a solid neoplasm.

In addition to many examples in the literature where heterogeneity of human cancers has been demonstrated by direct isolation, propagation, and characterization of clones, there are numerous studies in which multiple-clonality has been inferred by the analysis of genetic characteristics in multiple samples taken from different regions of the same neoplasm. These studies, although impressive, are less supportive of the Nowell hypothesis because they do not demonstrate that the genetic variants are actually viable clones, rather than reproductive end points, unable to propagate to a significant extent. Flow cytometry has revealed extensive intratumor

heterogeneity in DNA content/ploidy in multiple samples from individual breast cancers (Meyer and Wittliff, 1991; Beerman *et al.*, 1991; Fuhr *et al.*, 1991; Schvimer *et al.*, 1995), head and neck cancers (Jacob *et al.*, 1996), colorectal cancers (Wilson *et al.*, 1993), hepatocellular cancers (Okada *et al.*, 1995), stomach cancers (Sasaki *et al.*, 1993; Fujimaki *et al.*, 1996), and prostate cancers (Shankey *et al.*, 1995a). Likewise, conventional cytogenetics on multiple samples and/or interphase cytogenetic analysis by FISH (fluorescence *in situ* hybridization) has been used to demonstrate extensive genetic heterogeneity in gliomas (Coons *et al.*, 1995), soft tissue tumors (Orndal *et al.*, 1994), bladder cancers (Schapers *et al.*, 1993), prostate cancers (Alers *et al.*, 1995), and breast cancers (Balazs *et al.*, 1995; Fiegl *et al.*, 1995). Furthermore, a number of investigators have also reported multiclonality in individual human tumors as revealed by heterogeneity in specific mutations in oncogenes or suppressor genes (Mirchandani *et al.*, 1995; Konishi *et al.*, 1995; Giaretti *et al.*, 1996). Again, these studies indicate that different clones may be regionally distributed within a solid cancer.

Thus, there is extensive support from both animal and human studies for the *sine qua non* of Nowell's hypothesis, that individual cancers contain multiple, genetically and/or behaviorally distinct subpopulations or clones. The next requirement is the demonstration that the relative proportion of these subpopulations changes as the cancer evolves in a way that reflects the changing behavior seen in tumor progression.

C. Clonal Evolution of Cancer

The direct demonstration of changes in subpopulation distribution during tumor progression has been achieved only rarely. There are many reports of karyotypic differences among primary tumors and their metastases, or between tumors sampled at different times or from different areas believed to represent different stages of tumor progression (Mitelman, 1972; Alam *et al.*, 1992; Orndal *et al.*, 1994; Simmons *et al.*, 1995; Fiegl *et al.*, 1995; Katsura *et al.*, 1996; Gagos *et al.*, 1996), but generally these studies, although suggestive of clonal evolution, do not actually quantitate its extent or relate the changed pattern to biological behavior (Nowell, 1976).

In part, this deficiency in the tumor progression literature may be due to the lack of experimental tools with which to monitor accurately and quantitatively individual clones within a mixed tumor. Various techniques have been applied to the problem, with somewhat anomalous results. Ichikawa *et al.* (1989) mixed together morphologically and karyotypically distinct, androgen-dependent and independent subpopulations of the experimental Shionogi carcinoma and grew them either *in vitro,* with testosterone in the media, or *in vivo* in either male or female mice. *In vitro,* neither

population had a selective advantage. *In vivo,* the androgen-independent population came to predominate, irrespective of the gender of the host (the dependent population did not grow in female mice). Thus, this system reproduced the progression to hormone independence seen in many animal and human tumors of endocrine-sensitive histotypes, but the nature of the selection pressure was obscure.

Staroselsky *et al.* (1990) also used karyotypic differences to follow the fate of metastatic line K-1735 melanoma cells mixed together with nonmetastatic cells in subcutaneous tumors in mice. The metastatic line came to predominate in mice in which they were at least 5% of the original inoculum.

Other investigators have used flow cytometry to monitor the distribution of mixtures of clones that differ in DNA content. Aabo *et al.* (1994) mixed together BCNU (1,3-bis (2-chloroethy 1)-1-nitrosourea) sensitive and insensitive human small cell lung cancer lines, injected them into nude mice, and treated the mice with BCNU after the tumor had become well-established. Tumors that initially had a high predominance of the sensitive line responded to treatment but recurred. Only the resistant-line cells were detected in the recurrence.

Various investigators have utilized molecular genetic markers to detect individual clones within artificially mixed tumors. A common technique has been transfection of pSV2 neo, which inserts at a unique site for each clone, allowing its detection by restriction fragment analysis. With this approach, Waghorne *et al.* (1988) demonstrated in primary mouse mammary cancers the development of dominant clones that were then responsible for metastasis, a result that was similar to that of a later study using human breast carcinoma clones grown in nude mice (Price *et al.,* 1990). Staroselsky *et al.* (1992) used the same approach with a human renal cell carcinoma model to demonstrate that the site of primary implantation (kidney, colon, or subcutaneous) influenced which clones grew from a mixture and which clones were able to grow as visceral metastases from the different primaries. Collectively, these studies have been taken as evidence for the role of "clonal dominance" in metastasis, that is, that clones able to metastasize come to dominate the primary tumor prior to the metastatic event (clonal dominance will be discussed more fully below). However, using the same technique, but analyzing a larger number of tumors and tumor types, Moffett *et al.* (1992) found that although a single clone in a primary tumor could become dominant, this was not necessarily associated with metastasis. Further, primary tumors usually remained polyclonal even at late stages and individual lung metastases, although often clonal, could also be polyclonal. Lymph node metastases were always polyclonal. Moffett *et al.* reported great variability in clonal evolution from tumor to tumor, even those produced by the same polyclonal inoculum, and concluded that the development of metastases and the acquisition of

growth supremacy by a clone of cells within the primary were not a constant feature of progression to malignancy and that their results conformed with "much previous clinical and experimental information indicating that tumor cell behavior is disorderly and unpredictable."

Our own group has used another method to follow the fate of individual tumor subpopulations growing within a single neoplasm. We mix together tumor subpopulations that differ in drug resistance markers that enable us to quantitate the number of clonogenic cells of each type by plating in the appropriate selective medium (Miller *et al.*, 1987). As we describe below, our results are similar to those of Moffett *et al.* in that they reveal a variety of outcomes in subpopulation composition during tumor growth. Even when under clear and controllable selection pressure, in the form of chemotherapy to which one population is sensitive and the other resistant (note: the chemotherapy is unrelated to the drug resistance markers used to quantitate the subpopulation distribution), there is no uniform selection for the resistant subpopulation and no overall progression to resistance (B. E. Miller *et al.*, 1989).

Thus, at this time, there is no large body of evidence that definitively documents the clonal evolution of tumors over time such that there is a clear relationship between the emergence or extinction of particular clones and the expression by the tumor of new behavior that is relevant to the behavioral changes seen in tumor progression. Most of the evidence consists of single snapshots indicating that, at any one time, cancers are heterogeneous at the cellular level and that this heterogeneity may change over time, but the direction of change and its relationship to any particular selection pressure remain ill-defined. This is not to suggest that the Nowell hypothesis is fundamentally incorrect but rather that the relationships among individual clones within tumors may be more complex than originally envisioned.

D. Tumor Subpopulation Interactions

Our hypothesis that tumor cell subpopulations within the same neoplasm might interact to influence each other's behavior, thereby subverting the logical course of tumor progression, derives support from an old tradition in tumor biology that focuses on cancer as a diseased *tissue* rather than on an abnormal proliferation of aberrant *cells*. The theoretical basis for this concept has been beautifully summarized by Prehn (1991) in a Perspective for the journal *Cancer Research*. Historically, it was noted that growth of multiple tumors in the same experimental host often results in a mutual decrease in growth rate or other proliferation indices, compared to growth of the tumors in individual hosts (Browning, 1948; Makino, 1956; Cheshire,

1970; Bichel, 1972). A perhaps related phenomenon is the burst of metastatic growth that sometimes follows surgical removal of a primary cancer (Gorelik *et al.*, 1978; Keller, 1985; Fisher *et al.*, 1989). These observations have been variously attributed to immune alterations (Gorelik *et al.*, 1978) or growth regulators, such as "chalones" (Cheshire, 1970) or secreted growth inhibitory factors (Fisher *et al.*, 1989), by which a primary cancer controls competing metastatic growth. They may also be related to the phenomenon known as "concomitant immunity" in which a growing tumor may suppress growth of the same or a related tumor injected at a different site (Nowotny and Grohsman, 1973; Gorelik *et al.*, 1981; Niederkorn and Streilen, 1983).

A more direct indication that interactions between tumor subclones can affect growth comes from observations that cloned subpopulations may behave differently than the tumors from which they were derived, for example, being less transplantable (Woodruff *et al.*, 1982) or more metastatic (Edwards *et al.*, 1990). Many investigators (Giavazzi *et al.*, 1980) have remarked that tumor cells grown out from metastases may not be more metastatic than the primaries from which they came and have interpreted this as evidence against the hypothesis that metastases are produced by highly metastatic clones preexisting within the primary tumors. However, as discussed below, this conclusion is unwarranted in light of the evidence for clonal interactions affecting metastasis, as well as the possibility of instability ("dynamic heterogeneity") in the metastatic phenotype.

Our laboratory has been especially interested in the possibility that interactions between tumor subpopulations may affect tumor behavior and alter the course of progression. We view cancers as societies of tumor (and normal) cells in which the individual behaviors and potentials contribute to, but do not necessarily dominate, the behavior of the whole (Heppner, 1989). We have arrived at this concept based on a series of experiments utilizing the set of five tumor subpopulation lines, mentioned above, and derivatives thereof, established from a single mouse mammary cancer. These lines differ in basic behavioral properties, such as growth rate, immunogenicity, and ability to metastasize, as well as in sensitivity to a variety of chemotherapeutic drugs. We have developed a number of experimental protocols to observe the effect, in syngeneic mice or *in vitro,* of one subpopulation on the behavior of another, and vice versa, and to determine the mechanism that may underlie any particular interaction. Examples of our results include the following:

- The ability of a subpopulation line designated as 410 to depress the growth of subpopulation line 168. This interaction occurs *in vivo* and involves an immune response elicited by 410 which is active against both 410 and 168 (line 168 is nonimmunogenic). It does not require physical contact between the two subpopulations (Miller *et al.*, 1980).

- The ability of subpopulation lines 168 and 68H to alter each other's growth rates *in vitro* so that they grow at the same rate, even though by itself line 68H has twice the doubling time of line 168. The combined rate can be that of either line alone, not an average of the two (Heppner *et al.,* 1980).
- The ability of line 4T07 to inhibit strongly (that is, to dominate) line 168 both *in vivo* and *in vitro.* This is true even when tumors are initiated with inocula in which the ratio of 168 to 4T07 is 100:1 or greater. The mechanism of interaction requires intermingling of the cells, but not gap junction formation, suggesting the involvement of a transitory growth inhibitory factor. In this example of "clonal dominance," neither line is able to metastasize, further evidence that the ability to dominate is independent of the ability to metastasize (Samiei and Waghorne, 1991; Moffett *et al.,* 1992).
- The ability of line 410.4 to allow lines 67 and 168, neither of which can produce metastases in the lung by themselves, to cometastasize with metastatic 401.4 cells (Miller, 1983).
- The ability of line 168 tumors, which are relatively sensitive to cyclophosphamide (CY), to render insensitive line 410.4 tumors susceptible to CY action. The mechanism of interaction does not involve the immune response but seems to involve the ability of 168 tumors to enhance the metabolism of CY to active drug (Miller *et al.,* 1981).
- The ability of methotrexate (MTX)-sensitive line 4T07 cells to render insensitive line 66 cells sensitive to that drug, whereas insensitive line 168 cells decrease the sensitivity of 4T07 cells. In these experiments paired cell lines are mixed together and injected into mice 2 days prior to treatment. The clonogenic fraction of individual subpopulations is monitored as described above (B. E. Miller *et al.,* 1989).
- The ability of melphalan (MEL) - sensitive line 4T07 cells to markedly (2 log) enhance the number of relatively insensitive line 66 cells killed by that drug. This can be seen even when treatment is withheld until the tumors are grossly palpable. It does not depend upon host factors, since the effect can be mimicked *in vitro* and does not require cell–cell contact. Another MEL-sensitive line, 168FAR, however, cannot transfer sensitivity to 66 cells, indicating specificity in the mechanism.

Such examples of tumor subpopulation interaction have also been reported by other investigators, using different model systems. For example, Caignard *et al.* (1985) reported a growth interaction between two subpopulations isolated from a single, chemically induced rat colon cancer. One subpopulation, TS, inhibited tumor formation by line TR when the two were injected simultaneously, either as mixtures or in separate sites of the same rat. However, TS cells were able to form tumors if injected into rats already bearing established TR tumors. Both outcomes required the host immune system.

As already mentioned, a number of investigators have reported examples of clonal dominance in which one subpopulation can completely overgrow another, even when the initial inoculum contains only a very small number of dominating cells and when, by themselves, the two populations grow at the same rate (B. E. Miller *et al.*, 1988; Waghorne *et al.*, 1988; Price *et al.*, 1990; Staroselsky *et al.*, 1990, 1992; Samiei and Waghorne, 1991; Aabo *et al.*, 1995). The mechanisms for this interaction have been reported to be due to the immune system (Staroselsky *et al.*, 1990), but others, including ourselves, have not found evidence for immune involvement (Ichikawa *et al.*, 1989). Kerbel and Theodorescu (1990) presented evidence that, rather than dominance, the interaction could be the result of stimulation of the dominating line by the release of active TGF-β by the dominated line. However, this mechanism also does not explain our results with lines 168 and 4T07.

Subpopulation interactions affecting the ability to metastasize have also been reported by others. A metastatic clone of the SP1 mouse mammary tumor enabled nonmetastatic SP1 cells to metastasize spontaneously to lung (Waghorne *et al.*, 1988). Lin *et al.* (1993) reported cooperativity between ras- and sis-transformed clones of Balb/c 3T3 cells such that following intravenous injection into nude mice, a number of lung foci were detected in which cells of the two lines had colocalized. Cells of one line increased the survivability and subsequent growth of cells of the other, whereas, in reverse, the second line facilitated the ability of the other to grow from micro- to overt metastases. Jouanneau *et al.* (1994) co-injected into nude mice mixtures of cancer sublines that differed in production of acidic fibroblast growth factor. The factor-producing line increased the tumorigenicity and decreased the time to metastatic growth of the entire population.

Tumor subpopulation interactions affecting hormone responsiveness have also been reported. Thus, in both a rat mammary tumor model (Danielpour and Sirbasku, 1984) and the human MCF-7 breast line (Kasid *et al.*, 1987) it has been found that estrogen-independent sublines can support growth of estrogen-dependent cells in the absence of estrogen by constituitively producing growth factors (TGF-α, IGF-1) that are otherwise induced by estrogen in the dependent cells. On the other hand, Horvath *et al.* (1993) reported that an estrogen-independent tumor inhibited growth of an estrogen-sensitive tumor in the presence of low concentrations of estrogen.

Cell subpopulation interactions that affect sensitivity to chemotherapy have been described in a number of different models. Tofilon *et al.* (1984) investigated *in vitro* BCNU sensitivity of multicellular spheroids consisting of mixtures of sensitive (9L) and resistant (R_3) sublines of a rat brain tumor. The sensitivity of the 9L cells decreased as the percentage of R_3 increased such that the resistant cells did not come to dominate the mixture.

In studies with a human small cell lung carcinoma model, Aabo *et al.* (1994) mixed together a BCNU-sensitive, clonal-dominating line (592) with

a BCNU-resistant, subservient line (NYH) and injected the mixture into nude mice. BCNU treatment was begun 3 to 4 weeks later. A significant, but transient response was seen in the mixed tumors, initiated at 9:1 ratio of 592:NYH, in which only 592 cells were detectable, by flow cytometry, at the beginning of treatment. In the regrowing tumors, only NYH cells could be detected. Tumors initiated with a 1:1 ratio of the two cell types failed overall to respond to BCNU, although 592 cells were eradicated. Thus, treatment of the sensitive population resulted in emergence of the resistant population, which otherwise had been nearly totally dominated. This is similar to our experience with mixed tumors of the MTX-sensitive, dominant 4T07 subline and the MTX-insensitive, subservient 168 where the overall response pattern is closer to that of 168 tumors and less than that of 4T07 tumors (B. E. Miller *et al.,* 1989).

Frankfurt *et al.* (1991) carried out an intriguing *in vitro* study in which subpopulations of a human ovarian cancer line that differed in sensitivity to L-phenylalanine mustard (L-PAM) were mixed, treated with L-PAM for 1 hr, and assessed for DNA damage. The extent of damage was significantly less in the sensitive subline in the mixture than that when treated alone. The interaction required direct cell contact and apparently involved transfer of glutathione from resistant to sensitive cells, thereby allowing them to repair DNA damage. The results were confirmed by clonogenic assays.

Of course, as we have also found, interactions affecting drug sensitivity do not occur with every combination of subpopulations. Thus, Leith *et al.* (1988) found that mixed tumors of two subpopulations of a human colon carcinoma responded as expected based on their individual sensitivities in nude mice treated with mitomycin C.

In addition to modification of the expression of behavioral traits, a number of investigators have asked whether tumor subpopulation interactions could alter the basic foundation of tumor heterogeneity, that is, the propensity to produce new variant subpopulations. Poste *et al.* (1981) and Poste and Grieg (1982) found that clones isolated from various metastatic lines of experimental tumors, such as B16 melanoma, Lewis lung carcinoma, or UV 2237 fibro sarcoma, were individually unstable, rapidly producing variant subclones with diverse metastatic phenotypes upon passage *in vitro* or *in vivo.* However, cocultivation of mixtures of the clones resulted in a "stabilization" of their individual phenotypes and a quenching of variant formation. The interactions were specific in that B16 clones were not stabilized by Lewis lung cells or by syngeneic, normal cells.

Itaya *et al.* (1989) found that culture of individual clones of the mouse CT-26 colorectal cancer line resulted in extensive diversification of a variety of phenotypic properties and genotypic markers. During the same culture

period the parental line from which the clones were derived remained stable in all the measured properties.

The possibility that tumor subpopulation interactions can stabilize clonal diversification has both practical as well as theoretical consequences. If true, it could imply that cancers are by nature heterogeneous and that monoclonal origin is in itself a stimulus to the production of the cellular heterogeneity from which tumor progression is the ultimate result. The mechanisms of stabilization are unknown. However, a very recent paper by Glick *et al.* (1996) suggests a possibility. These investigators have demonstrated in a mouse keratinocyte system that TGF-β can suppress amplication of the carbamoyltransferase-dihydroorotase (CAD) gene induced by the drug *N*-phosphono-acetyl-L-aspartate (PALA). The effect requires an intact TGF-β type II receptor and is independent of proliferation and of the p53 and Rb genes. Thus, factors, like TGF-β, produced by one clone could perhaps control genetic diversification in another clone.

We have attempted to see whether subpopulation interactions can affect the rate of emergence of drug resistance by comparing the development of MTX resistance in cultures of mixtures versus individual clones of the mammary tumor sublines 66, 168TFAR, and 4T07 (Miller *et al.*, 1996). There is little evidence of such an interaction in this system. However, to our knowledge this is the only published test of clonal stabilization of the emergence of drug resistance and surely deserves further study.

As can be seen from the above, there is a reasonable amount of experimental evidence that tumor cell subpopulations and clones can interact to affect each other's behavior, and it is reasonable to postulate that such interactions may influence, and complicate, the course of tumor progression. It is clear, however, that there is little consistency in the mechanisms by which these interactions are effected. Indeed, not all subpopulations interact, at least in regard to the phenotypes tested. Where interactions do occur, they may involve the host, such as through the immune response or by metabolism of drugs, etc., or they may depend upon tumor properties. In this case, they may require cell–cell contact or they may involve production of a growth factor or inhibitor. From our work, we have seen examples of all these possibilities. Other suggested mechanisms of interaction from the literature include production of growth inhibitory factors (Horvath *et al.*, 1993), production of growth stimulatory factors, i.e., acidic fibroblast growth factor (Jounneau *et al.*, 1994), production by one population of proteases that facilitates establishment or invasion of a second population (Biswas *et al.*, 1978), suppression by one population of proteases produced by the other (Newcomb *et al.*, 1978), and alterations by one population of angiogenesis and/or necrosis that affect establishment or survival of the second population (Ichikawa *et al.*, 1989; Good *et al.*, 1990). [Note: Given the current interest in apoptosis, it is likely that suppression or stimulation

of apoptosis will be suggested as intercellular mechanisms of stimulation or inhibition, respectively.]

There is also no consistency in the direction of subpopulation interactions in regard to progression: the interaction can stimulate or inhibit proliferation, can stimulate or inhibit tumorigenicity or metastasis, or can increase or decrease sensitivity to chemotherapy. In part, this may reflect the experimental approach of testing only a few clones at a time, rather than the entire polyclonal population. However, it appears that the outcome of any particular interaction is just as likely to be the preservation of the heterogeneity as it is the establishment of the supremacy of a particular clone.

Perhaps the lesson from all this is that cancers, in addition to being heterogeneous and dynamic, share another characteristic with other types of societies, ours included. Individuals and groups in societies communicate, i.e., "interact," for all sorts of reasons. Some interactions are mutually beneficial, some mutually destructive, and some one-sided. The mechanisms of interaction (visual clues, olfactory clues, speech, writing, the internet, etc.) depend on the capabilities, i.e., the "phenotype," of the parties involved and on the environment in which they find themselves. From the point-of-view of understanding tumor progression, where the goal is to relate specific behavioral changes to alterations in the balance of the interacting populations with the environment, it may be that it is more fruitful to accept the holistic view of a cancer as an integrated tissue rather than to dissect out example after example of the mechanisms by which that integration is achieved.

As might be expected most of the literature on subpopulation interactions utilized either animal models or human cell lines in which the relationship among the tumor clones is quite artificial. That the results from these models have an application to clinical cancers, however, has been suggested by a number of clinical investigators based on their observations of spacial relationships among divergent neoplastic cells in the same cancer (Teixera *et al.*, 1995; Visscher *et al.*, 1995; Okcu *et al.*, 1996).

E. Origins of Cellular Heterogeneity

1. Genetic Instability

a. Cytogenetic and Molecular Genetic Abnormalities As already described, a basic tenet of the Nowell hypothesis is that acquired genetic instability is the underlying cause of tumor heterogeneity and progression. The nature of this genetic instability and its mechanisms continue to be very active research areas. Nowell himself focused on cytogenetic evidence

of instability, although he discussed many of the other possibilities as well (Nowell, 1976). Evidence of genetic alteration in cancer, as manifested at the chromosome level, is commonly reported, although these observations are usually made at one time, late in a tumor's evolution, and are difficult to correlate to progression (Rodriguez *et al.*, 1994). There are reports, however, that relate specific, nonrandom, sequential changes in chromosome number or structure to biological properties, such as age of tumor (Mitelman, 1971), acquisition of tumorigenicity (Kitchin and Sager, 1980), loss of hormone dependence (Isaacs *et al.*, 1982), and metastatic potential (Wolman *et al.*, 1985). However, in a direct test of the hypothesis that chromosomal instability is related to progression, Kendal *et al.* (1987) found no correlation between the rate of generation of marker chromosomal abnormalities and metastatic potential in a mouse melanoma cell model. The cytogenetic literature, which is vast, has been succinctly summarized in a recent review by Rodriguez *et al.* (1994).

Rodriguez *et al.* also summarized the still growing literature on the use of molecular genetic techniques to analyze chromosomal alterations. These techniques allow for analysis of much smaller samples of tissue and do not require cell growth in culture, both limitations of conventional cytogenetics. Two major conclusions are presented in their review: (1) that the type of genetic lesions associated with tumor development are related to the embryonal derivation of the precursor cell, with epithelial and neurogeneic tumors exhibiting deletions of genetic material (evidence of the importance of tumor suppressor genes), sarcomas and hematopoietic tumors exhibiting rearrangements (translocations), and germ cell tumors showing amplifications; and (2) that, as previously cited (Fearon and Vogelstein, 1990; Califano *et al.*, 1996), it has been possible to associate specific and different structural defects (allelic losses) with the progression of colorectal adenoma to carcinoma, Barrett's esophagus to esophageal cancer, early to late ovarian cancer, and other examples in the progression of bladder, gastric, breast, and hepatocellular cancer. Most of these observations are correlative, without direct proof of a specific role or mechanism for genes in the deleted regions vis-à-vis tumor progression. However, as more tumor suppressor genes are cloned and put into functional test systems, it is likely that this situation will change rapidly.

b. Mutation Frequency Nowell's hypothesis specifies that genetic instability is enhanced in cancer cells, relative to their normal counterparts, and that the degree of instability may increase with tumor progression. Testing these parts of the hypothesis presents a number of questions: (1) What is the normal baseline of genetic change? (2) What types of genetic changes are relevant to progression? (3) When is phenotypic instability too unstable to be equated with genetic instability? A number of investigators have

approached these questions by comparing the mutation rates in normal versus cancer cells.

A common technique is measurement of the rate of development of resistance to thioguanine, which reflects point mutations in the hypoxanthine–guanine phosphoribosyl transferase gene located on the X chromosome. Seshadri *et al.* (1987) compared the spontaneous mutation rates of lymphocytes from three normal individuals with those of three human neoplastic lymphocyte cell lines. Considerable variability was seen in both groups, but the rate of mutation/cell/generation was 2- to 120-fold greater in the leukemic cell lines than that in the normal lymphocytes. Using the same technique, Kaden *et al.* (1989) measured the mutation rate in a series of sublines from the tumorigenic Chinese hamster embryo fibroblast cell line CHEF/16 and the nontumorigenic line CHEF/18. The mutation rates of four lines ranged from 1.2×10^{-6} to 8.9×10^{-6} mutations/cell/generation, with only one tumorigenic line having a frequency significantly greater (2.5×10^{-5}) than that of the nontumorigenic CHEF/18 cells. These authors concluded that mutation rate per se did not correlate with tumorigenicity. A similar result was obtained by Eldridge and Gould (1992) who compared mutation frequencies in early cultures of normal and carcinomatous human breast samples.

Cifone and Fidler (1981) compared the spontaneous mutation frequency for development of both thioguanine and ouabain resistance in a set of mouse UV-fibrosarcoma clones that differed in the ability to produce lung colonies in the experimental metastasis assay. In all cases, the cells with the greatest metastatic potential had 3- to 7-fold greater mutation rates than did the poorly metastatic, but tumorigenic clones. Other comparisons between metastatic versus nonmetastatic tumor lines, however, failed to confirm these results as generally true. Thus, although the spontaneous mutation rates to thioguanine and ouabain resistance in our series of mouse mammary tumor lines varied 10- and 9-fold, respectively, there was no correlation with these rates and the ability to either spontaneously metastasize or form lung colonies after intravenous injection. There was a positive correlation, however, between metastatic ability and the mutation rates in response to the mutagen ethyl methylsulfonate (Yamashina and Heppner, 1985). Kendal and Frost (1986) also found no correlation between the rate of mutation to ouabain resistance in metastatic and nonmetastatic variants of NIH 3T3 or CBA SP-1 cells.

Recently, there was an elegant analysis of genetic instability, as measured by a point mutation in a bacteriophage λ transgene, in normal mammary gland, mammary tumors, and lung metastases in transgenic mice also carrying a polyomavirus middle T oncogene, targeted to expression in the mammary gland (Jakubezak *et al.*, 1996). The frequency of mutation in the λ

gene was not significantly different among normal mammary epithelium, primary mammary tumors, and their metastases.

Collectively, these studies do not offer support for the notion that genetic instability, as measured by a variety of general assays for point mutations, is associated with the progression of normal to tumor, or of tumor to metastatic, phenotypes.

*c. **Gene Amplification*** Another genetic defect that has been investigated as a correlate of tumor progression is gene amplification. The evidence that amplification is a relevant indicator of genetic instability is stronger and much more consistent than that for point mutations. Amplification of specific oncogenes has been associated with markers of poor prognosis in a variety of human cancers, including gastric, ovarian, breast, and esophageal cancer (Rodriguez *et al.,* 1994). Gene amplification as a mechanism of tumor heterogeneity and of drug resistance has been particularly studied in the case of MTX resistance due to amplification of the dihydrofolate reductase (DHFR) gene (Schimke *et al.,* 1985).

Tlsty (1990) compared the gene amplification potential of a large series of normal diploid, human, and rodent cells with that of their transformed counterparts. The test system involved two genes, DHFR and CAD, the amplification of which leads to resistance to MTX and PALA, respectively. In general, amplification was below detectable limits (10^{-8}) in the normal cells whereas all the transformed cells had amplification frequencies between 10^{-3} to 10^{-7} cell/generation. Similar results were reported for nontumorigenic versus tumorigenic rat liver epithelial cell lines (Tlsty *et al.,* 1989), and this association has been found to hold up in other tumor models as well (Glick *et al.,* 1996).

Comparisons between the rates of gene amplification of tumorigenic, but nonmetastatic cells versus metastatic cells likewise show a strong correlation with tumor progression. Cillo *et al.* (1987) measured generation of resistance to MTX and PALA in highly metastatic versus poorly metastatic mouse B16 melanoma cells and found an approximately fivefold higher frequency in the former. Gitelman *et al.* (1987) also reported enhanced amplified DNA (homogeneously staining regions) in metastatic versus nonmetastatic clones of the human melanoma line MeWo. Likewise, Radinsky *et al.* (1988) reported enhanced amplification and rearrangement of the v-ki-ras oncogene as a correlate of metastatic ability of clones of v-ki-ras transformed Balb/c 3T3 cells.

Mention has already been made of the recent finding that TGF-β_1, via transduction of the TGF-β type II receptor, is able to suppress amplification of the CAD gene in a model of keratinocyte carcinogenesis (Glick *et al.,* 1996). In this model, cells which carry a target deletion of the TGF-β_1 gene undergo both malignant transformation and gene amplification (in response

to PALA) at much higher frequencies that do TGF-β_1-expressing controls. Interestingly, inactivation of type II TGF-β_1 receptors has been recently associated in colon cancer cells with another indicator of genetic instability, microsatellite instability (Markowitz *et al.,* 1995).

d. "Mutator" Phenotypes and DNA Repair Genes The idea that an underlying cause of genetic instability in cancer may be mutations in genes associated with some step(s) in DNA repair is not new (Loeb, 1991), but the topic has taken on new life with the linkage of a particular manifestation of instability seen in the length of microsatellites, or short, repeated sequences of nucleotides, in the DNA of colorectal tumors from patients with hereditary nonpolyposis colorectal cancer (Leach *et al.,* 1993) and the recognition that the genes responsible are homologous to the yeast DNA-mismatch repair genes hMSH2 and LMLHI (Fishel *et al.,* 1993). These observations have triggered a stampede of investigations seeking evidence of microsatellite instability in nonhereditary cancers, and indeed it has been found, at varying frequencies, in pancreatic and gastric (Han *et al.,* 1993), endometrial (Duggan *et al.,* 1994), and breast cancer (Yee *et al.,* 1994), among others. The relationship of these replication error repair mutations to tumor progression remains unclear. Most studies have provided only snap-shot data on their frequency in a series of cancers, although Duggan *et al.* (1994) reported one case of endometrial cancer in which DNA of the primary tumor was stable while that of a metastasis was unstable. However, in their limited series, there was evidence of heterogeneity within primary tumors as well, so the significance of this one case, vis-à-vis progression, is uncertain. As might be expected from Foulds' Rule 1, there are also reports of heterogeneity in expression of microsatellite instability among multiple tumors of the same patient (Nakashima *et al.,* 1995). A recent report of microsatellite instability in nonneoplastic tissues from chronic inflammatory conditions, pancreatitis and ulcerative colitis, raises questions about the role of this type of instability in progression although it is intriguing in view of the possible involvement of inflammation in carcinogenesis (see below) (Brentnall *et al.,* 1996).

Other mutator phenotypes have been assessed for their effect on tumor progression. Damen *et al* (1989) reported no difference in the generation of metastatic variants in the thy-49 Chinese hamster mutator cell line or in the amplificator baby hamster kidney cell mutant lines YMP1 and YMP7, compared to their wild-type controls. McMillan *et al.* (1990) found that amplificator mutants of the B16 melanoma line actually produced fewer experimental lung metastases than did their parental controls. The molecular mechanisms responsible for the altered phenotypes in these lines are either not reported or do not involve a DNA repair defect (Meuth *et al.,* 1979).

Other genes that affect DNA stability or repair are potential contributors to genetic instability and tumor progression. These include the poly (ADP-ribose) polymerase (PARP) gene, disregulation of which has been recently reported to be associated with gene amplification and chromosomal loss of heterozygosity in breast cancer (Bieche *et al.,* 1996). PARP is involved in DNA excision repair. Likewise, the p53 gene is known to affect DNA repair and has been suggested to contribute to genetic instability (Livingstone *et al.,* 1992; Yin *et al.,* 1992; Fishel, 1996). However, a recent report failing to link p53 mutations with other measures of genetic instability (indeed, the opposite correlation was seen) in colorectal cancers casts doubt on its role in this regard, at least in this neoplasm (Kahlenberg *et al.,* 1996).

e. Genetic Damage and Reactive Oxygen Species The possibility that reactive oxygen species, produced by a variety of metabolic and inflammatory processes, are involved in tumorigenesis, by virtue of their ability to damage DNA, has been reviewed by Feig *et al.* (1994). As will be discussed, phagocytic cells are a major source of reactive oxygen species, but it has also been shown that tumor cells themselves can produce large amounts of hydrogen peroxide (Szatrowski and Nathan, 1991). This raises the possibility that tumor cells contribute to their own genetic instability by producing oxyradical mutagens. There is little evidence for this hypothesis. However, Malins *et al.* (1996) recently compared the levels of hydroxyl-radical-associated DNA damage in a series of human breast cancers that had not metastasized to lymph nodes to those in similarly diagnosed breast cancers with nodal involvement. DNA from the metastatic tumors exhibited greater than a twofold increase in hydroxyl radical damage, as well as greater base diversity. The source of the hydroxyl radicals was not identified, nor were primary and metastatic tumor foci analyzed from the same women. However, this report suggests that reactive oxygen species may contribute to genomic instability in a way that is associated with tumor progression.

f. Tumor Environment and Genetic Instability

i. Infiltrating Inflammatory Cells So far this discussion of the possible origin of genetic instability in cancer cells has focused on the cancer cells themselves as the source of genetic damage. However, cancers exist in the presence of host cells and other microenvironmental factors which may contribute to their genetic instability as well.

Inflammatory cells, neutrophils, macrophages, and eosinophils, produce oxyradicals able to cause DNA damage similar to that produced by other radical-generating reactions (Reid and Loeb, 1992; Dizdaroglu *et al.,* 1993). Activated leukocytes are mutagenic in the Ames assay (Weitzman and Stossel, 1981, 1982; Fulton *et al.,* 1984) and carcinogenic *in vitro* (Weitzman *et al.,* 1985). Activated macrophages are able to induce DNA strand breaks

in tumor cells (Chong *et al.,* 1989). It appears likely that they are also involved in the long-appreciated association between chronic inflammation, would healing, and susceptibility to carcinogenesis (Rosin *et al.,* 1994).

Solid cancers are frequently infiltrated with inflammatory cells, including macrophages. Some years ago we analyzed the phenotypes of macrophages isolated from tumors produced by metastatic versus nonmetastatic mouse mammary tumor lines and found that those from metastatic tumors exhibited greater cytotoxic activity (Loveless and Heppner, 1983) and were more activated (Mahoney *et al.,* 1985) than were those from nonmetastatic tumors. We tested macrophages from these tumors for their ability to induce thioguanine resistance variants in a mouse mammary tumor cell line and found that the metastatic tumor-associated macrophages were able to do so at a significant frequency. Inhibitor studies indicated that the hydroxyl radical was likely responsible (Yamashina *et al.,* 1986).

Interestingly, TNF-α, a product of many infiltrating inflammatory and lymphoid cells, has been shown to induce manganese superoxide diamutase (Wong and Goeddel, 1988), an enzyme which protects cells from oxyradical damage and which has been shown to reverse the malignant phenotype of human breast cells (Li *et al.,* 1995). Thus, tumor-infiltrating inflammatory cells may be involved in both inducing and suppressing genetic instability in cancers.

ii. Tumor Microenvironment Paquette and Little (1994) compared the level of genomic rearrangements in a set of clones isolated from $C_3H/10T2$ cells, transformed by exposure to X-rays *in vitro,* that were either grown *in vitro* for 25 passages or injected into syngeneic mice and grown *in vivo* for 3 to 5 months (the number of cell divisions was approximately the same under the two conditions). The two sets of clones were then subcloned and their DNA was analyzed by fingerprinting. The *in vivo* propagated clones exhibited a much greater frequency of genomic rearrangements than those propagated *in vitro.* Further study suggested that selection for more divergent clones *in vivo* could not explain the results and the authors speculated that some property of the *in vivo* environment contributed to enhanced genomic instability. A recent study by Reynolds *et al.* (1996) reported on the mutation frequency in a λ phage shuttle vector contained in a single chromosome of mouse LN12 tumor cells grown either *in vitro* or in nude mice. The frequency of mutations in the *in vivo* grown cells was five times that of the cultured cells, and there was a different pattern of mutations (deletions and transversions) as well.

As reviewed by Vaupel *et al.* (1989), the tumor microenvironment differs from that of normal tissue in important respects, including areas of hypoxia, acid pH, and poor nutrient supply. Several groups have tested whether these factors might affect genetic instability and tumor progression.

Rice *et al.* (1986) demonstrated that exposure of Chinese hamster ovary cells to transient hypoxia enhanced resistance to MTX and amplification of the DHFR gene. Likewise, Young and Hill (1988) reported that transient hypoxia resulted in DNA overreplication and increased metastatic potential in a number of mouse tumor cell lines.

The relevance of these findings to genetic instability *in vivo,* however, is unclear. Young and Hill (1990a,b) isolated tumor cells from different regions of several types of mouse tumors. These regions differed in degree of oxygenation. The cells were reoxygenated in culture and then tested for metastatic ability (Young and Hill, 1990a,b). Subpopulations isolated from hypoxic regions did demonstrate an increase in metastatic ability, but the increase was only transient. Likewise, their degree of DNA overreplication was minor, compared to that of cells exposed to hypoxia *in vitro.* It is difficult to interpret these studies in regard to tumor progression. The techniques utilized could not detect small areas of DNA amplification which could be relevant to progression. Furthermore, it is difficult, based on these studies, to predict the effect of chronic hypoxia, followed by periods of reoxygenation, and hypoxia again as may occur *in vivo.* It is also difficult to separate transient effects on metastatic ability (due to changes in cell cycle, cell size, etc.) from permanent, genetic alteration. However, the matter is far from settled. Stoler *et al.* (1992) have described an anoxia-inducible endonuclease which they suggest could contribute to genetic instability of cancer cells. Furthermore, in the study by Reynolds *et al.* (1996) described above, the authors were able to reproduce the shuttle vector mutation frequency and pattern seen in tumor cells grown *in vivo* by exposing the cells to hypoxia *in vitro.*

Other microenvironmental factors that could effect genetic instability and progression are acidic pH and glucose starvation. Schlappack *et al.* (1991) investigated the effects of both on drug resistance, metastatic ability, and DNA content. The results somewhat depended upon the cell line. Both KHT sarcoma and B16 melanoma cells showed enhanced ability to colonize lungs, but increased DNA content (detected by flow cytometry) was seen only in KHT cells. MTX resistance was only slightly affected by acidosis and not at all by glucose starvation. Again, these studies are inconclusive and suffer from a lack of sensitivity in the experimental techniques.

g. Stability versus Instability in Tumor Heterogeneity and Progression

Genetic models of tumor heterogeneity and progression are based on the idea that, by whatever mechanisms, new clonal variants *occasionally* arise within a tumor population, and if they are viable and conditions are favorable, they may come to be an important influence on the behavior of the tumor. Many experimental models of tumor heterogeneity consist of sets of subpopulations or clones isolated from the same tumor, which are then

independently grown for periods of time. The stability of these subpopulations and clones becomes an issue in regard to assessing the degree of stability necessary to be important in tumor progression. If a clone is to be influential in the behavior of a cancer, and it is to be responsive to selection, it must have a degree of stability, but how much? Data indicating that clones grown individually may be more unstable than those grown in polyclonal cultures have already been discussed. If generally applicable, this may indicate that there is a limit on the monoclonality of a tumor population such that new clones will inevitably arise to maintain heterogeneity. It may also indicate that *in vivo* clones are more stable than isolated measurements indicate.

It is clear, however, that there are quantitative differences among cancers and among cancer subpopulations in degree of instability (Nagase *et al.*, 1987; Dulbecco and Armstrong, 1988). It is also clear that changes will occur in cancer populations and, in the absence of selection pressure, there may be a "phenotypic drift" resulting in an unexpected change in the characteristics of the population (Welch and Nicolson, 1983).

In some systems, and for some phenotypes, the degree of clonal instability has been reported to be quite high, on the order of $>10^{-5}$/cell/generation. This has been especially noted in the metastatic phenotype (Chambers *et al.*, 1981; Harris *et al.*, 1982; Hill *et al.*, 1984; Ling *et al.*, 1985). In part, this may be due to the sensitivity of that phenotype to experimental variables (Chambers *et al.*, 1981; Young and Hill, 1990a,b). On the other hand, measured rates of variation in some, but not all (Volpe and Milas, 1988), tumor models have indicated that metastatic variants can be generated at unusually high rates and that the ability to classify a particular tumor subpopulation line as either highly metastatic or poorly so reflects the balance between the "on and off" rates of generation of variants, which in the former favors the metastatic phenotype and in the latter the nonmetastatic one. This concept was presented by Ling and associates (1985) as the "dynamic heterogeneity" model of tumor metastasis. It is complicated by the well-known phenomenon of "metastatic insufficiency" that is the very low frequency at which even highly metastatic cells succeed in forming metastases due to the complexity of events required to complete the metastatic cascade (Miller and Heppner, 1990). Furthermore, metastasis no doubt involves many genes, so analysis of variant formation frequency is difficult in the context of a genetic model. In short, the contention that tumor population variants may be too unstable to form the basis of tumor progression probably reflects a lack of appreciation of the complexities in the *in vivo* tumor environment, as well as those which determine the metastatic phenotype.

2. Epigenetic Mechanisms in Tumor Progression

a. Alterations in DNA Methylation Although, as seen above, there is substantial evidence for direct genetic alterations in tumor progression,

there is also literature on the possibility that "epigenetic" mechanisms, particularly alterations in DNA methylation, may likewise contribute to clonal diversity. The pattern of methylation is inheritable (Holliday, 1987; Jones, 1996) and alterations in methionine metabolism are oftentimes observed in cancer cells (Hoffman, 1984), suggesting that they are especially susceptible to disruption of the normal pattern of DNA methylation. If so, gene expression my be affected and if the genes are involved in neoplastic processes, or in other traits of importance to cancer behavior, this altered gene expression could play a role in the cellular diversity and progression of neoplasms.

Early reports focused on the hypomethylation of DNA or of specific genes in cancer, relative to normal tissues (Feinberg and Vogelstein, 1983a,b; Diala *et al.*, 1983; Flatau *et al.*, 1983), suggesting that this resulted in the enhanced expression of genes during tumor development and progression. Indeed, a number of investigators demonstrated that hypomethylating agents, such as 5-azacytidine, induced inheritable, although relatively (compared to mutations) unstable changes in a variety of relevant phenotypes, including immunogenicity (Frost *et al.*, 1984), tumorigenicity, and metastatic ability (Kerbel *et al.*, 1984; Olsson and Forchhammer, 1984) and growth rate and clonogenicity (Olsson *et al.*, 1985). Similar results were reported with agents that interfered with methonine metabolism (Liteplo and Kerbel, 1986).

Further studies, however, suggested that the hypomethylation results were more complicated than originally thought. Goelz *et al.* (1985) reported that genes in benign polyps were as hypomethylated as those in colon cancers, indicating that this change might precede cancer formation. Also, the so-called hypomethylating agents, 5-aza-2 deoxycytidine and 2′-deoxy-5 azacytidine, were found to have additional activities, making it difficult to separate direct genetic effects from alterations in gene expression (Frost *et al.*, 1987; Alvarez *et al.*, 1988).

Now the focus of this research has shifted from hypomethlation to methylation (Jones, 1996). Rather than being an epigenetic phenomenon, cytosine methylation is suspected of being responsible for a range of inactivating point mutations in tumor suppressor genes, such as p53 (Rideout *et al.*, 1990; Greenblat *et al.*, 1994). Jones (1996) has suggested that increasing methylation of tumor suppressor gene CpG sites might play a role in tumor progression. Clearly, these new data do not negate the hypothesis that epigenetic alterations in gene expression are involved in the generation of tumor heterogeneity and tumor progression, but they do require a new experimental approach to test it.

b. Adaptation If scientific truth were established by vote, there is no question that the result would be "election" of genetic instability as the driving force in tumor progression. However, scientific truth is not decided

that way (although research grant funding is). An alternative theory to explain tumor heterogeneity and progression is the adaptation of cells to perturbations in their environment. Since the range of adaptive responses will be limited by the hereditary potential of the cells, over time, selection of particular genotypes will occur, but particular environmental conditions will induce the behavior necessary to survive. In this view, the main proponents of which are Farber and Rubin (1991), cancer development is analogous to embryological development in which "cell lineage and environmental induction interact in a complex manner to form differentiated tissues," and "progression reflects an imbalance in the homeostatic interactions operating in an organism and mutations may be as much an effect as a cause of this imbalance" (Hennessey and Rubin, 1986).

The experimental basis for this view comes from Rubin's detailed, meticulous series of experiments on the "spontaneous transformation" or, to quote Rubin (1994a), "physiological induction" of focus formation or other changes in growth response in transformed BALB/c3T3 mouse fibroblasts. In these studies the cells are subjected to mild adversity (excess NaCl concentration, low serum supplementation, crowding). Cell cultures treated in these ways exhibit many of the properties considered to be the basis of tumor progression, including a high degree of interclonal heterogeneity (H. Rubin *et al.,* 1984; A. L. Rubin *et al.,* 1990), selection of particular phenotypes upon further passage of adapted clones, a process Rubin terms "progressive state selection" (Rubin and Xu, 1989; Yao *et al.,* 1990; Rubin *et al.,* 1990; Grundel and Rubin, 1991), and, in addition, conformance to Foulds' rules (Rubin, 1994b). The new behavior can be inherited over many generations (H. Rubin *et al.,* 1985), but there is a high rate of reversal in some highly transformed clones (A. L. Rubin *et al.,* 1992), incompatible with a mutational origin for the behavior change. Further evidence for an adaptive as opposed to a genetic origin in the new behavior is the rapidity with which change may be noted (Hennessey and Rubin, 1986) and cloning experiments that show that all, or nearly all, cells are affected (Rubin, 1994b).

Although the fact that in Rubin's system progression can take place in the absence of cell proliferation (Rubin, 1994b) may also seem to be evidence against a mutational origin, MacPhee (1995) has recently provided a mechanism by which mutation could occur under these conditions.

The adaptation versus mutation controversy has a long history in biological science. It is a stimulus to thought and to experimental ingenuity. Rubin's experiments show that the matter is not yet settled.

3. Differentiation and Tumor Progression

Rubin has compared tumor progression to embryogenesis, a normal developmental process. Pierce and Cox (1978) characterized cancer as a carica-

ture of normal tissue renewal. A possible source of tumor cell heterogeneity, and hence progression, may be subpopulation variants derived by differentiation from a developmental, neoplastic stem cell. Unlike normal tissue differentiation, these variants would have to retain their proliferative potential if they are to participate in tumor progression. (Note: morphological and histological heterogeneity of tumors may also reflect processes resulting in terminal differentiation.) Teratocarcinoma is an obvious example of tumor heterogeneity through differentiation (Pierce and Cox, 1978). However, a number of other tumor models have been described in which variant tumorigenic lines are generated from an apparent neoplastic stem cell. These include a rat tumor of peripheral nerve origin, RT4, which consists of a stem cell line and its three variants (Imada and Sueoka, 1978), the mouse myeloma line MOPC-315 (Daley, 1981), and the human salivary gland tumor line HSGc (Shirasuna *et al.,* 1986). A variety of breast tumor models have also been described. One of our mouse mammary tumor subpopulation lines, line 68H, gave rise to a family of clonal lines of either fusiform or epithelioid morphology (Hager *et al.,* 1981). The fusiform variants remained stable upon passage, whereas the epithelioid clones continued to give rise to more variants. Among all the clones there was great heterogeneity in *in vitro* growth properties, karyotype (although they all shared common marker chromosomes), and tumorigenicity. The histologies of the tumors produced by individual clones included glandular, sarcomatous, and angiomatous patterns. A similar mouse mammary tumor line was also described by Sonnenberg *et al.* (1986) and rat mammary tumor lines by Rudland *et al.* (1982) and Dulbecco *et al.* (1986). In this later model, there was an especially careful attempt to relate the heterogeneity of the tumor to differentiation by the mapping of a large number of markers associated with various stages of mammary gland development to discrete, heterogeneous areas within the tumor (Dulbecco *et al.,* 1986).

RAMA 25, a stem cell line derived from a dimethylbenzanthracene-induced rat mammary tumor (Bennett *et al.,* 1978), forms tumors in nude mice with areas resembling both fibrosarcoma and carcinoma. We have recently described a human breast epithelial cell line which forms persistent lesions in immune-deficient mice (Miller *et al.,* 1993). These lesions are preneoplastic, progressing to carcinoma in about 25% of cases, of which approximately 75% are adenocarcinomas and the remaining 25% are undifferentiated carcinomas or squamous carcinomas (Miller *et al.,* 1993; Dawson *et al.,* 1996).

4. Cell Fusion and Tumor Heterogeneity

Cell biologists have long used fusion between different cell types as a way to investigate gene chromosomal location of various characteristics. Upon

division of fused cells, there may be an unequal distribution of chromosomes and chromosome loss, resulting in the production of variant cell populations. Cell–cell fusion can occur *in vivo* (Goldenberg *et al.*, 1974; DeBaetselier *et al.*, 1981; Kerbel *et al.*, 1983; F. R. Miller *et al.*, 1988) and may be a mechanism for generating tumor heterogeneity and progression (Goldenberg *et al.*, 1974). Fusion of normal cells with nonmetastatic tumor cells has been reported to yield highly metastatic variants (DeBaetselier *et al.*, 1981; Kerbel *et al.*, 1983) and F. R. Miller *et al.* (1989) have produced a more aggressive variant by fusion *in vitro* of two, less aggressive tumor cell clones. How often this mechanism is actually involved in tumor progression is not known, but it provides an unusual potential for rapid generation of diversity by asymmetric chromosome segregation following fusion.

IV. Consequences of Tumor Heterogeneity and Progression

A. Practical Considerations in Experimented and Clinical Cancer Research

The theoretical aspects of tumor heterogeneity aside, it is apparent that it poses a number of practical challenges for practitioners of experimental and clinical cancer research. Those who use cell culture and animal models have to be aware of the possibility of change in their experimental tools over time, as well as of the necessity for sampling strategies, of both physical and statistical variety, to deal with intratumor heterogeneity.

The issue of adequate sampling is currently being addressed in the flow cytometry and clinical pathology community. Shankey *et al.* (1995b) have called upon flow cytometrists to adopt sampling standards, quantitative models, and statistical techniques to deal with intratumor heterogeneity in DNA content. Zhang and Xie (1995) suggested that a minimum of three samples from different parts of the same primary or metastasis be tested. Beerman *et al.* (1991) found from their analysis that an average of four samples was needed for reliable determinants of ploidy status in human breast cancer. Other investigators have likewise commented on the extensive ploidy heterogeneity and the need for multiple samples in breast (Fuhr *et al.*, 1991; Schvimer *et al.*, 1995), prostate (Warzynski *et al.*, 1995), and liver (Okada *et al.*, 1995) cancers.

The need for multiple samples implies regional or zonal distributions of different clones within the same neoplasm. That this can be the case has already been discussed. However, karyotypic analysis of multiple regions and interphase cytogenetics which can reveal clonal heterogeneity within

a single area have both shown that karyotypically different clones can be interspersed, even in the absence of histological heterogeneity (Teixeira *et al.*, 1995; Alers *et al.*, 1995).

Assessment of indices of proliferation have also revealed considerable heterogeneity among multiple samples of the same breast (Meyer and Wittliff, 1991; Siitonen *et al.*, 1993), colon (Wilson *et al.*, 1993), and esophogeal (Jenner *et al.*, 1996) cancer. Although this heterogeneity may be clonal in origin (Balazs *et al.*, 1995), it could also reflect differences in the vascular supply, leading to differences in the proliferation rate. Jenner *et al.* (1996) therefore recommended that multiple samples be taken from both central and edge regions of biopsy specimens.

Finally, as already remarked, molecular genetic markers may also be heterogeneously distributed within a neoplasm, again necessitating the need for multiple samples (Mirchandani *et al.*, 1995; Giaretti *et al.*, 1996).

B. Tumor Heterogeneity in Prognosis

Adequate sampling of tumor specimens is of course important clinically for obtaining accurate prognostic information. However, it may be that it will yield additional information, as well. If tumor heterogeneity is a consequence of genetic/phenotypic instability, it may be that the extent of tumor heterogeneity per se is an indication of the likelihood of tumor progression and therefore a useful prognostic factor. Several recent studies have addressed this hypothesis, with somewhat variable results.

Van der Poel *et al.* (1993) assessed the degree of variability in cell parameters associated with karyotype (nuclear size, chromatin) in 121 renal cell carcinomas. Heterogeneity in these features was an unfavorable sign (poorer survival times) in patients who had not developed metastatic disease at the time of diagnosis but was less predictive in patients who had. In contrast, Ljungberg *et al.* (1996) analyzed multiple samples of 200 cases of renal cell carcinoma and found no correlation between the extent of ploidy heterogeneity and survival. They did find, however, that at least four samples were necessary to achieve a 90% probability of detecting an aneuploid clone and that aneuploidy was a significantly adverse prognostic factor.

Takahashi *et al.* (1994) analyzed DNA content, by flow cytometry, of multiple samples of 41 cases of primary epithelial ovarian cancer. None of the eight patients with only diploid DNA had lymph node involvement, in contrast to 70% of those with DNA heterogeneity, defined as two or more different aneuploid stem lines. DNA heterogeneity also correlated with stage, whereas the presence of only one aneuploid population did not.

A similar study was performed by Sasaki *et al.* (1993) on 74 cases of undifferentiated stomach cancer. Heterogeneity in DNA content was sig-

nificantly associated with other markers of poor prognosis only in tumors that had invaded the muscle layer. No such correlation was seen for cancers confined within the muscle layer. Fujimaki *et al.* (1996) have also used flow cytometry to assess variation in DNA content in multiple samples of gastric cancer. They reported that DNA ploidy heterogeneity itself correlated with invasion depth and was a better correlate of prognosis than was the presence of aneuploidy.

Yildiz *et al.* (1994) reported that intratumor DNA ploidy heterogeneity, rather than DNA ploidy, was a correlate of unfavorable histology and poor survival in 44 patients with Wilm's tumor.

On the other hand, Paradiso *et al.* (1995) found that heterogeneity among multiple samples in the thymidine labeling index, a measure of cellular proliferation, had no prognostic significance in terms of disease-free survival greater than 5 years in a series of 101 operable node-negative breast cancer patients. As discussed above, however, proliferative heterogeneity is probably the result of tumor environmental factors in addition to clonal heterogeneity. Furthermore, overall survival in node-negative patients is quite favorable.

Clearly, at this time, it is not known whether heterogeneity will prove to be a generally useful prognostic indicator. At the very least, the above studies suggest that it will depend upon the particular clinical situations and the type of characteristics being assessed.

C. Tumor Heterogeneity and Treatment

As has already been discussed, tumor cell heterogeneity is seen in a number of characteristics that impact on cancer therapy, including sensitivity to chemotherapy, hormone therapy, radiation therapy, and hyperthermia, as well as in parameters important to biological therapy and immunotherapy (antigenicity, immunogenicity, and sensitivity to growth factors and hormones). Such heterogeneity poses grave challenges to the successful treatment of clinical cancer.

One of these challenges is the development of predictive assays for therapeutic response. Several of the earliest reports of tumor heterogeneity involved demonstrations that cancer cells taken from different pieces of the same primary cancer, or from different foci of metastatic disease, were markedly divergent in response to drugs *in vitro* (Hakansson and Trope, 1974a,b; Trope *et al.*, 1979; Siracky, 1979). The advent of clonogenic (soft agar) assays for drug sensitivity likewise gave confirmation of substantial differences in the sensitivity of primary versus metastatic tumors and among individual metastases from the same patient in a variety of human tumors and to a variety of drugs (Schlag and Schreml, 1982; Tanigawa *et al.*, 1984;

von Hoff *et al.*, 1986). Von Hoff (1985) reviewed the implications of intratumor heterogeneity for *in vitro* drug testing of human cancers and listed several areas in which it presented significant difficulty: heterogeneity among subpopulations in the ability to form colonies in culture, heterogeneity in the size of the clones formed, heterogeneity in these regards in clones from metastatic versus primary cancers, and heterogeneity in drug sensitivity among cells from different sources, as much as 50% between two areas of the same primary and with such divergence between primaries and their metastases that there was not even a significant correlation between the results. Heterogeneity has also been reported in predictive assays for radiation responsiveness (Britten *et al.*, 1996) and hyperthermia (Woo *et al.*, 1991).

In addition to intratumor heterogeneity in sensitivity versus resistance to chemotherapeutic drugs, there are also reports of differences in the mechanisms by which the individual subpopulations are resistant (Rosowsky *et al.*, 1985; Ferguson and Cheng, 1989; de Vries *et al.*, 1989).

Tumor heterogeneity also poses an immense challenge to the development of effective treatment protocols against clinical cancer (Goldie and Coldman, 1984; Schnipper, 1986; Heppner and Miller, 1989). Goldie and Coldman (1984) have considered at length the implications of intrapatient therapeutic heterogeneity and have developed mathematical and computer models of strategies to maximize cure. They have suggested two major strategies. The first is to begin treatment at the time of the smallest tumor burden possible. Of course this strategy is not unique to addressing the problem of heterogeneity, but in this context it is based on the idea that the emergence of drug resistance variants is a stochastic process, the likelihood of which increases with time. The earlier the treatment in the life of a tumor, the less the chance that variants will be present.

The second strategy is the use of combination chemotherapy (or multimodality therapy) in which one hopes that individual variants will respond to at least one in a cocktail of non-cross-resistant agents. The ideal is to use all the drugs at once, but this is limited by host toxicity. Thus, alternating regimes of combinations of drugs with noncompeting sites of toxicity is recommended. Dose is an important consideration; lowering the dose limits host toxicity, but it also increases the chance that marginally resistant variants that would perhaps have been killed by higher doses will be enriched as the more sensitive cells are killed. The Goldie and Coldman analysis suggests that combinations of multiple agents need to be given at maximum doses and at minimum intervals.

Clearly, these strategies are complex in concept and perilous in practice. Success depends, in part, on the rate and degree at which heterogeneity arises in a particular neoplasm. It is confounded by the interrelationships among tumor cell subpopulations (Heppner and Miller, 1989) and between

tumor cells and their environment (Leith and Michelson, 1990). Successful eradication of some variants may upset the balance of the "cellular society" and alter the course of tumor progression, not for the better, by allowing the escape of more aggressive subpopulations from interclonal control. If, as suggested by Poste and Grieg (1982), this control includes limitations on the generation of new variants, chemotherapy could, in effect, unleash an "Andromeda Strain" type of neoplastic instability.

Although most emphasis on the therapeutic implications of tumor heterogeneity has been placed on chemotherapy, the same considerations apply to other modalities. For example, numerous investigators have suggested the use of cocktails of different monoclonal antibodies, directed against different, heterogeneously distributed epitopes, for antibody-based therapy or imaging (Hand *et al.,* 1983; Olsson *et al.,* 1984; Roth *et al.,* 1984; Fargion *et al.*, 1986).

As we have described, it is possible to demonstrate tumor subpopulation interactions that have the effect of increasing the sensitivity of otherwise insensitive tumor clones to therapy (Heppner and Miller, 1989; B. E. Miller *et al.,* 1989) even in well-established tumors (Miller *et al.,* 1991). Other investigators have reported similar findings with hormone therapy (Robinson and Jordan, 1989). This raises the possibility that such interactions could be utilized in the design of more effective therapy. The multiplicity of mechanisms involved makes this approach problematic, but its clinical feasibility may have, in fact, been inadvertently demonstrated in a number of gene therapy experiments in which the results of the therapy seem to have been greater than expected based on the proportion of cells within the cancer that had been genetically altered (Moolten, 1986; Culver *et al.,* 1992; Freeman *et al.,* 1993; Roth *et al.,* 1996). This phenomenon, called the "bystander effect," may actually represent special cases of tumor subpopulation interactions in which the source of cellular heterogeneity is not nature but rather the therapist who has produced variant populations by introducing genes into only a fraction of the neoplastic population.

V. Concluding Remarks

As has been reviewed, tumor heterogeneity and progression are major features of neoplastic development and contribute adversely to our ability to control neoplastic diseases. For cancer researchers and clinicians, heterogeneity and progression represent problems that need to be solved or circumvented. However, it may be instructive to step back and ask what the significance of cancer heterogeneity and progression may be in a larger biological context. The analogy between progression and evolution has

already been mentioned, but how parallel are the processes and phenomena that have been discussed to situations in "normal," i.e., nonneoplastic, biology?

A. Heterogeneity of Normal Cell Populations

A great deal of the literature on tumor heterogeneity derives from studies in which tumor tissues are disaggregated, subpopulations and clones isolated by one means or another, and then the characteristics of these subcomponents analyzed. As we have seen, such studies demonstrate considerable variability. How do normal cells behave in similar circumstances?

Griffen *et al.* (1980) examined fibroblasts from human skin explants for the level of steroid 5α-reductase activity. Cultures from genital skin had high activity whereas activity in nongenital skin cultures was low. Clones derived from genital skin fibroblasts were extremely variable with activities ranging from barely detectable to very high. Clones from nongenital skin fibroblasts had uniformly low activity. High-activity clones gradually gave rise to mixed cultures of high- to low-activity clones. Low-activity clones remained stable.

Wier *et al.* (1987) studied the cellular and subcellular distribution of $[Ca^{2+}]_i$ as well as cellular appearance and contractile behavior in single, isolated rat cardiac ventricular cells. There were three subpopulations of cells that differed significantly in these parameters. The authors state that "experiments performed on suspensions of cells should be interpreted with caution."

Baroffio *et al.* (1995) examined the characteristics of isolated human muscle satellite cells (HMSC; prefusion cells) and their progeny in regard to a variety of muscle-associated markers. There was marked homogeneity among the HMSC themselves, but their clonal progeny consisted of at least four subpopulations, one of which appeared to have been "born in culture."

Ceriani *et al.* (1984) analyzed the variability in expression of a breast-associated surface antigen in cultures of normal human breast, human breast cancers, and apparently normal tissue peripheral to cancers. All three types of cells exhibited single-cell variability, although normal cells had the lowest coefficient of variation (48 ± 5%). Rates of development of quantitatively different variants were measured in clones of all three types of culture. Although the rate for cancer cells was highest (1.2×10^{-2}/cell/generation), the rates for normal cells (0.12×10^{-2}) and cancer-adjacent normal cells (0.3×10^{-2}) were not negligible.

Not all demonstrations of normal cell heterogeneity are in cell culture. Hughes *et al.* (1986) reported phenotypically distinct subpopulations, as

detected by a panel of monoclonal antibodies, among glands in normal mucosa from patients with colon cancer or various nonmalignant conditions.

Smeds *et al.* (1987) demonstrated *in situ* what appeared to be clones of cells with a very high proliferation potential in the follicular epithelium of mouse thyroids. Clonality was indicated by the labeling pattern of a single injection of [^{3}H]thymidine. These authors concluded the high intrinsic growth rate is a "stable, inheritable trait."

These examples of variability in normal tissues and in cultures of normal tissues indicate that cellular heterogeneity is not an exclusive property of neoplastic tissues. Of course, the demonstration of cellular variability does not address the mechanism by which it is generated, a conclusion that is as valid for neoplastic tissues as it is for normal ones.

B. Cellular Societies, Hierarchical Organizations, and Tumor Progression

If normal and neoplastic cells are fundamentally heterogeneous, it is also true that they are not independent units in multicellular organisms but function together to produce tissues and integrated cellular systems. For normal cells this conclusion is self-obvious. For neoplastic cells, its validity is demonstrated by the examples of tumor subpopulation and clonal interactions cited in this review. In both cases, the variability of the individual cellular units is subsumed through interrelationships with the whole. This is the principle of hierarchical organization in which the larger unit of organization (the tissue, the interacting polyclonal population) has a greater degree of predictability in behavior than do the smaller units of which it is composed (Rubin, 1994a). If this was not true of cancer, it would not be possible to develop any coherent view of the course of development, progression of disease, or strategies for treatment. For the most part, even in cancer, the hierarchy prevails, but a weakening of the interactions among the cellular units may eventually allow individual subpopulations to escape hierarchical control and become functionally independent. Breaking cell–cell bonds and intercellular communication is known to induce neoplastic behavior (Rubin, 1994a) and is believed to be involved in neoplastic development (Trosko *et al.*, 1983). From this perspective tumor progression is less a manifestation of selection and "survival of the fittest" than the breakdown of ordered control imposed by the "top level operations of the organism" that is normally relayed down to intermediate levels of greater variance (cells) and then to even lower levels (molecular) (Weiss, 1973; Rubin, 1994a). Again, from this perspective, the molecular alterations in gene structure and function that are taken as evidence of "genetic instabil-

ity" may be, in reality, the result, not the cause, of the loss of control at the top.

Irrespective of whether or not cancer progression is the result of the breakdown of hierarchical order or the accumulation of genetic and phenotypic heterogeneity, it *happens,* at the level of the whole tumor and in the composition of the cellular populations of which the tumor is made. Selection undoubtedly plays a role in these changes and selection pressures (such as hormones, drugs, microenvironmental factors, and organ-specific factors) help shape the cancer's course and outcome. Is selection the only role for the environment in this process or does it play a more instructive or inductive role, challenging cancer cells to adapt to new conditions, thereby assuring that whatever the gene composition of the most adaptable cells and whatever the characteristics these genes might confer, they will be better represented in succeeding generations than they were in the starting population?

Finally, variability may be a fundamental property of life but it need not signify a lack of order. That order may not be apparent, however, at least from the level from which we view the system. "Chaos" is the term used to describe "the presence of hidden order within apparent disorder" (Posadas *et al.,* 1996). It is becoming increasingly recognized that chaotic variability is a sign of health and breaks down in pathological states (Pool, 1989). Coffey and associates (Posadas *et al.,* 1996) have begun to use tumor heterogeneity as a way to analyze chaos in cancer and to see whether chaos can be controlled, even if it is not fully understood. Where these pioneering efforts will lead is itself uncertain, but how ironic it would be if the study of cancer, which to many is the epitome of disorder, will open our minds to an appreciation of the ultimate basis for order.

References

Aabo, K., Roed, H., Vindelov, L. L., and Spang-Thomson, M. (1994). A dominated and resistant subpopulation causes regrowth after response to 1,3-Bis (2-chloroethyl 1)-1-nitrosourea treatment of a heterogeneous small cell lung cancer xenograft in nude mice. *Cancer Res.* **54,** 3295–3299.

Aabo, K., Vindelov, L. L., and Spang-Thomson, M. (1995). Clonal dominance between subpopulations of mixed small cell lung cancer xenografts implanted ectopically in nude mice. *Eur. J. Cancer* **31A,** 222–229.

Abbruzzese, J. L., Abbruzzese, M. C., Hess, K. R., Raber, M. N., Lenzi, R., and Frist, P. (1994). Unknown primary carcinoma: Natural history and prognostic factors in 657 consecutive patients. *J. Clin. Oncol.* **12,** 1272–1280.

Alam, S. M., Whitford, P., Cushley, W., George, W. D., and Campbell, A. M. (1992). Aneuploid subpopulations in tumor invaded lymph nodes from breast cancer patients. *Eur. J. Cancer* **28,** 357–362.

Albino, A. P., Lloyd, K. O., Houghton, A. N., Oettgen, H. F., and Old, L. J. (1981). Heterogeneity in surface antigen and glycoprotein expression of all cell lines derived from different melanoma metastases of the same patient. *J. Exp. Med.* **154,** 1764–1778.

Alers, J. C., Krijtenburg, P. J., Vissers, C. J., Bosman, F. T., van der Kusast, T. H., and van Dekken, H. (1995). Cytogenetic heterogeneity and histologic tumor growth patterns in prostatic cancer. *Cytometry* **21,** 84–94.

Allalunis-Turner, M. J., Barron, G. M., Day, R. S., Dobler, K., and Urtasun, R. C. (1992). Heterogeneity in response to treatment with buthionine sulfoximine or interferon in human malignant glioma cells. *Int. J. Radiat. Oncol. Biol. Phys.* **22,** 765–768.

Alvarez, E., Elliott, B. E., Houghton, A. N., and Kerbel, R. S. (1988). Heritable high frequency modulation of antigen expression in neoplastic cells exposed to 5-aza-2′-deoxycytidine or hydroxyurea: Analysis and implications. *Cancer Res.* **48,** 2440–2445.

Azizi, E., Fiedman, J., Pavlotsky, F., Iscovich, J., Bornstein, A., Shafir, R., Trau, H., Brenner, H., and Nass, D. (1995). Familial cutaneous malignant melanoma and tumors of the nervous system—a hereditary cancer syndrome. *Cancer* **76,** 1571–1578.

Balazs, M., Matsumura, K., Moore, D., Pinkel, D., Grey, J. W., and Waldman, F. M. (1995). Karyotypic heterogeneity and its relation to labeling index in interphase breast tumor cells. *Cytometry* **20,** 62–73.

Baroffio, A., Bouhaton-Prallat, M.-L., Gabbiani, G., and Bader, C. R. (1995). Heterogeneity in the progeny of single human muscle satellite cells. *Differentation* **59,** 259–268.

Beerman, H., Smit, V. T. H. B. M., Kluin, P. M., Bonsing, B. A., Hermans, J., and Cornelisse, C. J. (1991). Flow cytometric analysis of DNA stemline heterogeneity in primary and metastatic breast cancer. *Cytometry* **12,** 147–154.

Bennett, D. C., Peachey, L. A., Durbin, H., and Rudland, P. S. (1978). A possible mammary stem cell line. *Cell* **15,** 283–298.

Bichel, P. (1972). Specific growth regulation in three ascitic tumors. *Eur. J. Cancer* **8,** 167–173.

Bieche, F., deMurcia, G., and Lidereau, R. (1996). Poly (ADP-ribose) polymerase gene expression status and genomic instability in human breast cancer. *Clin. Cancer Res.* **2,** 1163–1167.

Biswas, C., Moran, W. P., Bloch, K. J., and Gross, J. (1978). Collagenolytic activity of rabbit V2-carcinoma growing at multiple sites. *Biochem. Biophys. Res. Commun.* **80,** 33–38.

Boone, C. W., Vembu, D., White, B. J., Takeichi, N., and Parajpe, M. (1979). Karyotypic, antigenic, and kidney-invasive properties of cell lines form fibrosarcomas arising in C3H/10T1/2 cells implanted subcutaneously attached to plastic plates. *Cancer Res.* **39,** 2172–2178.

Brattain, M. G., Fine, W. D., Khalel, F. M., Thompson, J., and Brattain, D. E. (1981). Heterogeneity of malignant cells from human colonic carcinomas. *Cancer Res.* **41,** 1751–1756.

Brattain, M. G., Marks, M. E., McCombs, J., Finley, W., and Brattain, D. E. (1983). Characterization of human colon carcinoma cell lines isolated from a single primary tumour. *Br. J. Cancer* **47,** 373–381.

Brennan, M. J., Donigan, W. L., and Appleby, D. E. (1979). The variability of estrogen receptors in metastatic breast cancer. *Am. J. Surg.* **137,** 260–262.

Brentnall, T. A., Crispin, D. A., Bronner, M. P., Cherian, S. P., Hueffed, M., Rabinovitch, P. S., Rubin, C. E., Haggitt, R. C., and Boland, C. R. (1996). Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res.* **56,** 1237–1240.

Britten, R. A., Evans, A. J., Allalunis-Turner, M. J., Franko, A. J., and Pearcey, R. G. (1996). Intratumoral heterogeneity as a confounding factor in clonogenic assays for tumor radioresponsiveness. *Radiother. Oncol.* **39,** 145–153.

Brodt, P., Parker, R., Sarkar, P., and LaLa, P. K. (1985). Studies on clonal heterogeneity in two spontaneously metastasizing mammary carcinomas of recent origin. *Int. J. Cancer* **35,** 265–273.

Brown, J. L., Russell, P. J., Phillips, J., Wotherspoon, J., and Raghaven, D. (1990). Clonal analyses of a bladder cancer cell line: An experimental model of tumour heterogeneity. *Br. J. Cancer* **61,** 369–376.

Browning, H. C. (1948). Heterologous and homologous growth of transplants during the course of development of spontaneous mammary tumors in C3H mice. *JNCI, J. Natl. Cancer Inst.* **8,** 173–189.

Caignard, A., Martin, M. S., Michel, M. F., and Martin, F. (1985). Interaction between two cellular populations of a rat colonic carcinoma when inoculated to the syngeneic host. *Int. J. Cancer* **36,** 273–279.

Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S., Corio, R., Lee, D., Greenberg, B., Koch, W., and Sidransky, D. (1996). Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res.* **56,** 2488–2492.

Ceriani, R. L., Peterson, J. A., and Blank, E. W. (1984). Variability in surface antigen expression of human breast epithelial cells cultured from normal breast, normal tissue peripheral to breast carcinomas and breast carcinomas. *Cancer Res.* **44,** 3033–3039.

Chambers, A. F., Hill, R. P., and Ling, V. (1981). Tumor heterogeneity and stability of the metastatic phenotype of mouse KHT sarcoma cells. *Cancer Res.* **41,** 1368–1372.

Cheshire, P. J. (1970). The effect of multiple tumours on mammary tumour growth rates in the C3H mouse. *Br. J. Cancer* **24,** 542–547.

Chong, Y. C., Heppner, G. H., Paul, L. A., and Fulton, A. M. (1989). Macrophage-mediated induction of DNA strand breaks in target tumor cells. *Cancer Res.* **46,** 6652–6657.

Cifone, M. A., and Fidler, I. J. (1981). Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. *Proc. Natl. Acad. Sci. USA* **78,** 6249–6252.

Cillo, C., Dick, J. E., Ling, V., and Hill, R. P. (1987). Generation of drug-resistant variants in metastatic B16 mouse melanoma cell lines. *Cancer Res.* **47,** 2604–2608.

Coons, S. W., Johnson, P. C., and Shapiro, J. R. (1995). Cytogenetic and flow cytometry DNA analysis of regional heterogeneity in a low grade human glioma. *Cancer Res.* **55,** 1569–1577.

Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. (1992). In vivo gene transfer with retroviral-producer cells for treatment of experimental brain tumors. *Science* **256,** 1550–1552.

Czarnecki, D., Tait, B., Nicholson, I., and Lewis, A. (1994). Multiple non-melanoma skin cancer: evidence that different MHC genes are associated with different cancers. *Dermatology* **188,** 88–90.

Daley, M. J. (1981). Intratumor maturational heterogeneity with the murine myeloma MOPC-315. *Cancer Res.* **41,** 187–191.

Damen, J. E., Tagger, A. Y., Greenberg, A. H., and Wright, J. A. (1989). Generation of metastatic variants in populations of mutator and amplificator mutants. *JNCI, J. Natl. Cancer Inst.* **81,** 628–631.

Danielpour, D., and Sirbasku, D. A. (1984). New perspectives in hormone-dependent (responsive) and autonomous mammary tumor growth: role of autostimulatory growth factor. *In Vitro* **20,** 975–980.

Dawson, P. J., Wolman, S. R., Tait, L., Heppner, G. H., and Miller, F. R. (1996). MCF10AT: A model for the evolution of cancer from proliferative breast disease. *Am. J. Pathol.* **148,** 313–319.

DeBaetselier, P., Gorelik, E., Eshhar, Z., Ron, Y., Katzav, S., Feldman, M., and Segal, S. (1981). Metastatic properties conferred on non-metastatic tumours by hybridization of spleen B-lymphocytes with plasmacytoma cells. *JNCI, J. Natl. Cancer Inst.* **67,** 1079–1087.

de Vries, E. G. E., Meijer, C., Timmer-Bosscha, H., Berendsen, H. H., de Leij, L., Schaper, R. J., and Mulder, N. H. (1989). Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up. *Cancer Res.* **49,** 4175–4178.

Dexter, D. L., Kowalski, H. M., Blazar, B. A., Fligiel, Z., Vogel, R., and Heppner, G. H. (1978). Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res.* **38,** 3174–3181.

Diala, E. S., Cheah, M. S. C., Rowitch, D., and Hoffman, R. M. (1983). Extent of DNA methylation in human tumor cells. *JNCI, J. Natl. Cancer Inst.* **71,** 755–764.

Dizdaroglu, M., Olinski, R., Doroshow, J. H., and Akman, S. A. (1993). Modification of DNA bases in chromatin of intact target human cells by activated human polymorphonuclear leucocytes. *Cancer Res.* **53,** 1269–1272.

Dominguez, O. V., and Huseby, R. A. (1968). Heterogeneity of induced testicular interstitial cell tumors of mice as evidenced by steroid biosynthetic enzyme activities. *Cancer Res.* **28,** 348–353.

Duggan, B. D., Felix, J. C., Muderspach, L. I., Tourgeman, D., Zheng, J., and Shibuta, D. (1994). Microsatellite instability in sporadic endometrial carcinoma. *J. Natl. Cancer Inst.* **86,** 1216–1221.

Dulbecco, R., and Armstrong, B. (1988). Stochastic development of invasive potential in rat mammary tumors induced by N-methyl-N-nitrosourea. *Proc. Natl. Acad. Sci. USA* **85,** 8659–8663.

Dulbecco, R., Armstrong, B., Allen, W. R., and Bowman, M. (1986). Distribution of developmental markers in rat mammary tumors induced by N-nitrosourea. *Cancer Res.* **46,** 5142–5144.

Edwards, R. E., Hill, R. J., Brown, D. G., and Carthew, P. (1990). Phenotypic stability and metastatic behavior of serially xenografted rat mesothaliomas. *Br. J. Cancer* **62,** 201–208.

Eldridge, S. R., and Gould, M. N. (1992). Comparison of spontaneous mutagenesis in early passage human mammary cells from normal and malignant tissue. *Int. J. Cancer* **50,** 321–324.

Eng, C., Mulligan, L. M., Healey, C. S., Houghton, C., Frilling, A., Rave, F., Thomas, G. A., and Ponder, B. A. J. (1996). Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. *Cancer Res.* **56,** 2167–2170.

Fante, R., Roncucci, L., Di Gregorio, C., Tamassia, M. G., Losi, L., Benatti, P., Pedroni, M., Percesepe, A., De Pietri, S., and Ponz de Leon, M. (1996). Frequency and clinical features of multiple tumors of the large bowel in the general population and in patients with hereditary colorectal carcinoma. *Cancer* **77,** 2013–2021.

Farber, E., and Rubin, H. (1991). Cellular adapatations in the origin and development of cancer. *Cancer Res.* **51,** 2751–2761.

Fargion, S., Carney, D., Mulshine, J., Rosen, S., Bunn, P., Jewett, P., Cuttitta, F., Gazdar, A., and Minna, J. (1986). Heterogeneity of cell surface antigen expression of human small cell lung cancer detected by monoclonal antibodies. *Cancer Res.* **46,** 2633–2638.

Fearon, E. R., and Vogelstein, B. A. (1990). A genetic model for colorectal tumorigeneis. *Cell* **61,** 759–767.

Feig, D. I., Reid, T. M., and Loeb, L. A. (1994). Reactive oxygen species in tumorigenesis. *Cancer Res.* **54,** 1890s–1894s.

Feinberg, A., and Vogelstein, B. (1983a). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature (London)* **301,** 89–92.

Feinberg, A. P., and Vogelstein, B. (1983b). Hypomethylation of ras oncogenes in primary human cancers. *Biochem. Biophys. Res. Commun.* **111,** 47–54.

Ferguson, P. J., and Cheng, Y. (1989). Phenotypic instability of drug sensitivity in a human colon carcinoma cell line. *Cancer Res.* **49,** 1148–1153.

Fialkow, P. J. (1979). Clonal origin of human tumors. *Annu, Rev. Med.* **30,** 135–143.

Fidler, I. J., and Kripke, M. L. (1977). Metastasis results from pre-existing variant cells within a malignant tumor. *Science* **197,** 893–895.

Fiegl, M., Tueni, C., Schenk, T., Jakesz, R., Gnant, M., Reiner, A., Rudes, M., Pirc-Danoewinata, H., Marosi, C., Huber, H., and Drach, J. (1995). Interphase cytogenetics reveals a high

incidence of aneuploidy and intra-tumour heterogeneity in breast cancer. *Br. J. Cancer* **72,** 51–55.

Fishel, R. (1996). Genomic instability, mutators, and the development of cancer: Is there a role for p53? *J. Natl. Cancer Inst.* **88,** 1608–1609.

Fishel, R., Lescoe, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M., and Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75,** 1027–1038.

Fisher, B., Gunduz, N., Coyle, J., Rudock, C., and Saffer, E. (1989). Presence of a growth-stimulating factor in serum following primary tumor removal in mice. *Cancer Res.* **49,** 1996–2001.

Flatau, E., Bogenmann, E., and Jones, P. A. (1983). Variable 5-methylcytosine levels in human tumor cell lines and fresh pediatric tumor explants. *Cancer Res.* **43,** 4901–4905.

Foulds, L. (1969). "Neoplastic Development," Vol. 1. Academic Press, New York.

Foulds, L. (1975). "Neoplastic Development," Vol. 1. Academic Press, New York.

Frankfurt, O. S., Seckinger, D., and Sugarbaker, E. V. (1991). Intercellular transfer of drug resistance. *Cancer Res.* **51,** 1190–1195.

Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. (1993). The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* **53,** 5274–5283.

Frost, P., Liteplo, R. G., Donaghue, T. P., and Kerbel, R. S. (1984). Selection of strongly immunogenic "TUM-" variants from tumors at high frequency using 5-azacytidine. *J. Exp. Med.* **149,** 1491–1501.

Frost, P., Kerbel, R. S., Hunt, B., Man, S., and Pathak, S. (1987). Selection of metastatic variants with identifiable karyotypic changes from a nonmetastatic murine tumor after treatment with 2′-deoxy-5-azacytidine or hydroxyurea: Implications for mechanisms of tumor progression. *Cancer Res.* **47,** 2690–2695.

Fucci, L., Pirrelli, M., and Caruso, M. L. (1994). Carcinoma and synchronous hyperplastic polyps of the large bowel. *Pathologica* **86,** 371–375.

Fuhr, J. E., Frye, A., Kattine, A. A., and Van Meter, S. (1991). Flow cytometric determination of breast tumor heterogeneity. *Cancer* **67,** 1401–1405.

Fujii, H., Marsh, C., Carnes, P., Sidransky, D., and Gabrielson, E. (1996). Genetic divergence in the clonal evolution of breast cancer. *Cancer Res.* **56,** 1493–1497.

Fujimaki, E., Sasaki, K., Nakano, O., Chiba, S., Tazawa, H., Yamashiki, H., Orii, S., and Sugai, T. (1996). DNA ploidy heterogeneity in early and advanced gastric cancers. *Cytometry* **26,** 131–136.

Fulton, A. M., Loveless, S. E., and Heppner, G. H. (1984). Mutagenic activity of tumor-associated macrophages in Salmonella typhimurium strains TA98 and TA100. *Cancer Res.* **44,** 4308–4311.

Gagos, S., Iliopoulos, D., Tscleni-Balafouta, S., Agapitos, M., Antachupoulos, C., Kostakis, A., Karayannakos, P., and Skalkeas, G. (1996). Cell senescence and a mechanism of clonal evolution leading to continuous cell proliferation, loss of heterozygosity, and tumor heterogeneity: Studies on two immortal colon cancer cell lines. *Cancer Genet. Cytogenet.* **90,** 157–165.

Geradts, J., Richards, J., Edery, M., Pang, K., Larson, L., and Nandi, S. (1986). Heterogeneity in the hormonal responsiveness of clones derived from the 13762 NF rat mammary tumor. *Cancer Res.* **46,** 1920–1927.

Gerharz, C. D., Gabbert, H. E., Engers, R., Ramp, U., Mayer, H., Bicsalski, H. K., and Luley, G. (1989). Heterogeneous response to differentiation induction in different clonal subpopulations of a rat rhabdomyosarcoma cell line (BA-HAN-1). *Cancer Res.* **49,** 7132–7140.

Giaretti, W., Monaco, R., Pujic, N., Rapallo, A., Nigro, S., and Geido, E. (1996). Intratumor heterogeneity of K-ras α mutations in colorectal adenocarcinomas. *Am. J. Pathol.* **149,** 237–245.

Giavazzi, R., Alessandri, G., Spreafico, F., Garattini, S., and Mantovani, A. (1980). Metastasizing capacity of tumour cells from spontaneous metastases of transplanted murine tumours. *Br. J. Cancer* **42,** 462–472.

Gitelman, I., Dexter, D. F., and Roder, J. C. (1987). DNA amplification and metastases of the human melanoma cell line MeWo. *Cancer Res.* **47,** 3851–3855.

Glick, A. B., Weinberg, W. C., Wu, I. H., Quan, W., and Yuspa, S. A. (1996). Transforming growth factor B1 suppresses genomic instability independent of a G1 arrest, p53 and Rb. *Cancer Res.* **56,** 3645–3650.

Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* **228,** 187–198.

Goldenberg, D. M., Pavia, R. A., and Tsao, M. C. (1974). In vivo hybridization of human tumour and normal hamster cells. *Nature* (*London*) **250,** 649–651.

Goldie, J. H., and Coldman, A. J. (1984). The genetic origin of drug resistance in neoplasms: implications for systemic therapy. *Cancer Res.* **44,** 3643–3653.

Good, D. J., Polverini, P., Rastinejad, F., LeBeau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. (1990). A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. USA* **87,** 6624–6628.

Gorelik, E., Segal, S., and Feldman, M. (1978). Growth of a local tumor exerts a specific inhibitory effect on progression of lung metastases. *Int. J. Cancer* **21,** 617–625.

Gorelik, E., Segal, S., and Feldman, M. (1981). On the mechanism of tumor "concomitant" immunity. *Int. J. Cancer* **27,** 847–856.

Greenblat, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Res.* **54,** 4855–4878.

Griffen, J. E., Allman, D. R., Durrant, J. L., and Wilson, J. D. (1980). Variation in steroid 5α-reductase activity in cloned human skin fibroblasts. *J. Biol. Chem.* **256,** 3662–3666.

Grundel, R., and Rubin, H. (1991). Effect of interclonal heterogeneity on the progressive, confluence-mediated acquisition of the focus-forming phenotype in NIH-3T3 populations. *Cancer Res.* **51,** 1003–1013.

Hager, J. C., Miller, F. R., and Heppner, G. H. (1978). Influence of serial transplantation on the immunological–clinical correlates of BALB/cfC3H mouse mammary tumors. *Cancer Res.* **38,** 2492–2500.

Hager, J. C., Fligiel, S., Stanley, W., Richardson, A. M., and Heppner, G. H. (1981). Characterization of a variant-producing tumor cell line from a heterogeneous strain Balb/cfC3H mouse mammary tumor. *Cancer Res.* **41,** 1293–1300.

Hakansson, L., and Trope, C. (1974b). Cell clones with different sensitivity to cytostatic drugs in methylcholanthrene-induced mouse sarcomas. *Acta Pathol. Microbiol. Scand. Secti A* **82,** 41–47.

Hakansson, L., and Trope, C. (1974a). On the presence within tumours of clones that differ in sensitivity to cytostatic drugs. *Acta Pathol. Microbiol. Scand., Sect A* **82A,** 35–40.

Han, H.-J., Yanagisawa, A., Kato, Y., Park, J.-G., and Nakamura, Y. (1993). Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. *Cancer Res.* **53,** 5087–5099.

Hand, P. H., Nuti, M., Colcher, D., and Schlom, J. (1983). Definition of antigenic heterogeneity and modulation among human mammary carcinoma cell populations using monoclonal antibodies to tumor-associated antigens. *Cancer Res.* **43,** 728–735.

Harris, J. F., Chambers, A. F., Hill, R. P., and Ling, V. (1982). Metastatic variants are generated spontaneously at a high rate in mouse KHT tumor. *Proc. Natl. Acad. Sci. USA* **79,** 5547–5551.

Hart, I. R. (1984). Tumor cell hybridization and neoplastic progression. *In* "Cancer Invasion and Metastasis: Biological and Therapeutic Aspects" pp. 133–143. Raven Press, New York.

Hauschka, T. S. (1953). Methods of conditioning the graft in tumor transplantation. *JNCI, J. Natl. Cancer Inst.* **14,** 723–736.
Hennessey, T. L., and Rubin, H. (1986). Development and inheritance of osmotic tolerance in a line of spontaneously transformed Balb/c/3T3 cells. *Cancer Res.* **46,** 6041–6048.
Heppner, G. H. (1989). Tumor cell societies. *JNCI, J. Natl. Cancer Inst.* **81,** 648–649.
Heppner, G. H., and Miller, B. E. (1989). Therapeutic implications of tumor heterogeneity. *Semin. Oncol.* **16,** 91–105.
Heppner, G. H., Dexter, D. R., DeNucci, T., Miller, F. R., and Calabresi, P. (1978). Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mouse mammary tumor. *Cancer Res.* **38,** 3758–3763.
Heppner, G. H., Miller, B., Cooper, D. N., and Miller, F. R. (1980). Growth interactions between mammary tumor cells. *In* "Cell Biology of Breast Cancer" (C.M. McGrath, M.J. Brennan, and M.A. Rich, eds.), pp. 161–172. Academic Press, New York.
Hill, H. Z., Hill, G. J., Miller, C. F., Kwong, F., and Purdy, J. (1979). Radiation and melanoma response of B16 mouse tumor cells and clonal lines to in vitro irradiation. *Radiat. Res.* **80,** 259–276.
Hill, R. P., Chambers, A. F., Ling, V., and Harris, J. F. (1984). Dynamic heterogeneity: Rapid generation of metastatic variants in mouse B16 melanoma cells. *Science* **224,** 998–1001.
Hoffman, R. M. (1984). Altered methionine metabolism, DNA methylation and oncogene expression. *Biochim. Biophys. Acta* **738,** 49–87.
Holliday, R. (1987). The inheritance of epigenetic defects. *Science* **238,** 163–170.
Horvath, G., Ferno, M., Baldetori, B., and Johansson, M. (1993). Growth interaction between different tumor populations in human endometrial adenocarcinoma growing in nude mice. *In Vivo* **7,** 511–518.
Hostetler, L. W., and Kripke, M. L. (1988). Proportion of antigenic variants induced by in vitro uv irradiation differs in clones derived from a single tumor. *J. Immunol.* **140,** 666–670.
Hsu, S. H., Luk, G., Krush, A. J., Hamilton, S. R., and Hoover, H. H. (1983). Multiclonal origin of polyps in Gardner Syndrome. *Science* **221,** 951–953.
Hughes, N. R., Walls, R. S., Newland, R. C., and Payne, J. E. (1986). Gland to gland heterogeneity in histologically normal mucosa of colon cancer patients demonstrated by monoclonal antibodies to tissue-specific antigens. *Cancer Res.* **46,** 5993–5999.
Ichikawa, T., Akimoto, S., Hayata, S., and Shimazaki, J. (1989). Progression and selection in heterogeneous tumor composed of androgen-responsive Shionogi Carcinoma 115 and its autonomous subline (Chiba subline 2). *Cancer Res.* **49,** 367–371.
Imada, M., and Sueoka, N. (1978). Clonal sublines of rat neurotumor RT4 and cell differentiation. *Dev. Biol.* **66,** 97–108.
Isaacs, J. T., Wake, N., Coffey, D. S., and Sandberg, A. A. (1982). Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostatic adenocarcinoma system. *Cancer Res.* **42,** 2353–2361.
Itaya, T., Judde, J.-G., Hunt, B., and Frost, P. (1989). Genotypic and phenotypic evidence of clonal interactions in murine tumor cells. *JNCI, J. Natl. Cancer Inst.* **81,** 664–668.
Jacob, R., Welkoborsky, H. J., Mann, W. J., Hofken, F., Dienes, H. P., and Frieje, J. E. (1996). Heterogeneity of squamous cell carcinomas of the head and neck—Analysis of tumor biologic factors and proliferation rates. *Laryngoscope* **106,** 1170–1175.
Jakubezak, J. L., Merlino, G., French, J. E., Muller, W. J., Paul, B., Adhya, S., and Garges, S. (1996). Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage transgene target. *Proc. Natl. Acad. Sci. USA* **93,** 9073–9078.
Jamasbi, R. J., and Perkins, E. H. (1990). Biological heterogeneity and radiation sensitivity of in vitro propagated lung metastatic lines originated from a transplantable squamous cell carcinoma of BALB/c mouse. *In Vitro Cell Dev. Biol.* **26,** 222–228.

Jenner, K., Darnton, S. J., Billingham, L., Warfield, A. T., and Matthews, H. R. (1996). Tumour heterogeneity: A problem in biopsy assessment of the proliferation index of oesophageal adenocarcinomas. *J. Clin. Pathol. Mol. Pathol.* **49,** M61-M63.

Jones, P. A. (1996). DNA methylation errors and cancers. *Cancer Res.* **56,** 2463–2467.

Jouanneau, J., Moens, G., Bourgeois, Y., Poupon, M. F., and Thierry, J. P. (1994). A minority of carcinoma cells producing acidic fibroblast growth factor induces a community effect for tumor progression. *Proc. Natl. Acad. Sci. USA* **91,** 286–290.

Kaden, D., Gadi, I. K., Bardwell, L., Gelman, R., and Sager, R. (1989). Spontaneous mutation rates of tumorigenic and nontumorigenic Chinese hamster embryo fibroblast cell lines. *Cancer Res.* **49,** 3374–3379.

Kahlenberg, M. S., Stoler, D. L., Basik, M., Petrelli, N. J., Rodriquez-Bigas, M., and Anderson, G. (1996). p53 tumor suppressor gene status and the degree of genomic instability in sporadic colorectal cancer. *JNCI, J. Natl. Cancer Inst.* **88,** 1665–1670.

Kasid, A., Knabbe, G., and Lippman, M. E. (1987). Effect of v-rasH oncogene transfection on estrogen-independent tumorigenicity of estrogen-dependent human breast cancer cells. *Cancer Res.* **47,** 5733–5738.

Katsura, K., Sugihara, H., Nakai, S., and Fujita, S. (1996). Alteration of numerical chromosomal aberrations during progression of colorectal tumors revealed by a combined fluorescence in situ hybidization and DNA ploidy analysis of intratumoral heterogeneity. *Cancer Genet. Cytogenet.* **90,** 146–153.

Keller, R. (1985). Repression of lymphatic metastasis by a second implant of the same tumor. *Invasion Metastasis* **5,** 295–308.

Kendal, W. S., and Frost, P. (1986). Metastatic potential and spontaneous mutation rates: Studies with two murine cell lines and their recently induced metastatic variants. *Cancer Res.* **46,** 6131–6135.

Kendal, W. S., Wang, R.-Y., Hsu, T. C., and Frost, P. (1987). Rate of generation of major karyotypic abnormalities in relationship to the metastatic potential of B16 murine melanoma. *Cancer Res.* **47,** 3835–3841.

Kerbel, R. S., and Theodorescu, D. (1990). Tumor cell subpopulation interactions mediated by transforming growth factor β may contribute to clonal dominance of metastatically competent cells in primary tumors. *In* "Cell to Cell Interaction" (M. M. Burger, B. Sordat, and R. M. Zinkernagel, eds.), pp. 110–113. Karger, Basel.

Kerbel, R. S., Lagarde, A. E., Dennis, J. W., and Donaghue, T. P. (1983). Spontaneous fusion in vivo between normal host and tumor cells: Possible contribution to tumor progression and metastasis studied with a lectin-resistant tumor. *Mol. Cell. Biol.* **3,** 523–538.

Kerbel, R. S., Frost, P., Liteplo, R., Carlow, D. A., and Elliott, B. E. (1984). Possible epigenetic mechanisms of tumor progression: Induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. *J. Cell. Physiol. Suppl.* **3,** 87–97.

Kitchin, R. M., and Sager, R. (1980). Genetic analysis of tumorigenesis VI: Chromosome rearrangements in tumors derived from diploid premalignant Chinese hamster cells in nude mice. *Somatic Cell Genet.* **6,** 615–630.

Konishi, N., Hiasa, Y., Matsuda, H., Tao, M., Tsuzuki, T., Hayashi, I., Kitahori, Y., Shiraishi, T., Yatani, R., Shimazaki, J., and Lin, I. C. (1995). Intratumor cellular heterogeneity and alterations in ras oncogene and p53 tumor suppressor gene in human prostate carcinoma. *Am. J. Pathol.* **147,** 1112–1122.

Kripke, M. L., Gruys, E., and Fidler, I. J. (1978). Metastatic heterogeneity of cells from an ultraviolet light-induced murine fibrosarcoma of recent origin. *Cancer Res.* **38,** 2962–2967.

Law, L. W. (1952). Origin of the resistance of leukemia cells to folic acid antagonists. *Nature* (*London*) **169,** 628–629.

Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jan, J., Parsons, R., Peltomäki, P., Sistonen, P., Aaltonen, L. A., Nystro;u;m-Lahti, M., Guan, X. Y., Zhang, J., Meltzer, P. S.,

Yu, J. W., Kao, F. T., Chen, D. J., Cerosaletti, K. M., Fournrev, R. E. K., Todd, S., Lewis, T., Leach, R. J., Naylor, S. L., Weissenbach, J., Mecklin, J. P., Järvinen, H., Peterson, G. M., Hamilton, S. R., Green, J., Jass, J., Watson, P., Lynch, H. T., Trent, J. M., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1993). Mutations of a MutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75,** 1215–1225.

Leith, J. T., and Michelson, S. (1990). Tumor radiocurability relationship to intrinsic tumor heterogeneity and to the tumor bed effect. *Invasion Metastasis* **10,** 329–351.

Leith, J. T., Brenner, H. J., De Wyngaert, J. K., Dexter, D. L., Calabresi, P., and Glicksman, A. S. (1981). Selective modification of the X-ray response of two mouse mammary adenocarcinoma sublines by N,N-dimethylformamide. *Int. J. Radiat, Oncol. Biol. Phys.* **7,** 943–947.

Leith, J. T., Gaskins, L. A., Dexter, D. L., Calabresi, P., and Glicksman, A. S. (1982a). Alteration of the survival response of two human colon carcinoma subpopulations to X-irradiation by N,N-dimethylformamide. *Cancer Res.* **42,** 30–34.

Leith, J. T., Dexter, D. L., DeWyngaert, J. K., Zeman, E. M., Chu, M. Y., Calabresi, P., and Glicksman, A. S. (1982b). Differential responses to x-irradiation of subpopulations of two heterogeneous human carcinomas in vitro. *Cancer Res.* **42,** 2556–2561.

Leith, J. T., Heyman, P., DeWyngaert, J. K., Dexter, D. L., Calabresi, P., and Glicksman, A. S. (1983). Thermal survival characteristics of cell subpopulations isolated from a heterogeneous human colon tumor. *Cancer Res.* **43,** 3240–3246.

Leith, J. T., Bliven, S. F., Lee, E. S., Glicksman, A. S., and Dexter, D. L. (1984). Intrinsic and extrinsic heterogeneity in the responses of parent and clonal human colon carcinoma xenografts to photon irradiation. *Cancer Res.* **44,** 3757–3762.

Leith, J. T., Bliven, S. F., and Glicksman, A. S. (1985). Similarity of thermotolerance characteristics in heterogeneous human colon tumor subpopulations after exposure to fractionated heat doses (44°C). *Radiat. Res.* **104,** 128–139.

Leith, J. T., Faulkner, L. E., Bliven, S. F., Michelson, S. (1988). Compositional stability of artificial heterogeneous tumors *in vivo:* use of mitomycin C as a cytotoxic probe. *Cancer Res.* **48,** 2669–2673.

Li, J.-J., Oberley, L. W., St. Clair, D. K., Ridnour, L., and Oberley, T. D. (1995). Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase. *Oncogene* **10,** 1989–2000.

Lin, W.-C., O'Connor, K. L., and Culp, L. A. (1993). Complementation of two related tumor cell classes during experimental metastasis tagged with different histochemical marker genes. *Br. J. Cancer* **67,** 910–921.

Ling, V., Chamber, A. F., Harris, J. F., and Hill, R. P. (1985). Quantitative genetic analysis of tumor progression. *Cancer Metastasis Rev.* **4,** 173–194.

Linsley, P. S., Ochs, V., Horn, D., Ring, D. B., and Frankel, A. S. (1986). Heritable variation in expression of multiple tumor associated epitopes on a high molecular weight mucin-like antigen. *Cancer Res.* **46,** 6380–6386.

Liteplo, R. G., and Kerbel, R. S. (1986). Periodate-oxidized adenosine induction of murine thymidine kinase: role of DNA methylation in the generation of tumor cell heterogeneity. *Cancer Res.* **46,** 577–582.

Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T. D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70,** 923–935.

Ljungberg, B., Mehle, C., Sterling, R., and Roos, G. (1996). Heterogeneity in renal cell carcinoma and its impact on prognosis—A flow cytometric study. *Br. J. Cancer* **74,** 123–127.

Loeb, L. A. (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res.* **51,** 3075–3079.

Loveless, S. E., and Heppner, G. H. (1983). Tumor-associated macrophages of mouse mammary tumors. *J. Immunol.* **131,** 2074–2078.

Luria, S. E., and Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28,** 491–511.

Mackay, B., and Ordonez, N. G. (1993). Pathological evaluation of neoplasms with unknown primary tumor site. *Semin. Oncol.* **20,** 206–228.

MacPhee, D. G. (1995). Mismatch repair, somatic mutations, and the origins of cancer. *Cancer Res.* **55,** 5489–5492.

Mahoney, K. H., Miller, B. E., and Heppner, G. H. (1985). FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor-associated macrophages from metastatic and nonmetastatic mouse mammary tumors. *J. Leukocyte Biol.* **38,** 573–585.

Makino, S. (1956). Further evidence favoring the concept of the stem cell in ascite tumors of rats. *Ann. NY Acad. Sci.* **63,** 818–830.

Malins, D. C., Polissar, N. L., and Gunselman, S. J. (1996). Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage. *Proc. Natl. Acad. Sci. USA* **93,** 2557–2563.

Mantovani, A., Gravazzi, R., Allessandri, G., Spreafico, F., and Garattani, S. (1981). Characterization of tumor lines derived from spontaneous metastases of a transplanted murine sarcoma. *Eur. J. Cancer* **17,** 71–76.

Markowitz, S., Wang, J., Mycroff, L., Parsons, R., Sung, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. (1995). Inactivation of the type II TGF-receptor in colon cancer cells with microsatellite instability. *Science* **268,** 1336–1338.

Martin, F., Caignard, A., Jeannin, J. F., LeClerc, A., and Martin, M. (1983). Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. *Int. J. Cancer* **32,** 623–627.

McMillan, T. J., Kalebic, T., Stark, G. R., and Hart, I. R. (1990). High frequency of double drug resistance in the B16 melanoma cell line. *Eur. J. Cancer* **26,** 565–567.

Meuth, M., L'Heureux-Huard, N., and Trudel, M. (1979). Characterization of a mutator gene in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **76,** 6505–6509.

Meyer, J. S., and Wittliff, J. L. (1991). Beyond heterogeneity in breast carcinoma: Thymidine labelling index, steroid hormone receptors, DNA ploidy. *Int. J. Cancer* **47,** 213–220.

Miller, B. E., Miller, F. R., Leith, J., and Heppner, G. H. (1980). Growth interaction in vivo between tumor subpopulations derived from a single mouse mammary tumor. *Cancer Res.* **40,** 3977–3981.

Miller, B. E., Miller, F. R., and Heppner, G. H. (1981). Interactions between tumor subpopulations affecting their sensitivity to the antineoplastic agents cyclophosphamide and methotrexate. *Cancer Res.* **41,** 4378–4381.

Miller, B. E., Miller, F. R., Wilburn, D., and Heppner, G. H. (1987). Analysis of tumour cell composition in tumours composed of paired mixtures of mammary tumour cell lines. *Br. J. Cancer* **56,** 561–569.

Miller, B. E., Miller, F. R., Wilburn, D., and Heppner, G. H. (1988). Dominance of a tumor subpopulation line in mixed heterogeneous mouse mammary tumors. *Cancer Res.* **48,** 5747–5753.

Miller, B. E., Miller, F. R., and Heppner, G. H. (1989). Therapeutic pertubution of the tumor ecosystem in reconstructed heterogeneous mouse mammary tumors. *Cancer Res.* **49,** 3747–3753.

Miller, B. E., Machemer, T., Lehotan, M., and Heppner, G. H. (1991). Tumor subpopulation interactions affecting melphalan sensitivity in palpable mouse mammary tumors. *Cancer Res.* **51,** 4378–4387.

Miller, B. E., Matherly, L. H., Lehotan, M., and Heppner, G. H. (1996). Rates of development of methotrexate resistance in heterogeneous mouse mammary tumor cell cultures. *J. Exp. Ther. Oncol.* **1,** 30–38.

Miller, F. R. (1983). Tumor subpopulation interactions in metastasis. *Invasion Metastasis* **3,** 234-242.

Miller, F. R., and Heppner, G. H. (1979). Immunologic heterogeneity of tumor cell subpopulations from a single mouse mammary tumor. *JNCI, J. Natl. Cancer Inst.* **63,** 1457–1463.

Miller, F. R., and Heppner, G. H. (1990). Cellular interactions in metastasis. *Cancer Metastasis Rev.* **9,** 21–34.

Miller, F. R., Miller, B. E., and Heppner, G. H. (1983). Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: Heterogeneity in phenotypic stability. *Invasion Metastasis* **3,** 22–31.

Miller, F. R., McInerney, D., Rogers, C., and Miller, B. E. (1988). Spontaneous fusion between metastatic mammary tumor subpopulations. *J. Cell. Biochem.* **36,** 129–136.

Miller, F. R., Mohamed, A. N., and McEachern, D. (1989). Production of a more aggressive tumor cell variant by spontaneous fusion of two mouse tumor subpopulations. *Cancer Res.* **49,** 4316-4321.

Miller, F. R., Soule, H. D., Tail, L., Pauley, R. J., Wolman, S. R., Dawson, P. J., and Heppner, G. H. (1993). Xenograft model of human proliferative breast disease. *JNCI, J. Natl. Cancer Inst.* **85,** 1725-1732.

Mirchandani, D., Zheng, J., Miller, G. J., Ghosh, A. K., Shibata, D. K., Cote, R. J., and Roy-Burman, P. (1995). Heterogeneity in intratumor distribution of p53 mutations in human prostate cancer. *Am. J. Pathol.* **147,** 92–101.

Mitelman, F. (1971). The chromosomes of fifty primary Rous rat sarcomas. *Hereditas* **69,** 155–186.

Mitelman, F. (1972). Comparative chromosome analysis of primary and metastatic Rous sarcomas in rats. *Hereditas* **70,** 1–14.

Moffett, B. F., Baban, D., Bao, L., and Tarin, D. (1992). Fate of clonal linkages during neoplasia and metastasis studied with an incorporated genetic marker. *Cancer Res.* **52,** 1737–1743.

Moolten, F. L. (1986). Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. *Cancer Res.* **46,** 5276–5281.

Nagase, F., Jamshedur-Rahman, S. M., Yokochi, T., Kawashima, K., Isobe, K.-I., Yoshida, T., Nagura, E.-I., and Nakashima, I. (1987). Dynamics of generation of antigen loss variants from L1210 murine leukemia clones detected by a tumor-specific T-cell clone. *Cancer Res.* **47,** 6494–6499.

Nakashima, H., Inoue, H., Honda, M., Shibuta, K., Arinaga, S., Mori, M., Vee, H., Era, S., and Akiyoshi, T. (1995). The heterogeneity of microsatellite instability in multiple gastric cancers. *Am. J. Gastroenterol.* **90,** 653–655.

Natali, P. G., Cavaliere, R., Bigotti, A., Nicotra, M. R., Russo, C., Ng, A. K., Giacomini, P., and Ferrone, S. (1983). Antigeneic heterogeneity of surgically removed primary and autologous metastatic human melanoma lesions. *J. Immunol.* **130,** 1462–1466.

Newcomb, E., Silverstein, S. C., and Silagi, S. (1978). Malignant mouse melanoma cells do not form tumors when mixed with cells of a non-malignant subclone: Relationships between plasminogen activator expression by the tumor cells and the host's immune response. *J. Cell. Physiol.* **95,** 169–178.

Nicolson, G. L., Brunson, K. W., and Fidler, I. J. (1978). Specificity of arrest, survival and growth of selected metastatic variant cell lines. *Cancer Res.* **38,** 4105–4111.

Niederkorn, J. Y., and Streilen, J. N. (1983). Intracamerally induced concomitant immunity: mice harboring progressively growing intraocular tumors are immune to spontaneous metastases and secondary tumor challenge. *J. Immunol.* **131,** 2587-2594.

Noguchi, S., Matomura, K., Inaji, H., Imaoka, S., and Koyama, H. (1992). Clonal analysis of human breast cancer by means of the polymerase chain reaction. *Cancer Res.* **52,** 6594–6597.

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. *Science* **194,** 23–28.

Nowotny, A., and Grohsman, J. (1973). Mixed tumor challenge of strain specific and nonspecific TA3 mouse ascites in mammary adenocarcinomas. *Int. Arch. Allergy Appl. Immunol.* **44,** 434–440.

Ogawa, K., Solt, D. B., and Farber, E. (1980). Phenotypic diversity as an early property of putative preneoplastic hepatocyte populations in liver carcinogenesis. *Cancer Res.* **40,** 725–733.

Okada, S., Ishii, H., Nose, H., Okusaka, T., Kyogoku, A., Yoshimori, M., Sakamoto, M., and Hirohashi, S. (1995). Intra-tumoral DNA heterogeneity of small hepatocellular carcinoma. *Cancer* **75,** 444–450.

Okcu, A., Hofmann-Wellenhof, R., Woltsche, I., Smalle, J., and Kerl, H. (1996). Pathological findings suggestive of interclonal stabilization in a case of cutaneous melanoma. *Clin. Exp. Metastasis* **14,** 215–218.

Olsson, L., and Ebesen, P. (1979). Natural polyclonality of spontaneous AKR leukemia and its consequences for so-called specific immunotherapy. *JNCI, J. Natl. Cancer Inst.* **62,** 623–627.

Olsson, L., and Forchhammer, J. (1984). Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine, and characterization of an antigen associated with metastatic activity. *Proc. Natl. Acad. Sci. USA* **81,** 3389–3393.

Olsson, L., Sorensen, H. R., and Behinke, O. (1984). Intratumoral phenotypic diversity of cloned human lung tumor cell lines and consequences for analysis with monoclonal antibodies. *Cancer* **54,** 1757–1765.

Olsson, L., Due, C., and Dumant, M. (1985). Treatment of human cell lines with 5-azacytidine may result in profound alterations in clonogenicity and growth rate. *J. Cell Biol.* **100,** 508–513.

Orndal, C., Rydholm, A., Willen, H., Mitelman, F., and Mandahl, N. (1994). Cytogenetic intratumor heterogeneity in soft tissue tumors. *Cancer Genet. Cytogenet.* **78,** 127–137.

Paquette, B., and Little, J. B. (1994). In vivo enhancement of genomic instability in minisatellite sequences of mouse C3H/10T1/2 cells transformed in vitro by x-rays. *Cancer Res.* **54,** 3173–3178.

Paradiso, A., Mangia, A., Barletta, A., Fusilli, S., Marzullo, F., Schittulli, F., and DeLena, M. (1995). Heterogeneity of intratumour proliferative activity in primary breast cancers: Biological and clinical aspects. *Eur. J. Cancer* **31A,** 911–916.

Pierce, G., Aguilar, D., Hood, G., and Wells, R. S. (1984). Trophectoderm in control of murine embryonal carcinoma. *Cancer Res.* **44,** 3987–3996.

Pierce, G. B., and Cox, W. F. (1978). Neoplasms as cariactures of tissue renewal. *In* "Cell Differentiation and Neoplasia" (G.F., Saunders, ed.), pp. 57–66. Raven Press, New York.

Pool, R. (1989). Is it healthy to be chaotic? *Science* **243,** 604–607.

Posadas, E. M., Criley, S. R., and Coffey, D. S. (1996). Chaotic oscillations in cancer cells. *Cancer Res.* **56,** 3682–3688.

Poste, G., and Grieg, R. (1982). On the genesis and regulation of cellular heterogeneity in malignant tumors. *Invasion Metastasis* **2,** 137–176.

Poste, G., Doll, J., and Fidler, I. J. (1981). Interactions between clonal subpopulations affect the stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells. *Proc. Natl. Acad. Sci. USA* **78,** 6226–6230.

Poste, G., Tzeng, J., Doll, J., Grieg, R., Rieman, D., and Zeidman, I. (1982). Evolution of tumor cell heterogeneity during progressive growth of individual lung metastases. *Proc. Natl. Acad. Sci. USA* **79,** 6574–6578.

Prehn, R. T. (1991). The inhibition of tumor growth by tumor mass. *Cancer Res.* **51,** 2–4.

Price, J. E., Bell, C., and Frost, P. (1990). The use of a genotypic marker to demonstrate clonal dominance during the growth and metastasis of a human breast carcinoma in nude mice. *Int. J. Cancer* **45,** 968–971.

Radinsky, R., Kraemer, P. M., Proffitt, M. R., and Culp, L. A. (1988). Clonal diversity of the Kirsten-ras oncogene during tumor progression in athymic nude mice: Mechanisms of amplification and rearrangement. *Cancer Res.* **48,** 4941–4953.

Reid, T. M., and Loeb, L. A. (1992). Mutagenic specificity of oxygen radicals produced by human leukemia cells. *Cancer Res.* **52,** 1082–1086.

Reynolds, T. Y., Rockwell, S., and Glazer, P. M. (1996). Genetic instability induced by the tumor microenvironment. *Cancer Res.* **56,** 5754–5757.

Rhee, J. G., Schuman, V. L., Song, C. W., and Levitt, S. H. (1987). Difference in the thermotolerance of mouse mammary carcinoma cells in vivo and in vitro. *Cancer Res.* **47,** 2571–2575.

Rice, G. C., Hoy, C., and Schimke, R. T. (1986). Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **83,** 5978–5982.

Rideout, W. M., Coetzec, G. A., Olumi, A. F., and Jones, P. A. (1990). 5-Methycytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* **249,** 1288–1290.

Robinson, S. P., and Jordan, V. C. (1989). Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. *Cancer Res.* **49,** 1758–1762.

Rodriguez, E., Sreekantaiah, C., and Chaganti, R. S. K. (1994). Genetic changes in epithelial tumors. *Cancer Res.* **54,** 3398–3406.

Rofstad, E. K. (1992). Radiation sensitivity in vitro of primary tumors and metastatic lesions of malignant melanoma. *Cancer Res.* **52,** 4453–4457.

Rosin, M. P., Anwar, W. A., and Ward, A. J. (1994). Inflammation, chromosomal instability, and cancer: The schistomiasis model. *Cancer Res.* **54,** 1929s-1933s.

Rosowsky, A., Wright, J. E., Cucchi, C. A., Lippke, J. A., Tantravahi, R., Ervin, T. J., and Frei, E., 3rd (1985). Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low-level methotrexate resistance. *Cancer Res.* **45,** 6205–6212.

Roth, J. A., Restrepo, C., Scuderi, P., Baldwin, R. W., Reichert, C. M., and Hosoi, S. (1984). Analysis of antigenic expression by primary and autologous metastatic human sarcomas using murine monoclonal antibodies. *Cancer Res.* **44,** 5320–5325.

Roth, J. A., Nguyen, D., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Person, D. Z., Hong, W. K., Kemaki, R., Lee, J. J., Nesbitt, J. C., Pisters, K. M. W., Putnam, J. B., Schea, R., Shin, D. M., Walsh, G. L., Dolormente, M. M., Han, C.-I., Martin, F. D., Yen, N., Xu, K., Stephens, L. C., McDonnell, T. J., Mukhopadhyay, T., and Cai, D. (1996). Retrovirus-mediated wild-type p53 gene transfer to tumor of patients with lung cancer. *Nature Med.* **2,** 985–991.

Rubin, A. L., Yao, A., and Rubin, H. (1990). Relation of spontaneous transformation in cell culture to adaptive growth and clonal heterogeneity. *Proc. Natl. Acad. Sci. USA* **87,** 482–486.

Rubin, A. L., Sneade-Koenig, A., and Rubin, H. (1992). High rate of diversification and reversal among subclones of neoplastically transformed NIH 3T3 clones. *Proc. Natl. Acad. Sci. USA* **89,** 4183–4186.

Rubin, H. (1988). Uniqueness of each spontaneous transformant from a clone of BALB/c 3T3 cells. *Cancer Res.* **48,** 2512–2518.

Rubin, H. (1994a). Epigenetic nature of neoplastic transformation. *In* "Developmental Biology and Cancer," pp. 61–84. CRC Press, Boca Raton, FL.

Rubin, H. (1994b). Experimental control of neoplastic progression in cell populations: Fould's rules revisited. *Proc. Natl. Acad. Sci. USA* **91,** 6619–6623.

Rubin, H., and Xu, K. (1989). Evidence for the progressive and adaptive nature of spontaneous transformation in the NIH 3T3 cell line. *Proc. Natl. Acad. Sci. USA* **86,** 1860–1864.

Rubin, H., Arnstein, P., and Chu, B. M. (1984). High-frequency variation and population drift in a newly transformed clone of Balb/c/3T3 cells. *Cancer Res.* **44,** 5242–5248.

Rubin, H., Hennessey, T. L., Sanui, H., Arnstein, P., Taylor, D. O. N., and Chu, B. B. (1985). Inheritance of acquired changes in growth capacity of spontaneously transformed BALB/3T3 cells propagated in mice and culture. *Cancer Res.* **45,** 2550–2559.

Rudland, P. S., Gusterson, B. A., Hughes, C. M., Omerod, E. J., and Warburton, M. J. (1982). Two forms of tumors in nude mice generated by a neoplastic rat mammary stem cell line. *Cancer Res.* **42,** 5196–5208.

Samiei, M., and Waghorne, C. (1991). Clonal selection within metastatic SPI mouse mammary tumors is independent of metastatic potential. *Int. J. Cancer* **47,** 771–775.

Sasaki, O., Soejima, K., and Haraguchi, Y. (1993). DNA ploidy in undiffentiated carcinomas of the human stomach—With special reference to its heterogeneity and the relation between its intratumor distribution pattern and prognosis. *In Vivo* **7,** 363–368.

Schapers, R., Smects, W., Hopman, A., Pauwels, R., Geraedts, J., and Ramaekers, F. (1993). Heterogeneity in bladder cancer as detected by conventional chromosome analysis and interphase cytogenetics. *Cancer Genet. Cytogenet.* **70,** 56–61.

Schimke, R. T., Hill, A., and Johnston, R. N. (1985). Methotrexate resistance and gene amplification: An experimental model of tumor heterogeneity. *Br. J. Cancer* **51,** 459–465.

Schlag, P., and Schreml, W. (1982). Heterogeneity in growth pattern and drug sensitivity of primary tumor and metastases in the human tumor colony-forming assay. *Cancer Res.* **42,** 4086–4089.

Schlappack, O. K., Zimmerman, A., and Hill, R. P. (1991). Glucose starvation and acidosis: Effect on experimental metastatic potential, DNA content and MTX resistance of murine tumor cells. *Br. J. Cancer* **64,** 663–670.

Schnipper, L. E. (1986). Clinical implications of tumor-cell heterogeneity. *N. Engl. J. Med.* **314,** 1423–1431.

Schvimer, M., Lash, R. H., and Katzin, W. E. (1995). Intratumoral heterogeneity of DNA ploidy in breast carcinomas: A flow cytometric assessment of sampling techniques. *Cytometry* **22,** 292–296.

Seshadri, R., Kutlaca, R. J., Trainor, K., Matthews, C., and Morley, A. A. (1987). Mutation rate of normal and malignant human lymphocytes. *Cancer Res.* **47,** 407–409.

Shankey, T. V., Jin, J.-K., Dougherty, S., Flanigan, R. C., Graham, S., and Pyle, J. M. (1995a). DNA ploidy and proliferation heterogeneity in human prostate cancers. *Cytometry* **21,** 30–39.

Shankey, T. V., Jin, J.-K., Flanigan, R. C., and Pyle, J. M. (1995b). Analysis of heterogeneity in human tumors. *Cytometry* **22,** 159–160.

Shibeta, D., Schaeffer, J., Li, Z.-H., Capella, G., and Perucho, M. (1993). Genetic heterogeneity of the c-k-ras locus in colorectal adenomas but not in adenocarcinomas. *JNCI, J. Natl. Cancer Inst.* **85,** 1058–1063.

Shirasuna, K., Watani, K., Sugiyama, M., Marioka, S., and Miyazaki, T. (1986). Isolation and characterization of different clones including myoepithelial-like variants from a clonal neoplastic epithelial duct cell line of human salivary gland origin. *Cancer Res.* **46,** 1418–1426.

Siitonen, S. M., Isola, J. J., Rantala, I. S., and Helin, H. J. (1993). Intratumor variation in cell proliferation in breast carcinoma as determined by antiproliferating cell nuclear antigen monoclonal antibody and automated image analysis. *Am. J. Clin. Pathol.* **99,** 226–231.

Simmons, W. F., Liu, J., Knowles, D. M., and Inghirami, G. (1995). Breast cancer heterogeneity: evaluation of clonality in primary and metastatic lesions. *Hum. Pathol.* **26,** 210–216.

Siracky, J. (1979). An approach to the problem of heterogeneity of human tumour cell populations. *Br. J. Cancer* **39,** 570–577.

Slocum, H. K., Heppner, G. H., and Rustum, Y. M. (1985). Cellular heterogeneity of human tumors. *Biol. Responses Cancer* **4,** 183–248.

Smeds, S., Peter, H. J., Jortso, E., Gerber, H., and Studer, H. (1987). Naturally occurring clones of cells with high intrinsic proliferation potential within the follicular epithelium of mouse thyroids. *Cancer Res.* **47,** 1646–1651.

Sonnenberg, A., Daams, H., Calafat, J., and Hilgers, J. (1986). In vitro differentiation and progression of mouse mammary tumor cells. *Cancer Res.* **46,** 5913–5922.

Staroselsky, A., Pathak, S., and Fidler, I. J. (1990). Changes in clonal composition during in vivo growth of mixed subpopulations derived from the murine K-1735 melanoma. *Anticancer Res.* **10,** 291–296.

Staroselsky, A. N., Radinsky, R., Fidler, I. J., Pathak, S., Chernajovsky, Y., and Frost, P. (1992). The use of molecular genetic markers to demonstrate the effect of organ environment on clonal dominance in a human renal-cell carcinoma grown in nude mice. *Int. J. Cancer* **51,** 130–138.

Stoler, D. L., Anderson, G. R., Russo, C. A., Spina, A. M., and Beerman, T. A. (1992). Anoxia-inducible endonuclease activity as a potential basis of the genomic instability of cancer cells. *Cancer Res.* **52,** 4372–4378.

Szatrowski, T. P., and Nathan, C. F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **51,** 794–798.

Takahashi, Y., Takenada, A., Ishiguro, T., and Noda, Y. (1994). Intratumoral DNA heterogeneity correlated with lymph node involvement and surgical staging in epithelial ovarian cancer by flow cytometry. *Cancer* **73,** 3011–3014.

Talmadge, J., Wolman, S. R., and Fidler, I. J. (1982). Evidence for the clonal origin of spontaneous metastases. *Science* **217,** 361–363.

Talmadge, J. E., Benedict, K., Madsen, J., and Fidler, I. J. (1984). Development of biological diversity and susceptibility to chemotherapy in murine cancer metastases. *Cancer Res.* **44,** 3801–3805.

Tanigawa, N., Mizuno, Y., Hashimura, T., Honda, K., Satomura, K., Hikasa, Y., Niwa, O., Sugahara, T., Yoshida, O., Kern, D. H., and Morton, D. L. (1984). Comparison of drug sensitivity among tumor cells within a tumor, between primary tumor and metastases, and between different metastases in the human tumor colony-forming assay. *Cancer Res.* **44,** 2309–2312.

Tanooka, H. (1988). Monoclonal growth of cancer cells: experimental evidence. *Jpn. J. Cancer Res.* **79,** 657–665.

Tanooka, H., and Tanaka, K. (1982). Evidence for single-cell origin of 3-methylcholanthrene-induced fibrosarcomas in mice with cellular mosaicism. *Cancer Res.* **42,** 1856–1858.

Teixeira, M. R., Pandis, N., Bardi, G., Andersen, N., Mandahl, N., Mitelman, F., and Heim, S. (1994). Cytogenetic analysis of multifocal breast carcinomas: Detection of karyotypically unrelated clones as well as clonal similarities between tumour foci. *Br. J. Cancer* **70,** 922–927.

Teixeira, M. R., Pandis, N., Bards, G., Anderson, J. A., Mitelman, F., and Heim, S. (1995). Clonal heterogeneity in breast cancer: Karyotypic comparisons of multiple intra- and extra-tumorous samples from 3 patients. *Int. J. Cancer* **63,** 63–68.

Teixeira, M. R., Pandis, N., Bardi, G., Andersen, J. A., and Heim, S. (1996). Karyotypic comparisons of multiple tumorous and macroscopically normal surrounding tissue samples from patients with breast cancer. *Cancer Res.* **56,** 855–859.

Tlsty, T. D. (1990). Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl. Acad. Sci. USA* **87,** 3132–3136.

Tlsty, T. D., Margolin, B. H., and Lum, K. (1989). Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria-Delbrück fluctuation analysis. *Proc. Natl. Acad. Sci. USA* **86,** 9441–9445.

Tofilon, P., Buckley, N., and Deen, D. F. (1984). Effect of cell–cell interactions on drug sensitivity and growth of drug sensitive and-resistant tumor cells in spheroids. *Science* **226,** 862–864.

Tomasovic, S. P., Rosenblatt, P. L., Johnston, D. A., Tang, K., and Lee, P. S. Y. (1984). Heterogeneity in induced host resistance and its relation to synthesis of stress proteins in rat tumor cell clones. *Cancer Res.* **44,** 5850–5856.

Tomlinson, I. P. M., Novelli, M. R., and Bodmer, W. F. (1996). The mutation rate and cancer. *Proc. Natl. Acad. Sci. USA* **93,** 14800–14803.

Trope, C., Aspergen, K., Kullander, S., and Astedt, B. (1979). Heterogeneous response of disseminated human ovarian cancers to cytostatics in vitro. *Acta Obstet. Gynecol. Scand.* **58,** 543–546.

Trosko, J. E., Jone, C., and Chang, C. C. (1983). The role of tumor promoters on phenotypic alterations affecting intercellular communication and tumorigenesis. *Ann. NY Acad. Sci.* **407,** 316–327.

Tsuruo, T., and Fidler, I. J. (1981). Differences in drug sensitivity among tumor cells from parental tumors, selected variants, and spontaneous metastases. *Cancer Res.* **41,** 3058–3064.

Van der Poel, H. G., Mulders, P. F. A., Oosterhof, G. D. N., Schaafsma, H. E., Hendriks, J. C. M., Schalken, J. A., and Debruyne, F. M. J. (1993). Prognostic value of karyometric and clinical characteristics in renal cell carcinoma. *Cancer* **72,** 2667–2674.

Varani, J., Orr, W., and Ward, P. A. (1978). A comparison of the migration patterns of normal and malignant cells in two assay systems. *Am. J. Pathol.* **90,** 159–172.

Vaupel, P., Kallinowski, F., and Okunieff, P. (1989). Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res.* **49,** 6449–6465.

Visscher, D., Dolan, P., Ottosen, S., and Crissman, J. (1995). Geographic distribution of co-dominant DNA stemlines in breast carcinomas. *Cytometry* **21,** 14–17.

Vogelstein, B., Fearon, F. E., Hamilton, S. R., and Feinberg, A. P. (1985). Use of restriction fragment length polymorphism to determine the clonal origin of human tumors. *Science* **227,** 642–645.

Volpe, J. P., and Milas, L. (1988). Metastatic instability of murine tumor metastases: Dependence on tumor type. *Clin. Exp. Metastasis* **6,** 333–346.

von Hoff, D. D. (1985). Implications of tumor cell heterogeneity for in vitro drug sensitivity testing. *Semin. Oncol.* **12,** 327–331.

von Hoff, D. D., Clark, G. M., Forseth, B. J., and Cowan, J. D. (1986). Simultaneous in vitro drug sensitivity testing on tumor from different sites in the same patient. *Cancer* **58,** 1007–1013.

Waghorne, C., Thomas, M., Lagarde, A., Kerbel, R. S., and Breitman, M. L. (1988). Genetic evidence for progressive selection and overgrowth of primary tumors by metastatic cell populations. *Cancer Res.* **48,** 6109–6114.

Wallen, C. A., Ridinger, D. N., and Dethlefson, L. A. (1985). Heterogeneity of x-ray cytoxicity in proliferating and quiescent murine mammary carcinoma cells. *Cancer Res.* **45,** 3064–3069.

Wang, N., Yu, S. H., Liener, I. E., Hebbel, R. P., Eaton, J., and McKhann, C. F. (1982). Characterization of high-and-low -metastatic clones derived from a methylcholanthrene-induced murine fibrosarcoma. *Cancer Res.* **42,** 1046–1051.

Warzynski, M. J., Soechtig, C. E., Maatman, T. J., Goldsmith, L. C., Grobbel, M. A., Carothers, C. G., and Shockley, K. F. (1995). DNA heterogeneity determined by flow cytometry in prostatic adenocarcinoma—necessitating multiple site analysis. *Prostate* **27,** 329–335.

Weiss, P.A. (1973). "*The Science of Life.*" Futura Publications, Mt. Kisco, NY.

Weitzman, S. A., and Stossel, T. P. (1981). Mutation caused by human phagocytes. *Science* **212,** 546–547.

Weitzman, S. A., and Stossel, T. P. (1982). Effects of oxygen radical scavengers and antioxidants on phagocyte-induced mutagenesis. *J. Immunol.* **128,** 2770–2772.

Weitzman, S. A., Weitberg, A. B., Clark, E. P., and Stossel, T. P. (1985). Phagocytes as carcinogens: Malignant transformation produced by human neutrophils. *Science* **227,** 1231–1233.

Welch, D. R., and Nicolson, G. L. (1983). Phenotypic drift and heterogeneity in response of metastatic mammary adenocarcinoma cell clones to Adriamycin, 5-fluro-2-deoxyuridine and methotrexate treatment in vitro. *Clin. Exp. Metastasis* **1,** 317–325.

Welch, D. R., Milas, L., Tomasovic, S. P., and Nicholson, G. L. (1983). Heterogeneous response and clonal drift of sensitivities of metastatic 13762NF mammary adenocarcinoma clones to gamma radiation in vitro. *Cancer Res.* **43,** 6–10.

Welch, D. R., Krizman, D. B., and Nicolson, G. (1984a). Multiple phenotypic divergence of mammary adenocarcinoma cell clones. I In vitro and in vivo properties. *Clin. Exp. Metastasis* **2,** 333–355.

Welch, D. R., Evans, D. P., Tomasovic, S. P., Milas, L., and Nicolson, G. L. (1984b). Multiple phenotypic divergence of mammary adenocarcinoma cell clones. II. Sensitivity to radiation, hyperthermia and FUdR. *Clin. Exp. Metastasis* **2,** 357–371.

Wier, W. G., Cannell, M. B., Berlin, J. R., Marban, E., and Lederer, W. J. (1987). Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by Fura-2. *Science* **235,** 325–328.

Wikstrand, D. J., Bigner, S. H., and Bigner, D. D. (1983). Demonstration of complex antigenic heterogeneity in a human glioma cell line and eight derived clones by specific monoclonal antibodies. *Cancer Res.* **43,** 3327–3334.

Wilson, M. S., West, C. M. L., Wilson, G. D., Roberts, S. A., James, R. D., and Schofield, P. F. (1993). Intra-tumoral heterogeneity of tumour potential doubling times (Tpot) in colorectal cancer. *Br. J. Cancer* **68,** 501–506.

Wolman, S. R., McMorrow, L. E., Fidler, I. J., and Talmadge, J. E. (1985). Development and progression of karyotypic variability in melanoma K1735 following x-irradiation. *Cancer Res.* **45,** 1839–1844.

Wong, G. H., and Goeddel, D. V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factors: Possible protective mechanism. *Science* **242,** 941–943.

Woo, S. Y., Anderson, R. L., Kapp, D. S., Kerson, I. V., Rice, G. C., Krag, D. N., and Hahn, G. M. (1991). Heterogeneity of heat response in murine, canine and human tumors: influence on predictive assays. *Int. J. Radjat. Oncol. Biol. Phys.* **20,** 479–488.

Woodruff, M. F. A., Ansell, J. D., Forbes, G. M., Gordon, J. C., and Micklem, H. S. (1982). Clonal interaction in tumors. *Nature* (*London*) **229,** 822–824.

Yamashina, K., and Heppner, G. H. (1985). Correlation of frequency of induced mutation and metastatic potential in tumor cell lines from a single mouse mammary tumor. *Cancer Res.* **45,** 4015–4019.

Yamashina, K., Miller, B. E., and Heppner, G. H. (1986). Macrophage-mediated induction of drug resistant variants in a mouse mammary tumor cell line. *Cancer Res.* **46,** 2396–2401.

Yao, A., Rubin, A. L., and Rubin, H. (1990). Progressive state selection of cells in low serum promotes high density growth and neoplastic transformation in NIH 3T3 cells. *Cancer Res.* **50,** 5171–5176.

Yee, C. Y., Roodi, N., Verrier, C. S., and Parl, F. F. (1994). Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Res.* **54,** 1641–1644.

Yildiz, I., Jaffe, N., Aksoy, F., and El-Naggar, A. K. (1994). Regional DNA content heterogeneity in Wilm's Tumor: Incidence and potential clinical relevance. *Anticancer Res.* **14,** 1365–1370.

Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and Wahl, G. M. (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70,** 737–748.

Yokoyama, A., Muramatsu, T., Ohmori, T., Makuuchi, H., Higuchi, S., Matsushita, S., Yoshino, K., Maruyama, K., Nakano, M., and Ishii, H. (1996). Multiple primary esophageal and concurrent upper aerodigestive tract cancer and the aldehyde dehydrogenase-2 genotype of Japanes alcoholics. *Cancer* **77,** 1986–1990.

Young, S. D., and Hill, R. P. (1988). Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proc. Natl. Acad. Sci. USA* **85,** 9533–9537.

Young, S. D., and Hill, R. P. (1990a). Effects of reoxygenation on cells from hypoxic regions of solid tumors: Anticancer drug sensitivity and metastatic potential. JNCI, *J. Natl. Cancer Inst.* **82,** 371–380.

Young, S. D., and Hill, R. P. (1990b). Effects of reoxygenation on cells from hypoxic regions of solid tumors: analyses of transplanted murine tumors for evidence of DNA overreplication. *Cancer Res.* **50,** 5031–5038.

Yung, W.-K. A., Shapiro, J. R., and Shapiro, W. R. (1982). Heterogeneous chemosensitivities of subpopulations of human glioma cells in culture. *Cancer Res.* **42,** 992–998.

Zhang, X.-H., and Xie, T.-X. (1995). Heterogeneity of malignant tumors should be considered when performing flow cytometric DNA analysis. *Cytometry* **22,** 158–159.

Zhou, G.-H. K., Secrist, G. L., Brattain, M. G., and Mulder, K. M. (1995). Clonal heterogeneity of the sensitivity of human colon carcinoma cell lines to TGF isoforms. *J. Cell. Physiol.* **165,** 512–520.

Molecules Involved in Mammalian Sperm–Egg Interaction

S. B. McLeskey, C. Dowds, R. Carballada,[1] R. R. White, and P. M. Saling
Department of Obstetrics & Gynecology and Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

To achieve fertilization, sperm and egg are equipped with specific molecules which mediate the steps of gamete interaction. In mammals, the first interaction between sperm and egg occurs at an egg-specific extracellular matrix, the zona pellucida (zp). The three glycoproteins, ZP1, ZP2, and ZP3, that comprise the zp have been characterized from many species and assigned different roles in gamete interaction. A large number of candidate-binding partners for the zp proteins have been described; a subset of these have been characterized structurally and functionally. Galactosyltransferase, sp56, zona receptor kinase, and spermadhesins are thought to participate in the primary binding between sperm and zp and may initiate the exocytotic release of hydrolytic enzymes in the sperm head, the acrosome reaction. Digestion of the zp by these enzymes enables sperm to traverse the zp, at which time the proteins PH20, proacrosin, sp38, and Sp17 are thought to participate in secondary binding between the acrosome-reacted sperm and zp. Once through the zp, sperm and egg plasma membranes meet and fuse in a process reported to involve the egg integrin $\alpha_6\beta_1$ and the sperm proteins DE and fertilin. These molecules and the processes involved in gamete interaction are reviewed in this chapter within a physiological context.

KEY WORDS: Acrosome reaction, Cell adhesion, Egg, Exocytosis, Fertilization, Gamete, Mammals, Membrane fusion, Spermatozoa, Zona pellucida.

I. Introduction

The interactions between sperm and egg at fertilization are multiphasic and multicomponent events. Sperm first bind to an egg-specific extracellular

[1] Present address: Institut de Biologie du Developpement de Marseille, 13288 Marseille Cedex 9, France.

Copyright © 1998 by Academic Press.
All rights of reproduction in any form reserved.

matrix (ECM), the zona pellucida (zp). This primary interaction between sperm and ECM triggers a cellular response in the sperm, the exocytotic secretion of vesicular contents from the sperm head. The hydrolytic enzymes released by this reaction, termed the acrosome reaction, together with the sperm's own motility, allow it to traverse the zp during a secondary phase of sperm–zp interaction. Sperm–egg plasma membrane interaction then ensues, involving two steps, binding and fusion. Membrane fusion triggers cellular responses in the egg: exocytosis of egg cortical granules and activation of development. Elucidating the molecular details of these mechanisms is a principal goal of current research in fertilization.

The fundamental mechanisms that underlie these events are conserved among all cells, independent of their somatic or germ line origin. Sperm–zp binding involves adhesion of a cell to an ECM as seen in axonal growth, lymphocyte homing, and endothelial cell function. The acrosome reaction resembles regulated exocytosis triggered by conserved signaling pathways observed in a large variety of cell types, such as neurons and mast cells. Sperm–egg membrane fusion employs cell–cell adhesion pathways and viral fusion characteristics. However, unique features of fertilization are the specialized cells, the gametes, and how these cells use variations of these biological themes to drive reproduction.

In the last decade, there has been considerable effort to identify proteins and pathways utilized during gamete interaction, and the molecular basis of this process is beginning to be understood. In this review, we try to summarize new data on sperm–egg interaction beginning with a general overview of gamete structure and the steps of gamete interaction followed by a discussion of individual molecules that function in these processes. Although it is our goal to be comprehensive, different events of gamete interaction and the relevant molecules involved are treated unevenly, reflecting the extent of molecular analysis available.

A. The Egg

At ovulation, most mammalian eggs are surrounded extracellularly by two layers, have not yet completed meiosis, and the maternal chromosomes are aligned and arrested at metaphase II (Fig. 1). The egg's plasma membrane is covered by microvilli except for the area overlying the mitotic spindle (Phillips and Shalgi, 1980). Corresponding to the microvillar region is a subjacent layer of cortical secretory granules (Wassarman and Albertini, 1994); it is in this area of the egg surface that fusion with the sperm plasma membrane occurs. Fertilization triggers the resumption of meiosis and the regulated release of the cortical granules which fuse with the overlying egg

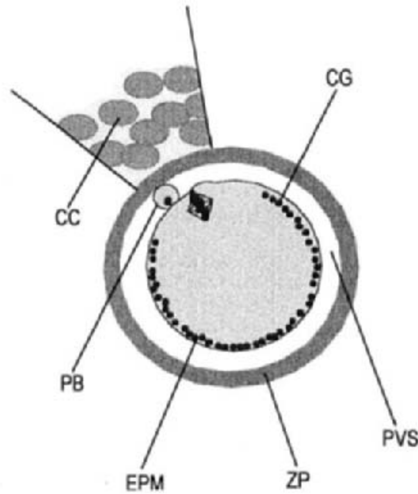


FIG. 1 Schematic representation of the mammalian egg at the time of fertilization. The maternal chromosomes aligned at the metaphase plate, the zona pellucida envelope, and a portion of the cumulus layer are depicted. CC, cumulus cells; PB, polar body; EPM, egg plasma membrane; ZP, zona pellucida; PVS, perivitelline space; CG, cortical granules.

plasma membrane and deposit their contents into the perivitelline space (Yanagimachi, 1994).

Surrounding the egg is the zp, an egg-specific ECM that is secreted by the growing oocyte, is present in all mammalian oocytes, and is a key regulator of fertilization (Yanagimachi, 1994). Structurally, the zp is composed of three sulfated glycoproteins, ZP1, ZP2, and ZP3, intertwined to form a three-dimensional matrix. The zp is always present at the time of gamete interaction and has been shown to possess specific ligands for cognate sperm receptors (Wassarman and Litscher, 1995). Interaction between sperm and zp stimulates the acrosome reaction, liberating acrosomal enzymes necessary for digestion through the zp. In addition to its role during initial interaction between sperm and egg, the zp also serves several other critical functions in reproduction: mediation of species-specificity during gamete interaction (O'Rand, 1988), prevention of polyspermy after fertilization (Bleil and Wassarman, 1980), and protection of the developing embryo prior to implantation (Mintz, 1962; Modlinski, 1970). Mice with eggs that lack zp are completely infertile, verifying the critical role of this ECM in the process of reproduction (Liu *et al.*, 1996; Rankin *et al.*, 1996).

A somatic cell layer, the cumulus, also accompanies the egg into the oviduct at ovulation (Fig. 1). The cumulus layer has both cellular and acellular components. The cellular component is a subset of follicular granu-

losa cells; its acellular component is a hyaluronic acid-rich ECM secreted by the cumulus cells (Camaioni *et al.,* 1996). Potential functions of the cumulus layer include presentation of an enlarged, sticky surface thought to facilitate pickup by oviductal fimbriae and trapping of sperm once within the oviduct (Bedford, 1994; Camaioni *et al.,* 1996; Eppig *et al.,* 1996). However, these functions do not appear essential for fertilization since the cumulus layer, unlike the zp, is not universally present at the time of mammalian gamete interaction. In some species (e.g., bovine, ovine), it is shed soon after ovulation and the sperm is not thought to encounter this layer.

B. The Sperm

The sperm is a highly polarized and specialized cell with a tripartite structure of head, midpiece, and tail that is conserved among all mammals studied (Eddy and O'Brien, 1994). Interaction with the egg is restricted to the sperm head, which can be distinguished into two major domains due to the location of a large secretory vesicle, the acrosome (Fig. 2). As a consequence of sperm interaction with the zp, exocytosis of this granule occurs, termed the acrosome reaction (AR). Exocytosis occurs over the acrosomal region but stops short of its terminal portion, differentiating the acrosome into two segments, the principal segment and the equatorial segment (Fig. 2). This structural distinction has a functional counterpart since the principal segment is involved in initial binding to the zp (Mortillo and Wassarman,

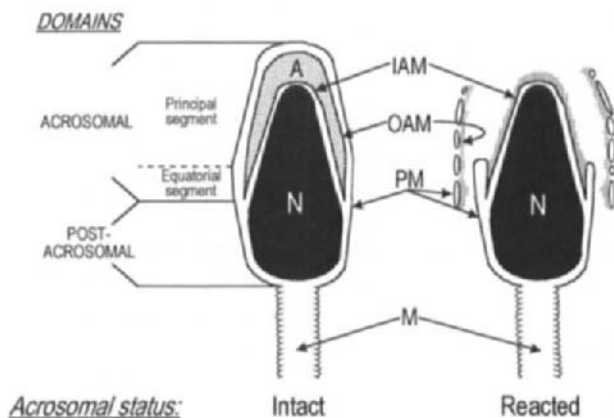


FIG. 2 Schematic representation of the mammalian sperm head as a function of the acrosome reaction. The major domains of the sperm head are indicated. IAM, inner acrosomal membrane; OAM, outer acrosomal membrane; PM, plasma membrane; N, nucleus; M, midpiece.

1991) whereas the equatorial segment is responsible for initiating fusion with the egg plasma membrane (Bedford *et al.*, 1979).

Before interaction with the egg can occur, sperm must penetrate the cumulus layer. To achieve this, sperm are required to first undergo physiological alterations known collectively as capacitation (Austin, 1951; Chang, 1951). If capacitated, sperm are able to penetrate through the cumulus layer, whereas uncapacitated sperm are excluded from entry (Saling, 1989). Despite considerable work, which has been reviewed comprehensively (Florman and Babcock, 1991; Benoff, 1993; Bedford, 1994; Baldi *et al.*, 1995; Fraser, 1995), a satisfying definition for capacitation has not yet been achieved. These recent reviews detail the changes known to occur during this process, including changes in (*a*) the lipid composition of the plasma membrane leading to a net cholesterol decrease; (*b*) many membrane proteins, including their redistribution, modification, or removal; (*c*) sperm motility resulting in a hyperactivated pattern; (*d*) oxidative metabolism; and (*e*) the tyrosine phosphorylation of several sperm proteins. However, whether any of these alterations are a cause or a consequence of capacitation is unclear. The process is reversible and occurs either in the female reproductive tract or in a defined medium *in vitro* (Florman and Babcock, 1991). The result is that capacitated sperm are able to fertilize eggs without delay, in particular by gaining competence for triggered acrosomal exocytosis (Bedford, 1983). The relationship of capacitation to regulated exocytosis appears to be unique to sperm; other secretory cells such as chromaffin or nerve cells do not require such an identified period of preparation for exocytotic stimulation. However, most other cells are not maintained with secretory vesicles anchored at the plasma membrane for the extended period found in sperm.

C. Gamete Interactions: An Overview

Since the cumulus layer is of somatic origin, the first interaction between sperm and egg occurs at the zp surface via receptors in the sperm plasma membrane; in the mouse the zp glycoprotein involved at this stage has been identified as ZP3 (Wassarman and Litscher, 1995; Saling, 1996). Both direct and indirect evidence suggests that this interaction causes aggregation of the receptors present in the sperm membrane and triggers acrosomal exocytosis (Leyton and Saling, 1989a; Aarons *et al.*, 1991; Macek *et al.*, 1991). Controversy revolves around the identity of the sperm's ZP3 receptors, and several candidate proteins will be discussed below. Irrespective of the specific receptors involved, there is general consensus that sperm interaction with ZP3 stimulates profound changes in the sperm cell, including the

activation of tyrosine kinase- and G protein-mediated signaling cascades and ion channels (Yanagimachi, 1994; Saling, 1996).

The consequence of such cellular activation is exocytosis of the acrosomal contents, the array of hydrolytic enzymes mentioned above. Exposure of these enzymes enables the sperm to digest a path through the zp matrix. Sperm proteins localized within the acrosomal granule are also thought to stabilize contact between the acrosome-reacted sperm and the zp during transit through this matrix. Of additional importance is the strong flagellar beat of the sperm which facilitates this penetration (Stauss *et al.,* 1995); indeed, sperm motility is likely to be an absolute requirement at this stage of gamete interaction.

After traversing the zp, the sperm reaches the egg plasma membrane. The plasma membrane overlying the equatorial segment of the acrosome (Fig. 2) is thought to be the site of initiation of fusion with the egg membrane (see below for further discussion); once initiated, fusion progresses until the sperm head and flagellum are incorporated into the egg cytoplasm. On the egg plasma membrane, fusion is restricted to the microvillar region overlying the cortical granules (Yanagimachi, 1994).

After fertilization, the zp is modified to prohibit the binding, AR, and further penetration of sperm (Austin and Braden, 1956; Barros and Yanagimachi, 1971). This block to polyspermy is thought to be mediated by the contents of the egg cortical granules (Fig. 1) (Barros and Yanagimachi, 1972). These granules fuse with the egg plasma membrane following fertilization and extrude their contents into the perivitelline space. A consequence of cortical granule exocytosis in the mouse is the modification of at least two zp glycoproteins, ZP2 and ZP3. ZP2 is proteolyzed from a 120- to a 90-kDa form, termed $ZP2_f$, by a non-trypsin-like protease (Moller and Wassarman, 1989). Similarly, ZP3 is altered to $ZP3_f$, which has altered biological activity, but its conversion is not detectable by a modified molecular weight or isoelectric point (Bleil and Wassarman, 1983). Similar postfertilization modifications of zp glycoproteins have been demonstrated for the rat [Repin and Akimova (1976) cited in Yanagimachi (1994)], the pig (Hedrick *et al.,* 1987; Hatanaka *et al.,* 1992), and the human (Shabanowitz and O'Rand, 1988; Moos *et al.,* 1995).

II. The Zona Pellucida and Its Interaction with Sperm

A. The Zona Pellucida

Structurally, the zp is a glycoprotein coat that forms during oogenesis in all mammalian eggs and, in mature oocytes, can vary in thickness from

<2 μm in marsupials to 27 μm in cows (Dunbar and Wolgemuth, 1984). The zp has a fibrous, sponge-like appearance made up of as little as 4 ng of total protein in the mouse to as much as 30 ng in the pig (Wassarman, 1988). Despite its complex appearance, in most species the zp is composed of only three glycoproteins, ZP1, ZP2, and ZP3 (Harris *et al.,* 1994). All three components are heavily and heterogeneously glycosylated and migrate as broad bands in one- and two-dimensional polyacrylamide gel electrophoresis (Wassarman, 1988). In the intact mouse zp, these glycoproteins have been proposed to be arranged in filaments of ZP2:ZP3 heterodimers assembled by ZP1 homodimers (Greve and Wassarman, 1985).

The zp proteins for which the corresponding genes have been cloned and characterized are shown in Fig. 3. Each is shown with its specific name used in the literature but, based on sequence information, is grouped into one of the three families: ZP1, ZP2, or ZP3. Hereafter in this review, each will be referred to by its species name and family name, e.g., rabbit ZP1, not RC55 or rabbit 55. In some species, such as the mouse and pig, the zp proteins, initially purified from native material, were characterized extensively and their cDNAs were cloned and sequenced subsequently. In other species, such as the cat and marmoset, primary information has been obtained by cloning techniques, and these proteins have yet to undergo comprehensive physiological or biochemical analysis. A few zp proteins have been identified experimentally, such as hamster ZP1 and ZP2, but the sequences of their cDNAs have not yet been reported. Here, we will discuss only those zp proteins for which sequence information is available.

1. Genes That Encode the Zona Pellucida Proteins

Nucleotide sequences are available for cDNAs that encode 26 zp proteins (Table I). The nucleotide and protein similarity among family members is shown in Fig. 4. Overall, the nucleotide sequence shows higher homology than the protein sequence. Genomic clones have been isolated for eight of these proteins and allow analysis of the genomic structure of these sequences. Each gene family is similar in intron/exon structure: the ZP1 family contains 12 exons, the ZP2 family has 18 exons, and the ZP3 family has 8 exons. Human ZP2 and cynomolgus macaque ZP2 have sequences from an additional exon in the C-terminal end (Liang and Dean, 1993; J. Harris, personal communication). The length of the chromosomal region comprising the locus of these genes varies from as few as 6.5 kb (mouse *ZP1*) to as many as 18.3 kb (human *ZP3*) (Chamberlin and Dean, 1990; Epifano *et al.,* 1995a). For those that have been examined, all but human *ZP3* are single-copy genes (Epifano and Dean, 1994). Human *ZP3* is partially duplicated on the same chromosome as a fusion between a gene homologous to a rat nuclear pore membrane protein (*POM121*) and exons

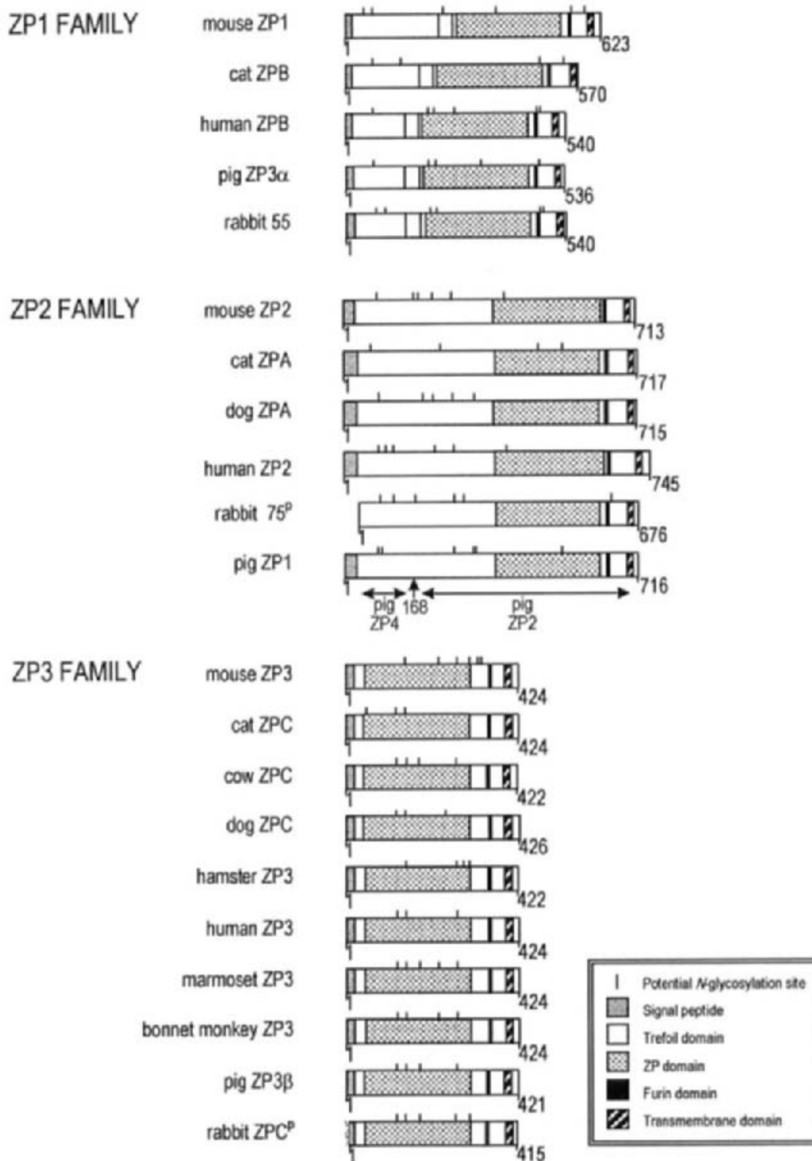


FIG. 3 Zona pellucida family domain organization. Motifs were determined by (1) Swiss-Protein database entries; (2) the PROSITE database at http://expasy.hcuge.ch/sprot/scnpsit1.html for N-glycosylation sites, zp, and Trefoil-(P-)-domain signatures and the algorithms at http://psort.nibb.ac.jp/form.html (a WWW server for analyzing and predicting protein-sorting signals coded in amino acid sequence) for signal peptide and transmembrane domain predictions; and (3) by homology with other family members. The pig zp proteins ZP2 and ZP4 correspond to residues 36–168 (ZP4) and 169–716 (ZP2) of the pig ZP1 cDNA shown here. [P]Partial or incomplete sequences. Only those sequences entered in GenBank are shown.

5–9 of human *ZP3;* this transcript is expressed ubiquitously and is termed *POM-ZP3* (Kipersztok *et al.,* 1995).

In general, the *ZP* genes are transcribed and translated only in oocytes. Mouse ZP2 mRNA appears to be expressed at low levels in resting oocytes, whereas mouse ZP1 and ZP3 are expressed only in growing oocytes (Epifano *et al.,* 1995b). These transcripts accumulate in a coordinated fashion during the growth of the oocyte and reach maximal levels in midsized (50–60 μm) oocytes. Biosynthesis and secretion of the zp by growing oocytes match this transcriptional activation; the coordinate regulation of these genes suggests that they are controlled by similar transcriptional regulators (Epifano *et al.,* 1995b). Coordinate regulation of the zp proteins is also seen in other species; however, in some species, granulosa cells appear to express some of the *ZP* genes as well (Lee and Dunbar, 1993; Grootenhuis *et al.,* 1996; Kölle *et al.,* 1996; Martinez *et al.,* 1996). Further analysis of this topic will better define the specific expression of the *ZP* genes in different species.

The zp proteins appear to have evolved from a common ancestral gene. Fish possess *ZP*-like genes for proteins that are components of their vitelline envelopes [white flounder, *wf*♀ (Lyons *et al.,* 1993), medaka, LSF41 (Murata *et al.,* 1995), goldfish, ZP3 (Chang *et al.,* 1996), and carp, ZP3 (Chang *et al.,* 1996)]. The region of similarity encompasses the ZP domain (see below). Interestingly, *wf*♀ and the medaka egg envelope protein, as well as egg envelope proteins from some species of trout, are synthesized in the liver. These proteins are then transported to the oocyte to become a part of the vitelline envelope (Lyons *et al.,* 1993). Thus, although nucleotide sequence analysis suggests a common ancestor, gene expression appears to be regulated differently in these groups.

2. Zona Pellucida Protein Sequence Analysis

The sequences determined for 26 zp proteins have served to elucidate the relationships among family members (Figs. 3 and 4, Table I). To date, ZP1, ZP2, and ZP3 family members have been cloned from cat, cow, mouse, human, pig, rabbit, and cynomolgus macaque. The five remaining sequences are found in the ZP2 and ZP3 families (Table I). In general, the ZP3 family displays a greater degree of structural conservation compared with that of the ZP1 or ZP2 families (Fig. 3). For those sequences available in GenBank, the following features are shared:

i. short 5′ and 3′ untranslated regions;
ii. N-terminal hydrophobic signal peptides;
iii. potential N- and O-linked glycosylation;

TABLE I
Zona Pellucida Family Members

Family member	Sperm binding	AR inducer	Calc. MW (kDa)	App. MW (kDa)	GenBank Accession No.	Sequence reference
ZP1						
Mouse ZP1			67	200[a,b]	U20448	Epifano *et al.* (1995a)
Cat ZPB			61		U05776	Harris *et al.* (1994)
Cow ZPB[c]			—	88[d]	N/A[e]	Harris *et al.* (1994)
Human ZPB			57	150[f]	U05781	Harris *et al.* (1994)
Cyno. macaque ZPB[c]			—		N/A[e]	J. D. Harris, personal communication
Pig ZP3α	+[g]		57	55[h]	L11000	Yurewicz *et al.* (1993a)
Rabbit 55 kDa	+[i]		57	75[j,k]	M58160	Schwoebel *et al.* (1991)
ZP2						
Mouse ZP2	+[l]		76	120[a]	M34148	Liang *et al.* (1990)
Cat ZPA			76		U05777	Harris *et al.* (1994)
Cow ZPA[c]			—	102[d]	N/A[e]	Harris *et al.* (1994)
Dog ZPA			76		U05779	Harris *et al.* (1994)
Human ZP2			78	100[f]	M90366	Liang and Dean (1993)
Cyno. macaque ZPA[c]			—		N/A[e]	J. D. Harris, personal communication
Pig ZP1	±[m]		76	90[n]	S74651	Taya *et al.* (1995)
Rabbit 75 kDa[c]			75	85[j,k]	L12167[o]	Lee *et al.* (1973)
ZP3						
Mouse ZP3	+[p]	+[p]	44	83[a]	M20026	Ringuette *et al.* (1988)
Cat ZPC			44		U05778	Harris *et al.* (1994)
Cow ZPC			44	73[d]	U05775	Harris *et al.* (1994)
Dog ZPC			45		U05780	Harris *et al.* (1994)
Hamster ZP3	+[q]	+[q]	44	56[q]	M63629	Kinloch *et al.* (1990)
Human ZP3	+[r]	+[r]	44	65[f]	M35109	Chamberlin and Dean (1990)
Marmoset ZP3			44		S71825	Thillai-Koothan *et al.* (1993)

Protein	Sperm binding	Acrosome reaction	Calculated MW	Apparent MW	GenBank Accession No.	Reference
Cyno. macaque ZPC[c]			—		N/A[e]	J. D. Harris, personal communication
Bonnet monkey ZP3			44		X82639	Kolluri *et al.* (1995)
Pig ZP3β	±[s]		44	55[h]	L22169	Harris *et al.* (1994)
Rabbit ZPC[c]			43		U05782[t]	Harris *et al.* (1994)

Note. ZP1, ZP2, and ZP3 family members are listed with their common nomenclature, each was evaluated for sperm-binding activity, acrosome reaction inducing function, calculated molecular weight, apparent molecular weight, GenBank Accession No., and sequence literature reference. Calculated molecular weights were determined by Protein (DNAstar, Inc., Madison, WI) and do not include the signal peptide sequence.

[a] Van Duin *et al.* (1993).

[b] Mouse ZP1 is a dimer.

[c] Partial or incomplete sequences.

[d] Florman and First (1988).

[e] Partial clones: cow ZPA and cynomolgus macaque ZPA encode the C-terminal $\frac{1}{4}$ of the protein; cow ZPB, cynomolgus macaque ZPB, and ZPC encode the C-terminal $\frac{1}{2}$ of the protein.

[f] Moos *et al.* (1995).

[g] Sacco *et al.* (1989).

[h] Yurewicz *et al.* (1987).

[i] Prasad *et al.* (1996).

[j] Lee *et al.* (1993).

[k] The apparent molecular weights shown for rabbit 55 kDa and rabbit 75 kDa were derived from endo-β-galactosidase-treated rabbit zp.

[l] Bleil *et al.* (1988).

[m] Tsubamoto *et al.* (1996).

[n] Yurewicz *et al.* (1983).

[o] The clone for rabbit 75 kDa is missing the N-terminal ~49 codons. However, N-terminal sequencing of the purified protein yielded a peptide whose sequence was KQLQPSDPAF plus a 17-amino-acid overlap with the predicted amino acid sequence of the clone. Addition of these 10 amino acids to the N terminus of the protein sequence predicted by the clone yields a 676-amino-acid protein which is missing ~33–39 amino acids. These amino acids encode the presumptive signal peptide.

[p] Bleil and Wassarman (1983).

[q] Moller *et al.* (1990).

[r] Van Duin *et al.* (1994).

[s] Yurewicz *et al.* (1993b).

[t] The rabbit ZPC clone is missing the initiating ATG and ~3 codons within the signal peptide.

Amino Acid Similarity (%)

Nucleotide Similarity (%)

	1	2	3	4	5	6	7		ZP1 Family
1		33	32	33	30	43	42	1	mouse ZP1
2	43		67	67	64	73	72	2	cat ZPB
3	40	74		72	73	70	90	3	human ZP1
4	41	75	79		71	83	40	4	pig ZP3α
5	39	79	79	78		75	71	5	rabbit 55
6	48	79	80	87	79		70	6	cow ZPB^P
7	48	78	93	76	77	79		7	cyno. macaque ZPB^P
	1	2	3	4	5	6	7		

Amino Acid Similarity (%)

Nucleotide Similarity (%)

	1	2	3	4	5	6	7	8		ZP2 Family
1		58	59	57	55	62	64	50	1	mouse ZP2
2	70		84	67	72	66	80	64	2	cat ZPA
3	70	89		67	71	67	81	64	3	dog ZPA
4	69	78	77		63	68	62	94	4	human ZP2
5	68	82	81	75		64	82	55	5	pig ZP1
6	73	79	78	77	76		74	63	6	rabbit 75^P
7	72	87	87	70	88	82		59	7	cow ZPA^P
8	59	70	70	96	66	70	66		8	cyno. macaque ZPA^P
	1	2	3	4	5	6	7	8		

Amino Acid Similarity (%)

Nucleotide Similarity (%)

	1	2	3	4	5	6	7	8	9	10	11		ZP3 Family
1		64	64	66	79	67	67	67	66	65	65	1	mouse ZP3
2	71		73	79	64	70	68	71	73	65	70	2	cat ZPC
3	67	80		74	64	72	71	71	83	68	67	3	cow ZPC
4	68	86	78		66	71	68	70	76	66	65	4	dog ZPC
5	85	72	68	70		68	68	68	65	66	69	5	hamster ZP3
6	72	78	76	76	74		91	93	74	69	91	6	human ZP3
7	71	76	75	73	74	92		88	72	68	87	7	marmoset ZP3
8	72	77	75	74	74	95	92		73	68	98	8	bonnet monkey ZP3
9	69	80	85	78	71	78	76	76		68	68	9	pig ZP3β
10	64	69	70	67	66	70	70	70	69		60	10	rabbit ZPC^P
11	72	77	75	75	74	95	91	99	76	70		11	cyno. macaque ZPC^P
	1	2	3	4	5	6	7	8	9	10	11		

iv. a hydrophobic domain near the C terminus with characteristics of a transmembrane domain;
v. a canonical furin proteolytic processing signal, R-X-R/K-R, altered to S-R-R-R (human and rabbit ZP1) and S-R-R-N (cow ZP1), upstream of the putative transmembrane domain; and
vi. a ZP-signature domain, altered in mouse ZP1 and cat ZP3.

Functions for the furin proteolytic processing signal and the putative transmembrane domain have not yet been determined. However, Yurewicz *et al.* (1993a) have suggested that processing at the furin site may allow the zp proteins to move to the outer edge of the zp as this region grows larger during oocyte maturation.

Two sequence motifs, the zp domain and the Trefoil (or P-) domain, have been recognized in zp family members and may help to determine the three-dimensional structure of these proteins. The zp domain (Bork and Sander, 1992) consists of ~260 amino acids and has thus far been recognized only in the zp proteins, transforming growth factor β receptor III, uromodulin, and pancreatic secretory granule glycoprotein GP-2 (Bork and Sander, 1992). This domain is contained in the PROSITE database (Bairoch, 1991) as an ~40-amino-acid signature. When the zp proteins are used to search this database, all family members except mouse ZP1 and cat ZP3 conform to the signature sequence. However, nonconformity for these latter two sequences is restricted to only 1 amino acid (residue 459 of mouse ZP1, residue 204 of cat ZP3); overall alignment of these proteins with the other family members indicates that, despite this discrepancy in the signature motif, the zp domain is intact in these proteins. Further analysis of the zp domain from the ZP1, ZP2, and ZP3 families indicates that the ZP1 and ZP2 families are more similar to one another (35–42%) than either family is to the ZP3 family (17–25%). The zp protein families show a sequence and genomic structural similarity to fish vitelline envelope proteins in this area (see above) (Epifano *et al.*, 1995a).

The Trefoil domain is a 45-amino-acid cysteine-rich region found originally in a family of small polypeptides called the Trefoil family. Of the zp proteins, only the ZP1 family features this domain. In addition to the ZP1s, this family also includes a group of gastrointestinal polypeptides, frog skin

---

FIG. 4 Zona pellucida family cDNA nucleotide and amino acid similarities. Boxes above the diagonal compare amino acid similarity; boxes below compare nucleotide similarity. Shaded boxes indicate a C-terminal partial sequence alignment with 25–50% of the cDNA available for comparison. Similarities were determined by the program MegAlign (DNAstar, Inc., Madison, WI) and are defined as similarity $(i,j) = 100 \times$ sum of the identity matches/length $-$ gap residues (i) $-$ gap residues (j). Protein and DNA sequences were aligned identically. P-Partial or incomplete sequences.

proteins and mucins, and the vertebrate enzymes sucrase–isomaltase and lysosomal α-glucosidase (Hoffman and Hauser, 1996). This domain is also featured in the PROSITE database (Bairoch, 1991) as a 22-amino-acid signature. All of the ZP1 family members conform strictly to this motif except mouse ZP1, which deviates from the signature at amino acids 235 and 240. The overall alignment of mouse ZP1 in this area, however, suggests that a Trefoil domain does exist in a slightly modified form in mouse ZP1 (P. Bork, personal communication).

The crystal structure for a dual Trefoil domain-containing protein, porcine pancreatic spasmolytic polypeptide, has been solved and reveals a disulfide-linked three-looped Trefoil motif containing an 8- to 10-Å cleft which could accommodate part of an oligosaccharide chain (Gajhede *et al.*, 1993; De *et al.*, 1994). Many Trefoil domain-containing proteins demonstrate high resistance to proteolytic degradation (Thim, 1989). A mechanism for this resistance is proposed to be associated with the domain's extensive crosslinking and short secondary structure. These elements limit the flexibility of the motif and serve to shield it from access to protease active sites (Gajhede *et al.*, 1993). For the ZP1 family, it is tempting to speculate that this domain could be involved in zp assembly by linking ZP1 to neighboring oligosaccharides via the Trefoil cleft or in maintaining zp ultrastructure during proteolytic attack by acrosomal enzymes or cortical granule enzymes during fertilization.

3. ZP1, ZP2, and ZP3

The most extensive investigation of sperm–zp interactions has been carried out in the mouse (Saling, 1989; Wassarman and Litscher, 1995). That work started at the cellular level and continued at a molecular level once the zp proteins were identified and purified. The collective conclusions from those studies, which are amplified below, are:

i. primary sperm–zp interaction is mediated by ZP3 and specific ZP3 receptors in the sperm plasma membrane;
ii. acrosomal exocytosis is triggered in sperm bound to ZP3;
iii. secondary sperm–zp interaction ensues between the acrosome-reacting/reacted sperm and ZP2; and
iv. ZP1 does not interact directly with sperm.

These findings have served as the fundamental paradigm for understanding gamete interaction in other mammalian species. Thus, ZP3 proteins are predicted to serve as the ligand during primary binding and to trigger the AR; likewise, ZP2 proteins are predicted to exhibit secondary binding activity with acrosome-reacted sperm. In this framework based on protein function, ZP1 has remained the neglected cousin. However, data concerning

zp proteins from several species have emerged recently, and it may be useful to reevaluate the collective findings on zp protein function to discern basic principles.

a. Primary Binding—Sperm Interaction with ZP3 In the mouse, ZP3 binds to specific receptors in the sperm plasma membrane during the initial interaction between sperm and zp. These ligand–receptor complexes serve dual roles: (*i*) to adhere sperm to the zp and (*ii*) to elicit a cellular response in sperm, the AR. The bifunctional nature of the interaction between sperm and ZP3 has been investigated extensively in the mouse. The mouse ZP3 glycoprotein consists of a 44-kDa polypeptide with three or four N-linked oligosaccharides and an undetermined number of O-linked oligosaccharides, generating a glycoprotein with an observed average molecular weight of 83 kDa (Wassarman and Litscher, 1995). The sperm-binding sites of mouse ZP3 have been attributed to O-linked oligosaccharides located in the C-terminal half of the molecule (Florman and Wassarman, 1985; Rosiere and Wassarman, 1992). In particular, O-linked oligosaccharides linked to amino acids between Cys_{328} and Asp_{343} have been suggested to play an important role in sperm binding (Kinloch *et al.,* 1995). Competition studies have attempted to examine the oligosaccharide structure important for binding sperm to mouse ZP3. Small glycopeptides (1.5–6 kDa) obtained following pronase digestion of mouse ZP3, O-linked oligosaccharides derived from alkaline reduction of mouse ZP3, and synthetic oligosaccharide constructs have been shown to inhibit binding of mouse sperm to mouse zp (Florman *et al.,* 1984; Florman and Wassarman, 1985; Litscher *et al.,* 1995). The ability of an oligosaccharide to compete for sperm binding depends upon its size and branching pattern, as well as the terminal sugar moiety at the nonreducing end of the oligosaccharide (Litscher *et al.,* 1995). However, the identity of the terminal sugar is controversial. Bleil and Wassarman (1988) suggest that terminal galactose is critical, whereas the work of Miller *et al.* (1993) implicates the sugar *N*-acetylglucosamine at the nonreducing end of the oligosaccharide chain by demonstrating that its modification by egg cortical granule *N*-acetylglucosaminidase renders ZP3 unable to bind sperm.

These isolated sugars and sugar–peptide moieties compete for sperm binding with an ID_{50} in the 1–10 μM range, whereas intact, purified mouse ZP3 competes 10- to 100-fold more effectively, with an ID_{50} of 100 nM (Litscher *et al.,* 1995). Furthermore, none of these smaller molecules mimics the ability of intact mouse ZP3 to induce the AR (Florman *et al.,* 1984; Florman and Wassarman, 1985; Litscher *et al.,* 1995). The smallest defined region of mouse ZP3 that retains both sperm-binding and AR-triggering ability is a 21-kDa C-terminal fragment produced by digestion of mouse ZP3 with papain and subsequent removal of N-linked oligosaccharides by

N-glycanase (Litscher and Wassarman, 1996). Regarding the ZP3 polypeptide backbone, potential functions include orienting the three-dimensional presentation of the sperm-binding O-linked oligosaccharides and serving a direct role in sperm binding. The latter suggestion arises from observations concerning the ability of antibodies raised against ZP3 peptides to block interaction with sperm (Bagavant *et al.*, 1993a,b).

In contrast to this thorough analysis of mouse ZP3 function, not all ZP3 homologs are even known to bind sperm and initiate the AR. Only 4 of the known 11 members of the ZP3 family have been evaluated for these functions at present (Table I). Confounding the mouse ZP3 role as the exclusive ligand for primary binding, in the porcine system, *both* ZP1 and ZP3 have been shown to possess sperm-binding capacity (see below) (Sacco *et al.*, 1989; Bagavant *et al.*, 1993a,b; Töpfer-Petersen *et al.*, 1993; Yurewicz *et al.*, 1993b,c).

b. Secondary Binding—Sperm Interaction with ZP2 After the AR occurs, the plasma membrane of the sperm is no longer the outermost membrane surface available to bind and interact with the zp. ZP3 receptors on the sperm plasma membrane that adhered sperm to zp prior to the AR are lost from the sperm during acrosomal exocytosis (Fig. 2). At this stage of interaction, termed secondary binding, a second mouse zp protein, mouse ZP2, has been proposed to mediate interactions between sperm and zp (Bleil and Wassarman, 1986). Whereas mouse ZP3 binds preferentially to acrosome-intact sperm heads, mouse ZP2 binds preferentially to the inner acrosomal membrane of acrosome-reacted sperm heads (Bleil *et al.*, 1988; Mortillo and Wassarman, 1991). Antibodies against mouse ZP2 compete for binding of acrosome-reacted sperm to eggs, but do not compete for binding of acrosome-intact sperm to eggs (Bleil *et al.*, 1988). Soybean trypsin inhibitor mimics the action of anti-mouse ZP2 antibodies by reducing the number of acrosome-reacted sperm bound to eggs, implying that a trypsin-like proteinase might be involved in sperm binding to mouse ZP2 (Bleil *et al.*, 1988).

c. ZP1—Structural Role or Sperm Binding? Mouse ZP1, a homodimer made up of 100-kDa monomers, is thought to play a structural role in the zp; it has been proposed to crosslink ZP2:ZP3 filaments to make up the three-dimensional zp matrix (Greve and Wassarman, 1985). In contrast to this restricted structural role, considerable work on porcine ZP1 has delineated a sperm-binding role, similar to that of murine ZP3 (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993a,b). Porcine ZP1 binds to sperm membranes, and N-linked oligosaccharides in the N-terminal region of the glycoprotein have been suggested to be important (Yonezawa *et al.*, 1995; Nakano *et al.*, 1996), in contrast to the O-linked oligosaccharides of ZP3 discussed

above. In addition, porcine ZP3 is also involved in primary binding by enhancing porcine ZP1's ability to bind to sperm membranes; the combination of ZP1 + ZP3 is eightfold more effective than ZP1 alone (Yurewicz *et al.*, 1993b). Other experiments also suggest a direct role for porcine ZP3 in sperm–zp binding since an anti-porcine ZP3 antibody blocks sperm–zp interaction; this antibody was prepared against a 25-mer peptide that is thought to be rich in O-linked oligosaccharide (Bagavant *et al.*, 1993b). It is useful to note, however, that the ZP1 and ZP3 proteins in the pig copurify and must be modified chemically to achieve separation (Yurewicz *et al.*, 1987). Consequently, the function of each of these proteins in isolation has yet to be analyzed fully. In addition to these results using pig gametes, rabbit ZP1 has also been suggested to play a sperm-binding role (Prasad *et al.*, 1996), questioning the suitability of mouse ZP1 as a general model for the function of this family of zp proteins. In this regard, it is useful to remember that mouse ZP1 is <40% similar to its other family members, each of which share >60% similarity (Fig. 4).

4. Recombinant Zona Pellucida Proteins

Until very recently, experimentation in the field of sperm–zp interaction has been limited by the paucity of eggs that can be recovered from most mammals. This technical limit has restricted the types of experiments that can be performed. For this reason, it is particularly valuable that many zp family members have been cloned and characterized. By expressing these proteins recombinantly, they can be produced and utilized in appropriate quantities. While the work done with these recombinant proteins will require verification by parallel studies using native material, development of these bioactive reagents continues to greatly enhance work in the field of sperm–zp interaction.

Several laboratories have begun to produce biologically active zp proteins *in vitro* (Kinloch *et al.*, 1991; Beebe *et al.*, 1992; Van Duin *et al.*, 1994; Barratt and Hornby, 1995; Burks *et al.*, 1995). These reagents are particularly critical for investigation of human sperm–zp interaction. The ZP3 family of proteins has been the subject of most of the work in this area: (*i*) human ZP3 has been expressed in bacteria (Barratt and Hornby, 1995), and in mammalian CHO (Van Duin *et al.*, 1994; Barratt and Hornby, 1995) and COS cells (Burks *et al.*, 1995); (*ii*) mouse ZP3 has been expressed in mouse L cells, monkey CV-1 cells (Beebe *et al.*, 1992), and embryonic carcinoma cells (Kinloch *et al.*, 1991); (*iii*) hamster ZP3 has been expressed in embryonic carcinoma cells (Kinloch *et al.*, 1991); and (*iv*) bonnet monkey ZP3 has been expressed in *Escherichia coli* (Kaul *et al.*, 1996). The only other recombinant zp proteins produced have been from the pig: porcine ZP2 has been expressed in *E. coli* (Tsubamoto *et al.*, 1996), and in CHO and 293T cells

(Yamasaki *et al.*, 1996), whereas porcine ZP1 has been expressed as a β-galactosidase fusion protein in *E. coli* (Gupta *et al.*, 1996b).

Use of these recombinant proteins has permitted an exploration of the role of glycosylation in ZP3 bioactivity. Bacterially expressed human ZP3-glutathione-S-transferase fusion protein is not glycosylated and is unable to induce the AR in human sperm (Barratt and Hornby, 1995). However, human ZP3 expressed in CHO cells produced via two different expression systems is able to induce the AR in human sperm (Van Duin *et al.*, 1994; Barratt and Hornby, 1995). Expression of rabbit ZP1 in a baculovirus expression system produced protein which was glycosylated by Sf9 insect cells, but not to the extent found in intact zp. This protein could bind to sperm and inhibit sperm–zp interaction, but its ability to trigger the AR was not reported (Prasad *et al.*, 1995, 1996). The role of recombinant zp proteins as experimental reagents and as tools in contraceptive vaccine development (Prasad *et al.*, 1996) can be predicted to continue vigorously in the coming years.

5. Summary

The zp is an egg-specific ECM that consists of three structurally conserved glycoproteins, is universally present at the time of fertilization, and regulates sperm function. Work in the mouse suggests that O-linked oligosaccharides of ZP3 feature prominently in primary binding to sperm whereas ZP2 serves as ligand for sperm binding once the AR has occurred (secondary binding). Mouse ZP1 has not been found to display sperm-binding properties and is thought to perform a scaffolding role in zp matrix organization. More recent work in other species, such as the pig and rabbit, reveals that ZP1 may also function as a primary ligand for sperm binding. Analysis of recombinantly expressed proteins is likely to facilitate determining whether a universal paradigm exists for mammalian sperm interaction with zp.

B. Sperm Proteins That Interact with the Zona Pellucida

The previous section detailed research identifying the zp proteins that interact with sperm. These proteins serve as ligands for receptors on the sperm. Several candidate zp-binding sperm proteins have been identified and can be grouped as primary or secondary receptors. Primary binding candidates are responsible for initial interaction with the zp and initiation of the AR; secondary binding candidates adhere the sperm to the zp following the AR and during the transit of the sperm through the zp. Thus, a sperm protein involved in primary interaction with the zp will be found on the sperm's plasma membrane overlying the acrosome, the region of the

sperm head which first contacts the zp. In mice, the zp ligand for this interaction is ZP3. A sperm protein involved in secondary interaction with the zp is anticipated to be present on or associated with the surface of the acrosome-reacted sperm (inner acrosomal membrane; see Fig. 2). Current evidence in the mouse suggests that ZP2 serves as the ligand at this stage. Molecules proposed to be involved in zp-regulated exocytosis of the acrosome are expected to modulate signaling cascades such as G proteins, tyrosine kinases, or ion channels, all of which are active in acrosomal exocytosis. Due to the zp modifications that constitute the block to polyspermy, sperm receptors necessary for fertilization are not expected to interact with zp proteins recovered from fertilized eggs.

From the preceding discussion of zp proteins, it would appear as though the identification of sperm protein binding partners for each of the zp proteins would be straightforward. Indeed, many studies have aimed at this goal and resulted in the identification of potentially relevant enzymatic activities (Cornwall *et al.*, 1991), antigens recognized by monoclonal antibodies (Brucker *et al.*, 1992; Alves *et al.*, 1995), or sperm-associated components (Robinson *et al.*, 1987). In most cases, however, molecular characterization of the candidate receptor has not yet been reported; although likely to be interesting in the context of gamete interaction, sperm components in this category will not be considered further here. Several other sperm proteins are also potentially important in interaction with the zp, such as FA-1, which blocks sperm–zp interaction in competition studies (Naz and Ahmad, 1994; Kadam *et al.*, 1995); however, sequence information has not yet been reported and we will not consider them further.

One limitation to the identification of binding partners for individual zp proteins has been production of sufficient amounts of purified native zp proteins. The mouse has served as the prototype for analysis of individual zp proteins, yet dealing with mouse sperm offers several drawbacks such as the limited number of sperm that can be obtained and their fragility. In other species, such as the pig or rabbit, in which sperm are plentiful or robust, the purification of isolated native zp proteins is difficult due to inherent properties of the zp proteins. Consequently, whole solubilized zp have been used by several investigators, enabling the identification of zp-binding proteins without regard to the particular zp protein(s) involved in the interaction. Recently, Hardy and Garbers (1995) reported a cDNA sequence that encodes a porcine sperm membrane protein, designated zonadhesin (Table II), which binds (whole) pig zp in a species-specific manner and whose expression is restricted to the testis. Interestingly, both the human and mouse *Zonadhesin* genes are located on the same chromosome as their respective *ZP3* gene (D. L. Garbers, personal communication), suggesting a possible chromosomal linkage of some fertilization-specific molecules, although neither the localization of zonadhesin on the

TABLE II
Sperm Proteins That Interact with the Zona Pellucida

Sperm protein	App. MW (kDa)[a]	Species	Accession No.[b,c]	Sequence reference
Galactosyltransferase	54	Mouse	JO3880[b]	Shaper *et al.* (1988)
PH-20	41 + 27[d]	Guinea pig	X56332[b]	Lathrop *et al.* (1990)
Proacrosin	52–55	Human	Y00970[b]	Baba *et al.* (1989b)
Sp17	17–26	Rabbit	Z20655[b]	Richardson *et al.* (1994)
sp38	38	Pig	D17572[b]	Mori *et al.* (1995)
sp 56	56	Mouse	U17108[b]	Bookbinder *et al.* (1995)
Spermadhesin				
AQN-1			P26322[c]	Sanz *et al.* (1992b)
AQN-3	12–16		P24020[c]	Sanz *et al.* (1991)
AWN[e]		Pig	P26776[c]	Sanz *et al.* (1992a)
Zonadhesin	105 + 45[f]	Pig	U40024[b]	Hardy and Garbers (1995)
ZRK	95	Human	L08961[b]	Burks *et al.* (1995)

Note. These sperm proteins are listed with respect to their apparent size, the species from which sequence information was derived, and the database accession number and literature reference for the sequence information.

[a] The reported size of each protein is based on disulfide-reducing SDS gels.

[b,c] The accession numbers provided are from [b]GenBank or [c]SwissProt.

[d] Following the AR, the 64-kDa PH-20 protein is cleaved into 41- and 27-kDa disulfide-linked fragments.

[e] AWN-1 and AWN-2 have the same nucleotide sequence, but AWN-2 is acetylated at the N terminus.

[f] Zonadhesin was originally purified from epididymal boar sperm as a 150-kDa protein without disulfide-reducing agents.

sperm nor its zp ligand has been defined. Since bioactive recombinant zp proteins are now becoming available, attempts to isolate sperm proteins that bind to individual zp proteins are finally reasonable and should lead to significant progress on this topic.

1. Candidate Sperm Proteins Involved in Primary Binding to the Zona Pellucida

An additional caveat to identifying sperm proteins involved in primary binding to the zp is their loss from the cell. By definition, proteins involved in primary binding are located on the principal segment of the plasma membrane overlying the sperm's acrosome and will be lost from the cell

as a consequence of acrosomal exocytosis (see Figs. 2 and 5). Despite this cautionary note, investigators have been successful in identifying sperm proteins involved in primary zp binding, and four candidates are diagramed in Fig. 5 and discussed below.

a. Galactosyltransferase (GalTase) The first sperm protein reported as a primary zp binding candidate was β1-4 galactosyltransferase (GalTase; Fig. 5) (Shur and Hall, 1982). This enzyme is found not only in the Golgi apparatus and endoplasmic reticulum, where it functions as a biosynthetic glycosyltransferase, but it is also found on the surface of several cell types (Evans *et al.*, 1995b), including sperm (Shaper *et al.*, 1990). The intracellular and surface forms differ by only 13 residues at the cytoplasmic N-terminal (Table II) (Shaper *et al.*, 1988). On the cell surface, GalTase apparently does not use its enzymatic activity in its receptor function but, rather, operates as a lectin, recognizing terminal *N*-acetylglucosamine-containing oligosaccharide ligands. The initial suggestion that GalTase may be involved in zp adhesion arose from a correlation between fertilizing ability and levels of GalTase activity in certain *t*-haplotype sperm (Shur and Bennett, 1979).

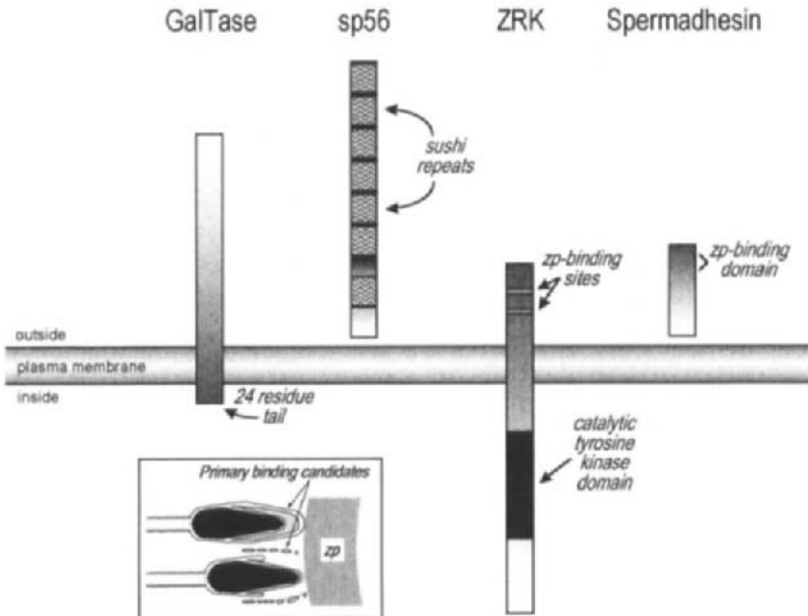


FIG. 5 Proposed structures of candidate sperm proteins involved in primary binding to the zp. Shading depicts amino → carboxy termini (dark → light). The inset represents the location of these proteins on the sperm head during interaction with the zp.

Subsequently, it was observed that sperm–egg binding is inhibited by purified GalTase, GalTase inhibitors, and anti-GalTase antibodies (Shur and Neely, 1988). Use of galactosylated zp proteins indicates that GalTase interacts specifically with ZP3 (Miller *et al.*, 1992), and sperm from mice that overexpress a surface GalTase transgene bind more ZP3 than wild-type sperm (Youakim *et al.*, 1994).

In addition to its role as a sperm adhesion molecule, GalTase is suggested to possess signal transduction functions as well. Sperm from transgenic mice that overexpress surface GalTase bind more ZP3 than controls and a greater proportion of these sperm undergo the AR (Youakim *et al.*, 1994). Shur and co-workers found that aggregation of sperm surface GalTase by bivalent antibodies activates G proteins, and synthetic peptides derived from the cytoplasmic domain of the long form of GalTase associate with heterotrimeric G_i proteins (Gong *et al.*, 1995). Consistent with the putative function of GalTase as a signal transducing receptor for ZP3, zp from unfertilized eggs increases GTP-γ-^{35}S binding to sperm membranes twofold, while zp from two-cell embryos does not (Gong *et al.*, 1995). Though considerable evidence supports a role for GalTase in sperm interaction with the zp, its suggested role in AR triggering has been challenged recently, and the precise regionalization of GalTase on the sperm surface at the time that the sperm meets the zp is ambiguous (Cardullo and Wolf, 1995).

Recent work from Shur and co-workers has focused on generating a GalTase knockout mouse and analyzing its phenotype (B. D. Shur, personal communication). GalTase-null sperm are capable of fertilizing eggs *in vivo,* though litters are smaller and gestation times are longer than those for wild-type controls, documenting the fact that GalTase is not essential for fertilization. Shur and co-workers are considering the idea that the entire mechanism of GalTase–ZP3 binding and signaling interactions is used by mouse gametes to optimize the process of fertilization (B. D. Shur, personal communication).

b. sp56 A second ZP3-binding candidate is a 56-kDa peripheral membrane protein, sp56. This protein was originally identified in mouse sperm by its ability to become covalently associated with purified mouse ZP3 (Bleil and Wassarman, 1990) or with ^{125}I-labeled ZP3 glycopeptides (Cheng *et al.*, 1994) via a photoactivatable, radiolabeled crosslinker. Analysis of the binding of monoclonal antibodies specific for sp56 using light and electron microscopy has localized the protein to the dorsal region of the mouse sperm head, appropriate for a role in primary binding. Furthermore, purified sp56 binds to zp surrounding mouse eggs, but not embryos, and inhibits sperm–egg binding *in vitro* (Bookbinder *et al.*, 1995).

Recently, a cDNA encoding sp56 has been cloned and sequenced (Bookbinder *et al.*, 1995), revealing a deduced protein which contains seven Sushi

repeats (Table II). Dual cysteine residues in each repeat are consistent with the numerous intramolecular disulfide linkages hypothesized from electrophoretic studies (Fig. 5). Sushi domains (also called short consensus repeats) are motifs of ~60 amino acids found in many blood complement system proteins and other molecules of widely varied function. These motifs often occur in tandem arrays of five or more (Reid and Day, 1989). Database homology searches indicate that sp56 is most similar to the α chain of complement 4B-binding protein, C4BPα, which also contains Sushi repeats (Hessing, 1991), and the region of homology is confined to this motif. Both human and mouse C4BPα are ~44% identical to mouse sp56. No putative transmembrane-spanning regions are present in sp56, consistent with previous data which suggested that sp56 is a peripheral membrane protein (Cheng *et al.,* 1994). Northern blot analysis of mouse tissues detects a 2-kb RNA transcript for sp56 in the testis, but not in other tissues. Correspondingly, protein immunoblot analysis with an anti-sp56 monoclonal antibody localizes the sp56 protein to the testis and epididymis, but not to other tissues. *In situ* hybridization of sp56 mRNA demonstrates its expression only in round spermatids, and immunohistochemistry reveals the sp56 polypeptide in round and elongating spermatids and in spermatozoa.

Using Northern blot and immunohistochemical analysis, sp56 does not appear to be highly conserved among mammalian species. It is found in the mouse and hamster, but not in the guinea pig or human (Bookbinder *et al.,* 1995). Of interest is a correlation between the occurrence of sp56 and the specificity of sperm–egg binding for that species. Both mouse and hamster sperm bind the zp of mouse eggs, and sperm from both of these species contain sp56 transcripts and anti-sp56 antigens. However, human and guinea pig sperm, neither of which contain detectable sp56 message or protein, do not bind mouse eggs (Bookbinder *et al.,* 1995). Although the closer evolutionary relationship of mice and hamsters may explain a greater similarity among these proteins, examination of additional species with regard to the expression of sp56 and zp binding will be important in validating this hypothesis.

Ongoing studies suggest that sp56 is present on the mouse sperm surface as a 62-kDa protein, tethered together to form a homomultimeric protein by intermolecular disulfide linkages in the C termini. Preliminary studies suggest that association between the homomultimeric sp56 complex and ZP3 on the zp surface results in the release of sp56, while the crosslinked C termini remain sperm-associated. Continuing work is aimed at examining whether these products may play a role in signal transduction (J. D. Bleil, personal communication).

c. Zona Receptor Kinase (ZRK) A third ZP3-binding candidate molecule, and one which has intrinsic signaling potential, is a 95-kDa transmem-

brane receptor tyrosine kinase known as ZRK. Tyrosine phosphorylation in sperm is important for fertilization since inhibition of protein tyrosine kinase activity prevents acrosomal exocytosis (Leyton *et al.,* 1992) and blocks fertilization. Consistent with observations in mice (Leyton and Saling, 1989b; Leyton *et al.,* 1992), a 95-kDa protein is the major phosphotyrosine-containing protein identified in human sperm, and its level of phosphotyrosine increases with capacitation (Burks *et al.,* 1995). Use of the monoclonal antibody mAb 97.25 has independently implicated a 95-kDa human sperm protein in sperm–zp interaction (Moore *et al.,* 1987); this human sperm protein is also tyrosine-phosphorylated. Live human sperm, probed with mAb 97.25, demonstrate that this antigen is located on the sperm surface in the acrosomal region, appropriate for a role in gamete interaction. Although Kopf and co-workers (Kalab *et al.,* 1994) have suggested that the 95-kDa ZP3-binding protein in mouse sperm is a novel hexokinase, these two proteins appear to be completely distinct (Leyton *et al.,* 1995). Two antibodies, anti-phosphotyrosine and mAb 97.25, were used in series to screen a human testis expression library and a novel clone reactive with both probes was isolated and termed hu9 (Table II) (Burks *et al.,* 1995). Based on intracellular subdomain structure and signature motifs, *huq* is a member of the *axl* family of receptor tyrosine kinases (RTKs) (Fig. 5) and is most similar (61%) to another recently isolated *axl* family member, *c-mer* (Graham *et al.,* 1994).

Among the evidence that supports the identity of *hu9* as encoding human ZRK is work with a rabbit antipeptide antibody generated against *hu9*-deduced intracellular residues 539–553, called K16. Not only does this antibody immunoprecipitate a 95-kDa phosphotyrosine-containing protein from extracts of capacitated human sperm, but mAb 97.25 also immunoprecipitates a 95-kDa band which is recognized by K16, indicating that the same tyrosine-phosphorylated protein is recognized by both antibodies. Immune complex kinase assays, using K16, show that human ZP3 is a ligand capable of stimulating sperm kinase activity and that K16 immunoprecipitates a zp-responsive kinase, further suggesting that *hu9* encodes ZRK. The putative receptor function of the *hu9*-encoded polypeptide was examined in a human hemi-zona assay (Durkman *et al.,* 1988). Two peptides (residues 57–71 and 94–105 of the *hu9*-encoded extracellular domain) competitively inhibit human sperm–zp interaction, blocking binding by 69 and 80%, respectively (Burks *et al.,* 1995).

Examination of the intracellular domain sequence encoded by *hu9* reveals that many of the tyrosine residues are located in consensus motifs predicted to bind SH2-containing proteins to activated RTKs (Songyang *et al.,* 1994). SH2-containing proteins that are involved directly in signaling, such as phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PI 3-K), as well as adaptor proteins, such as Grb-2, Shc, and 3BP2, which link RTKs

with G protein signaling pathways, are thus predicted to bind to ZRK. PLCγ1 has been identified in both mouse (Tomes *et al.*, 1996) and human (Saling *et al.*, 1996) sperm, where immunostaining demonstrates a periacrosomal location. Catalytically active PLC is immunoprecipitated by both anti-PLCγ1 and antiphosphotyrosine antibodies and, in human sperm, PLCγ1 coprecipitates with ZRK. Stimulation of mouse sperm with solubilized mouse zp or recombinant mouse ZP3 increases PIP_2–PLC enzymatic activity, leading to increased IP_3 production in a pathway that depends on tyrosine phosphorylation (Tomes *et al.*, 1996). Another SH2-containing signaling protein that associates with and is activated by RTKs is PI 3-K (Varticovski *et al.*, 1994). Consisting of a heterodimer with a regulatory 85-kDa subunit and a catalytically active 110-kDa subunit, PI 3-K catalyzes the formation of phosphorylated phosphoinositides, the appearance of which often correlates with the activation of differentiated cellular functions, such an granule secretion (Stephens *et al.*, 1993). Both subunits of PI 3-K are found in sperm (Saling *et al.*, 1996). Reciprocal immunoprecipitation and immunoblotting assays suggest that ZRK and the 85-kDa subunit are associated physically and tyrosine-phosphorylated in activated sperm. PI 3-K stimulation appears relevant to acrosomal exocytosis, since incubation of human sperm with wortmannin, a membrane-permeable inhibitor of PI 3-K (Yano *et al.*, 1993), results in inhibition of human ZP3-stimulated ARs (Saling *et al.*, 1996). These data suggest that both PLCγ1 and PI 3-K constitute key elements in the cascade(s) that couples sperm binding to ZP3 with regulated acrosomal exocytosis and emphasize the signaling potential associated with ZP3 stimulation of ZRK.

d. Spermadhesins Spermadhesins make up a family of small (12–16 kDa) sperm-associated proteins, characterized chiefly in boar sperm, which bind a number of ligands including zp glycoproteins (Fig. 5). Although the majority of spermadhesin molecules are lost from the sperm surface during capacitation, a population is retained and these have been shown to bind both solubilized and of zp (Töpfer-Petersen and Calvete, 1995). The spermadhesins implicated in sperm–zp binding include AWN-1, which is secreted by the rete testes and found on epididymal sperm, and AQN-1 and AQN-3, which are adsorbed at ejaculation from the seminal plasma (Table II). Interestingly, only specific isoforms of these three spermadhesins, those which lack N-glycosylation at Asp_{50}, have affinity for zp proteins, a finding which has led to the identification of a potential zp-binding region (Calvete *et al.*, 1993a,b, 1994). Indirect immunofluorescence, using anti-AWN-1 antibodies, suggests that AWN is localized in the physiologically relevant site for a role in primary zp binding: on the acrosomal cap of fertile sperm attached to intact zp (Dostàlovà *et al.*, 1995). The spermadhesins are not found exclusively in pigs; immunoreactive moieties are found on dog and horse sperm and purified horse AWN differs at only three residues from pig AWN (Töpfer-Petersen *et al.*, 1995). It is exciting to look forward to

further work on this protein family to determine whether spermadhesins are present universally on mammalian sperm and are essential for sperm–zp binding.

2. Candidate Sperm Proteins Involved in Secondary Binding to the Zona Pellucida

Secondary binding occurs following acrosomal exocytosis. Thus, sperm proteins that participate in this stage of gamete interaction will be found within the acrosomal vesicle and become available for interaction with the zp only upon dispersion of the acrosomal matrix. Since the zp ligand involved at this stage is not established unequivocally, our classification of sperm proteins in this category is based first on the ability of a candidate protein to bind to the zp and, second, on an intra-acrosomal localization.

a. PH-20 PH-20 is a glycosyl phosphatidylinositol-anchored membrane protein initially found on guinea pig sperm (Phelps *et al.*, 1988). In these cells, PH-20 actually exists in two populations: on the sperm surface in the postacrosomal region of the sperm head as well as within the acrosome. Paralleling these two areas of localization, the PH-20 polypeptide displays two distinct activities (Fig. 6) and PH-20 appears to play two independent roles during sperm–egg interaction. Although originally identified using a

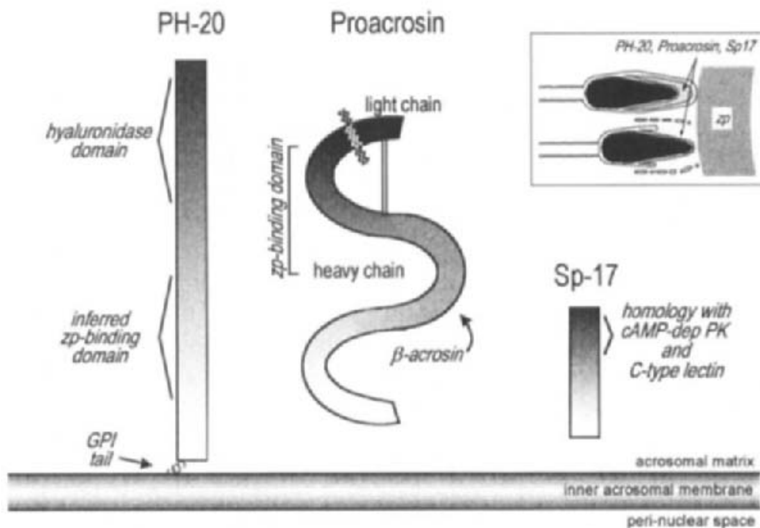


FIG. 6 Proposed structures of candidate sperm proteins involved in secondary binding to the zp. Shading depicts amino → carboxy termini (dark → light). The inset represents the location of these proteins on the sperm head during interaction with the zp.

monoclonal antibody that blocks zp binding of acrosome-reacted, but not acrosome-intact, guinea pig sperm (Primakoff *et al.*, 1985; Myles *et al.*, 1987), PH-20 has more recently been found to also demonstrate hyaluronidase activity (Hunnicutt *et al.*, 1996) and is thought to be important in degrading hyaluronic acid during transit through the cumulus layer (Lin *et al.*, 1994). Presumably, the postacrosomal surface population of PH-20 is utilized during cumulus penetration, while the intra-acrosomal population participates in secondary binding to the zp. PH-20 is a highly immunogenic protein that has been useful in contraceptive vaccine development (Primakoff *et al.*, 1988) and many aspects of its biology have been reviewed recently (Primakoff, 1994). The *PH-20* gene has been observed in a wide variety of mammals and clones have been reported for guinea pig (Table II) (Lathrop *et al.*, 1990), cynomolgus macaque, and human (Lin *et al.*, 1993).

b. Proacrosin A protein long known to be involved in sperm–zp interaction, the zymogen proacrosin is found ubiquitously within the acrosome of mammalian sperm. Due to this localization, proacrosin is most likely to participate in fertilization at the level of secondary binding following the AR (Jones, 1990; Töpfer-Petersen *et al.*, 1995; Töpfer-Petersen, 1996). As a consequence of the AR, the proacrosin molecule undergoes cleavage to yield the sperm's major trypsin-like serine protease, acrosin (Fig. 6) (Töpfer-Petersen, 1996). This activation of proacrosin to the biologically active enzyme appears to be regulated by the zp itself (Töpfer-Petersen and Cechová, 1990). Due to its demonstrated zp-binding and proteolytic activities (Jones and Brown, 1987; Töpfer-Petersen and Henschen, 1987, 1988; Urch and Patel, 1991), proacrosin has been thought to play roles in both sperm binding and penetration of the zp. Determination of the structure of proacrosin (Table II) (Baba *et al.*, 1989b) has revealed a high degree of homology across mammalian species; for example, pig and human proacrosin demonstrate 69% identity (Baba *et al.*, 1989a). Biochemical (Töpfer-Petersen *et al.*, 1990; Jansen *et al.*, 1995) and molecular (Jansen *et al.*, 1995; Richardson and O'Rand, 1996) analysis of proacrosin has mapped residues critical to zp binding (Fig. 6). Recently, Tsubamoto and colleagues (1996) used a blot-overlay technique to demonstrate binding between porcine ZP2 and proacrosin, supporting the involvement of ZP2 in secondary binding. Despite these advances, the physiological role of proacrosin has been questioned by the observation that proacrosin-null mice are fertile, though the time necessary for sperm penetration of the zp is increased (Baba *et al.*, 1994). In boars, a molecule that is structurally similar to proacrosin, sp38, has been shown to have many similar binding characteristics. A cDNA encoding sp38 was cloned recently and reported also to demonstrate an intra-acrosomal regionalization (Table II). Testis-specific expression of sp38 in conjunction with the ability of specific oligopeptides from both sp38 and acrosin to inhibit sp38–zp protein binding suggests that sp38 could play a physiological, and perhaps redundant, role in porcine sperm–egg interactions (Mori *et al.*, 1995).

c. ***Rabbit Sperm Autoantigen and Sp17*** Another family of sperm proteins identified by zp-binding properties is the rabbit sperm autoantigens (RSAs) (O'Rand, 1988). The RSAs are a family of low-molecular-weight proteins found on the surface of sperm and spermatogenic cells, and anti-RSA antibodies inhibit sperm–egg interaction *in vivo* and *in vitro* (O'Rand, 1988). Using antisera against unfractionated RSAs to probe a rabbit testis cDNA expression library, two cDNAs were identified, cloned, and sequenced; these cDNAs generated identical protein products of 146 amino acids with a predicted molecular weight of 17 kDa, and the encoded protein was named Sp17 (Table II) (1994). Interestingly, the N terminus of Sp17 shows similarity to the proposed dimerization domain of testis cAMP-dependent protein kinase (Oyen *et al.*, 1989; Taylor *et al.*, 1990), and it also contains key residues determined necessary for a C-type lectin galactose-binding domain (Drickamer and Taylor, 1993) (Fig. 6); however, the significance of these features in Sp17 function has not been addressed. Northern blot analysis detects RNA transcripts encoding Sp17 in both human and mouse testes, but not in any somatic tissue tested. cDNAs encoding mouse and human Sp17 have recently been sequenced, establishing the close homology within this family (Kong *et al.*, 1995; Lea *et al.*, 1996). Western blot analysis with an anti-Sp17 antibody prepared against bacterially expressed recombinant Sp17 recognizes multiple rabbit sperm proteins; Richardson et al. (1994) have concluded that these represent various post-translationally modified or proteolytically processed forms of the Sp17 protein. Using a solid-phase assay, bacterially expressed Sp17 binds not only rabbit zp, but also dextran and dextran sulfate (Richardson *et al.*, 1994), the latter two compounds serving as a carbohydrate source that mimics sperm-binding moieties of the zp (O'Rand, 1988; Parrish *et al.*, 1989). Rabbit Sp17 has recently been expressed in COS cells for further analysis of its role in zp binding (Yamasaki *et al.*, 1995). Analysis of complexes generated by crosslinking ^{125}I-Sp17 with solubilized rabbit zp suggests that both rabbit ZP1 and rabbit ZP3 associate with Sp17 and that unique sets of Sp17 family members interact with the different zp proteins (Yamasaki *et al.*, 1995).

Antisera prepared against bacterially expressed Sp17 or against a Sp17 peptide do not stain the surface of live, acrosome-intact sperm, but they do localize to the apical region of fixed, acrosome-intact sperm heads, suggesting an acrosomal distribution. Detailed observation of such images has led Richardson *et al.* (1994) to propose that during the AR, Sp17 becomes exposed on the rabbit sperm surface and moves posteriorly on the sperm head while aggregating into punctate foci; finally, Sp17 remains associated with both the acrosomal ghost and with the equatorial region of the head of AR sperm. These characteristics suggest that Sp17 is involved in secondary binding to the zp, yet the zp protein-binding profile recently

reported for Sp17 expressed in COS cells (see above) (Yamasaki *et al.*, 1995) is at odds with conclusions from studies in the mouse (Bleil and Wassarman, 1990) which predict that ZP2 is the ligand involved in secondary binding.

3. Summary

A large number of sperm proteins have been reported to interact with the zp, and consensus has not yet been reached on the sperm proteins that are necessary for fertilization in any particular mammalian species. However, one aspect of the analysis has been facilitated by recognizing that sperm interaction with the zp consists of at least two different stages, before and after the AR. The rather recent categorization of zp binding proteins of sperm as potentially involved in primary versus secondary binding begins to simplify this topic. Despite this clarification, recent knockout experiments (Baba *et al.*, 1994; B. D. Shur, personal communication) and biochemical analysis (Thaler and Cardullo, 1996) demonstrate that sperm–zp interaction is a complex series of interactions, with more than a single sperm protein serving as the zp binding partner during primary or secondary binding. Redundancy may be built into this system, considering the importance of the biological event involved, or the principal players in the process may not have identified yet. Our emerging ability to analyze these interactions at a molecular level will assist in generating agreement about sperm proteins that are essential for fertilization within and among mammalian species.

III. The Egg Plasma Membrane and Its Interaction with Sperm

After binding and penetrating the zp, the sperm quickly traverses the perivitelline space and reaches the egg plasma membrane. There, the sperm binds and membrane fusion is initiated. The entire sperm is then incorporated into the egg. Interaction of the sperm and egg plasma membranes involves at least two distinct steps, binding and fusion. The first step, binding, brings the two membranes into molecular contact. Although binding can occur over the entire surface of the egg plasma membrane, fusion rarely occurs in the area overlying the mitotic spindle (Fig. 7A) (Shalgi and Phillips, 1980b; Phillips and Shalgi, 1982; Talansky *et al.*, 1991). This region of the egg membrane appears to have altered characteristics: it is devoid of microvilli, it lacks underlying cortical granules, and it displays a different glycosylation (Johnson *et al.*, 1975; Wolf and Ziomek, 1983). Binding may involve any region of the sperm membrane, including the inner acrosomal

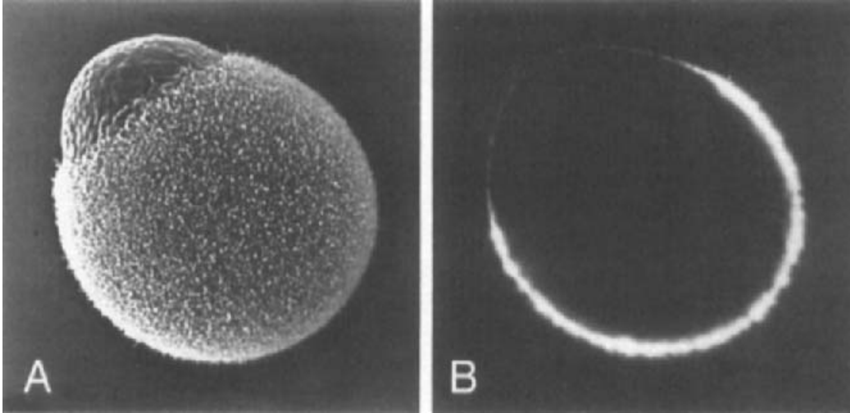


FIG. 7 Localization of microvilli and integrins on the egg surface. (A) Scanning electron micrograph of an unfertilized mouse egg, reproduced with permission from Phillips and Shalgi (1980). (B) Indirect integrin immunofluorescence using a pan-integrin antiserum. The image is taken from Almeida *et al.* (1995b) with permission and has been rotated with respect to the original in this representation.

membrane (Yanagimachi, 1994), suggesting that it may be mediated by nonspecific cellular interactions. The second step, fusion, has stricter requirements compared to those for binding. Fusion events only occur at specific cellular domains (see below) and also demand precise temperature, pH, and ionic conditions. In the hamster, fusion has been noted to be more temperature-sensitive than the events preceding and following it. Binding and postfusion events can take place at temperatures below 10°C, while fusion is inhibited reversibly (Hirao and Yanagimachi, 1978). Similarly, at pH 6.0, fusion, but not binding, in hamster sperm is inhibited reversibly (Yanagimachi *et al.*, 1980). Extracellular divalent cation dependence has been demonstrated in several species; calcium is required but magnesium, barium, and strontium may substitute (Yanagimachi, 1978). Collectively, these characteristics suggest that the steps of binding and fusion are two distinct processes and may be mediated by two distinct sets of molecules.

Most work in mammalian sperm–egg fusion has been conducted using eutherian (placental) mammals. In this group, in contrast to nonmammals and noneutherian mammals, the plasma membrane of the sperm head fuses with the egg plasma membrane and the inner acrosomal membrane is incapable of fusion (see Fig. 2) (Yanagimachi, 1994). (In noneutherian animals, the inner acrosomal membrane is the site of fusion initiation.) The specific region responsible for initiating fusion is controversial yet has important implications for identifying sperm proteins responsible for membrane fusion. Originally thought to be the postacrosomal region (Ya-

nagimachi and Noda, 1970), the site of fusion initiation is now generally agreed to be the plasma membrane overlying the equatorial segment as shown, first, in rabbit sperm by electron microscopy (Moore and Bedford, 1978; Bedford *et al.*, 1979) and, more recently, in human and bovine sperm by the use of lipid vesicles (Arts *et al.*, 1993). Some investigators maintain that fusion can be initiated in the postacrosomal segment (Vigil, 1989) or that fusion occurs simultaneously in both segments (Oura and Toshimori, 1990). The conclusions of the latter work, conducted using hamster gametes, may be attributable to the fact that the equatorial segment extends more rostrally in this species than in others (Yanagimachi, 1994). The equatorial segment, otherwise, seems to be essential for fusion with the egg plasma membrane although it should be recognized that only a few species have been examined rigorously.

The AR is an absolute requirement for fusion but not for binding (Yanagimachi, 1994). Acrosome-intact sperm cannot fuse with eggs which have had the zp removed experimentally (Yanagimachi and Noda, 1970). This observation emphasizes that the AR serves not only to permit sperm to traverse the zp but is also responsible for profound changes in the membrane, including the appearance and migration of proteins. It has been proposed that various substances released during the AR might alter the plasma membrane. The presence of inhibitors of acrosin (Tesarik *et al.*, 1990) and metalloendoproteases (Díaz-Pérez and Meizel, 1992) during the AR inhibits subsequent fusibility. However, these potential candidates do not appear to be the basis for acquisition of fusion competence since treatment of acrosome-intact hamster sperm with crude acrosomal extracts does not provoke fusibility, nor can fusion competence be attained artificially (Longo and Yanagimachi, 1993). An alternative explanation is that the AR might allow a necessary component in the medium to penetrate the plasma membrane. A calcium influx that occurs during the AR is one possible mechanism (Monroy, 1985) and may act through calpactin II (Berruti, 1991) and calpain II (Schollmeyer, 1986), two Ca^{2+}-activated proteins present in the acrosome.

Notably, whereas sperm motility is required for penetration of both the cumulus and zp matrices, fusion does not appear to require sperm motility. The weakly motile and immotile sperm of individuals with Kartagener's syndrome are able to fertilize eggs (Aitken *et al.*, 1983).

A. Egg Plasma Membrane Proteins Involved in Gamete Interaction

In situ, sperm bind only to the egg microvilli (Shalgi and Phillips, 1980a; Phillips and Shalgi, 1982; Talansky *et al.*, 1991; Santella *et al.*, 1992), suggest-

ing that these structures may play an important role in sperm–egg binding and fusion. The microvillar structure, however, is not required. At pH 6.0, egg microvilli flatten; sperm attach to the flat microvillus-free egg plasma membrane and, if the pH is raised to 7.3, fuse with the membrane despite the absence of microvilli (Yanagimachi *et al.*, 1980). These observations suggest that receptors for sperm in the egg membrane are normally associated with microvilli but that this is not a necessary relationship. These receptors are present in the membrane relatively early in egg maturation, since growing hamster oocytes acquire the capacity for fusion just as they begin to form microvilli (Zuccotti *et al.*, 1991). The fusibility of the egg plasma membrane increases as the oocyte grows; by the germinal vesicle stage, the egg plasma membrane is able to incorporate sperm in all species analyzed: hamster (Zuccotti *et al.*, 1991), pig (Polge and Dziuk, 1965), mouse (Niwa and Chang, 1975), dog (Mahi and Yanagimachi, 1976), and human (Lopata and Leung, 1988).

Egg fusibility, however, is transient and it is lost after fertilization, presumed to be the consequence of loss or modification of sperm receptors. The time at which fusibility is lost varies. In rabbits, mice, and humans, the egg plasma membrane becomes refractory to spermatozoa after fusion with the first sperm (Austin, 1961; Wolf, 1978; Sengoku *et al.*, 1995). In the hamster, although the ability to fuse is reduced after fertilization, it is not completely lost until the eight-cell stage (Zuccotti *et al.*, 1991). Loss of fusibility depends on fertilization and cannot be mimicked by activation or triggering of the cortical granule exocytosis (Horvath *et al.*, 1993). Sperm interaction with the egg plasma membrane appears less species-specific than interaction with the zp, although some specificity is retained. For example, mouse eggs only fuse with mouse sperm; however, hamster eggs fuse with sperm from all species tested (Yanagimachi, 1994) but display the greatest affinity for hamster spermatozoa (Yanagimachi, 1981).

The nature of egg plasma membrane receptors for sperm has been elusive until recent years. The first assays to identify these receptors were based on inhibition of sperm–egg fusion using a variety of agents, such as sugars (Boldt *et al.*, 1989b; Okabe *et al.*, 1989), trypsin inhibitors (Wolf, 1977), enzymatic treatment (Boldt *et al.*, 1988; Kellom *et al.*, 1992), or small peptides (Bronson and Fusi, 1990). These studies led to the identification of putative receptors, which are summarized below.

1. Egg Integrins

Integrins are a large family of cell-surface proteins that serve as receptors for cell–cell and cell–matrix interactions. At present, more than 20 different integrin heterodimers are known. Each integrin is composed of noncova-

lently associated α and β subunits (Fig. 8). The α subunits have large extracellular domains containing three to four divalent cation-binding domains, a single transmembrane region, and a short cytoplasmic region (Ruoslahti, 1996a,b). β Subunits have large extracellular domains with 48–56 cysteine residues, most of which are clustered in four repeated motifs. The cytoplasmic domains are relatively short (~65 amino acids) with the exception of β_4 which is ~1000 amino acids long (Hogervorst *et al.,* 1990; Suzuki and Naitoh, 1990). All integrin subunits are glycosylated and at least some of them can be phosphorylated (Ruoslahti, 1996a,b).

Ligands for integrins include collagen, laminin, vitronectin, fibronectin, and RGD-containing peptides (Ruoslahti, 1996a,b). It is noteworthy that some integrins are promiscuous and bind to more than one ligand. Moreover, some integrins are dual receptors for both secreted as well as cell-associated ligands (Ruoslahti, 1996a,b), which may be relevant in understanding the putative role of integrins in sperm–egg interaction.

The binding of an integrin to its ligand is not restricted to a role in adhesion, and it is becoming clear that integrins play a larger role in regulating signal transduction mechanisms (Burridge and Chrzanowska-Wodnicka, 1996). Thus, activation of integrins can trigger the phosphorylation of intracellular proteins (Yamada and Miyamoto, 1995), changes in intracellular pH (Schwartz *et al.,* 1991), reorganization of the cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996), and increased gene transcription (Hynes, 1991). Integrins are involved in such diverse processes as the formation of focal contacts, cell migration on extracellular matrices, platelet aggregation,

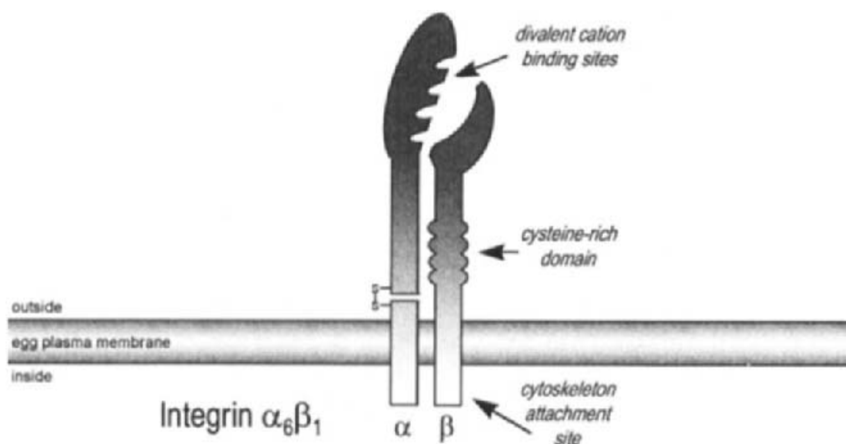


FIG. 8 Proposed structure of integrin $\alpha_6\beta_1$ a candidate egg protein involved in fusion with sperm.

and embryo implantation (Burridge and Chrzanowska-Wodnicka, 1996; Ruoslahti, 1996a,b).

The involvement of integrin-like molecules in sperm–egg fusion is suggested by several lines of evidence. First, RGD peptides, known ligands for many integrins, inhibit the fusion of human and hamster sperm to hamster eggs (Bronson and Fusi, 1990; Fusi *et al.,* 1993). Second, integrins are expressed on the surface of unfertilized eggs coincident with the distribution of microvilli in the fusogenic region of the egg membrane (Fig. 7). Finally, the most intriguing suggestion for the role of an integrin as a receptor for sperm arises from the identification of an integrin-binding disintegrin domain in fertilin, a sperm protein proposed to participate in fusion (see below) (Blobel *et al.,* 1992).

Among the integrins expressed on the egg surface, recent work has identified $\alpha_6\beta_1$ as the integrin that serves as a sperm receptor. Antibodies that block $\alpha_6\beta_1$ function inhibit sperm–egg binding in a dose-dependent fashion, while non-function-blocking antibodies do not have any effect. Moreover, peptides that inhibit other integrins, but do not inhibit $\alpha_6\beta_1$, do not inhibit either sperm binding or fusion. Sperm bind to transfected cells that express $\alpha_6\beta_1$; such binding is inhibited by peptides that contain the disintegrin domain present in fertilin (see below) (Almeida *et al.,* 1995b). Thus, a role for $\alpha_6\beta_1$ integrin in mouse sperm interaction with the egg plasma membrane is strongly supported. Moreover, since interaction between an integrin and its ligand can trigger signal transduction pathways, it is also possible that this interaction between sperm and egg membranes activates signals to stimulate subsequent events, such as membrane fusion and egg activation.

Despite firm evidence for the importance of $\alpha_6\beta_1$ integrin in interaction with sperm, some aspects of its role are not yet clear. As mentioned earlier, the ability of the egg membrane to fuse with sperm is a transient phenomenon. However, expression of $\alpha_6\beta_1$ continues after fertilization since it can be detected continuously on the unfertilized egg through fertilization to the blastocyst stage. Furthermore, a monoclonal antibody that blocks sperm–egg fusion by inhibiting $\alpha_6\beta_1$ also inhibits the migration of trophoblast cells, indicating that $\alpha_6\beta_1$ may also play a role in implantation (Sutherland *et al.,* 1993). Since an individual integrin can recognize several different ligands, it has been proposed that $\alpha_6\beta_1$ has both a cellular ligand (fertilin) and an acellular one (laminin, present in the uterine stroma) and that fertilization alters the affinity of $\alpha_6\beta_1$ (Almeida *et al.,* 1995a). However, since fusibility in the mouse egg is lost soon after fertilization (Wolf, 1978), $\alpha_6\beta_1$ or some other factor(s) must be altered as a consequence of fertilization.

2. Other Candidate Molecules

Although integrins are important in sperm–egg fusion, there are several lines of evidence to suggest that they are not the only sperm receptors present in the egg plasma membrane. Nevertheless, there is little information about other candidate receptors for sperm in the egg plasma membrane. Rochwerger *et al.* (1992) used a sperm-associated protein in the rat that is involved in fusion (protein DE; see below) as a probe to detect complementary sites in the egg plasma membrane. The distribution of the egg receptor for DE matches that of a protein involved in fusion (on the entire egg surface except the region overlying the mitotic spindle) and purified DE protein is able to inhibit sperm–egg fusion (Rochwerger *et al.*, 1992). In addition, DE-binding sites are first detected on the growing oocyte when fusibility appears (Cohen *et al.*, 1996), suggesting a correlation between these phenomena. However, the structure of the DE-binding protein on the egg has not yet been reported.

A 94-kDa mouse egg surface protein has also been proposed to be involved in gamete membrane fusion events. This protein can be visualized by surface radioiodination of mouse eggs and is lost following trypsin or chymotrypsin treatment, correlating with a loss of sperm attachment and penetrability (Boldt *et al.*, 1989a). Moreover, protease-treated eggs recover penetrability after 3–6 h of culture *in vitro,* an interval which coincides with reappearance of the 94-kDa protein at the egg surface (Kellom *et al.*, 1992). Myles (1993) has suggested that the 94-kDa protein may be the β subunit of an integrin, since it is the appropriate size, but there is no experimental evidence for this, as of yet.

3. Summary

Various lines of evidence suggest that integrins at the egg surface may be involved in fusion with the sperm plasma membrane, and accumulating data point to the integrin $\alpha_6\beta_1$ as an excellent candidate. Not only is $\alpha_6\beta_1$ expressed with appropriate temporal and spatial characteristics, but it also displays an alteration in ligand affinity following fertilization, consistent with the behavior of eggs. Since sperm attachment to the egg plasma membrane can be distinguished from sperm fusion, it is likely that other proteins on the egg plasma membrane are also important in this interaction, but these have yet to be characterized.

B. Sperm Interaction with the Egg Plasma Membrane

Most of the attempts to identify sperm molecules involved in sperm–egg plasma membrane binding and fusion have used antibodies to various sperm

proteins to inhibit sperm binding or fertilization. Several candidate molecules have been identified in this manner: DE (Cuasnicú *et al.*, 1984a), M29 and M37 (Saling *et al.*, 1985), fertilin [PH-30 (Primakoff *et al.*, 1987)], OBF13 (Okabe *et al.*, 1988), and MH61 (Okabe *et al.*, 1990). For a recent review of these molecules, see Myles (1993). In addition to its proposed role in zp binding, GalTase (see above) has also been suggested to participate in gamete membrane fusion due to redistribution to the equatorial segment following the AR (Lopez and Shur, 1987). Finally, Allen and Green (1995) have recently identified two guinea pig sperm proteins (34 and 44 kDa) located at the equatorial segment using monoclonal antibodies that recognize epitopes which appear *de novo* following the AR, a characteristic predicted for a sperm component involved in gamete fusion.

Considerable evidence for a role in sperm–egg fusion has accumulated for two of these candidates, protein DE and fertilin, which are diagramed in Fig. 9 and discussed below.

1. DE

DE is a 32-kDa protein that is abundantly secreted in the proximal epididymis of the rat in response to androgens and associates with the sperm

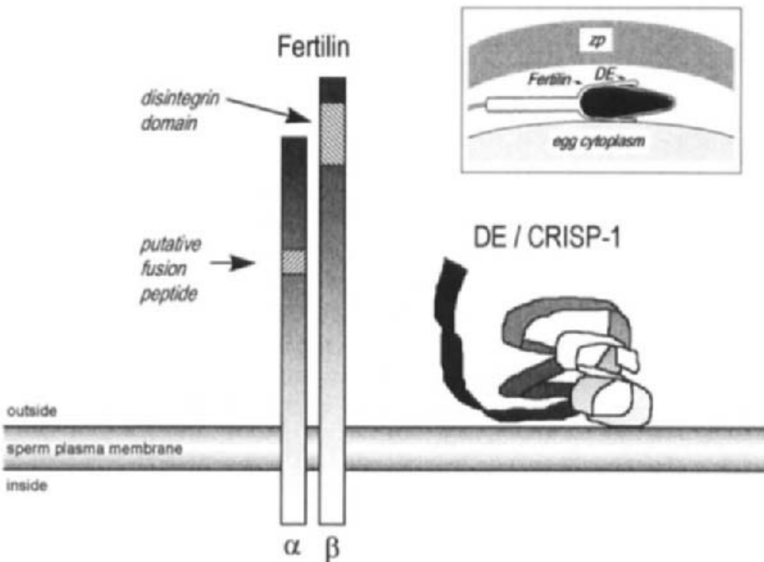


FIG. 9 Proposed structures of candidate sperm proteins involved in fusion with the egg plasma membrane. Shading depicts amino → carboxy termini (dark → light). The inset represents the location of these proteins on the sperm head during interaction with the egg.

surface (Garberi *et al.*, 1979; Kohane *et al.*, 1980; Sanjurjo *et al.*, 1990). DE is found on the dorsal region of the sperm head, where it was hypothesized to covalently bind to specific sperm receptors via disulfide bonds (but see below) (Tezon *et al.*, 1985; Brooks, 1987). Immunofluorescence studies indicate that, following the AR, DE relocalizes to the equatorial segment (Rochwerger and Cuasnicú, 1992). A role in fertilization for DE was first suggested when an anti-DE polyclonal antibody significantly reduced the percentage of rat eggs fertilized *in vitro* (Cuasnicú *et al.*, 1984b).

Relocalization of protein DE to the fusogenic region of the acrosome-reacted sperm, together with the finding that an anti-DE polyclonal antibody inhibits sperm penetration of rat eggs (Cuasnicú *et al.*, 1990), implies a role in sperm–egg fusion for DE. Further, purified DE inhibits fertilization in a concentration-dependent manner without an effect on sperm–egg binding (Rochwerger *et al.*, 1992), suggesting a role in fusion that is independent of and subsequent to binding. These results from studies *in vitro* have been partially corroborated by studies *in vivo*. Both male and female rats immunized with DE demonstrate reduced fertility (Cuasnicú *et al.*, 1990). In a related set of experiments, sperm were exposed to sera from DE-immunized animals prior to intrauterine insemination of superovulated female rats; in this case, the percentage of fertilized eggs was reduced compared to that of controls in which unexposed sperm were used (Perez Martinez *et al.*, 1995). Since observations *in vitro* indicated that these sera display no effect on sperm viability, motility, or ability to undergo the AR (as measured by relocalization of DE), the decrease in fertility may be attributed to interference with sperm–egg fusion.

Rat sperm protein DE (Table III) has actually been studied independently by several other groups and named acidic epididymal glycoprotein [AEG (Lea and French, 1981)], sialoprotein (Faye *et al.*, 1980), 32-kDa rat epididymal protein (Wong and Tsang, 1982), and protein IV (Pan and Li, 1982). More recently, two murine homologs of DE have also been identified:

TABLE III

Sperm Proteins That Interact with the Egg Plasma Membrane

Sperm protein	App. MW (kDa)	Species	GenBank Accession No.	Sequence reference
DE	30–32	Rat	X04643	Brooks *et al.* (1986)
Fertilin	44 (α)	Guinea pig	Z11719	
	60 (β)		Z11720	Blobel *et al.* (1992)

Note. These sperm proteins are listed with respect to their apparent size (based on disulfide-reducing SDS gels), the species from which sequence information was derived, and the GenBank accession number and literature reference for the sequence information.

cysteine-rich secretory protein-1 [CRISP-1 (Haendler *et al.*, 1993; Eberspaecher *et al.*, 1995)] and mouse epididymal protein-7 (Rankin *et al.*, 1992). In addition to expression in the epididymis, CRISP-1 is also present in the vas deferens, seminal vesicle, prostate, and salivary gland (Eberspaecher *et al.*, 1995). Like other CRISP family members, DE shares a highly conserved cluster of cysteines near the C terminus. Other family members are the secretory proteins CRISP-2 [also Tpx-1 (Kasahara *et al.*, 1989; Mizuki *et al.*, 1992)] and CRISP-3 [also AEG-2 (Mizuki and Kasahara, 1992; Haendler *et al.*, 1993)], found in the testis and salivary gland, respectively. The CRISP-2 homologs TPX-1 (Kasahara *et al.*, 1989) and Autoantigen 1 (Foster and Gerton, 1996) were the first human and guinea pig CRISP family members identified.

Very little is known about the function of the CRISP proteins, although a compact structure for these secreted proteins is suggested due to their conserved cysteine residues (Fig. 9) (Eberspaecher *et al.*, 1995). The absence of free cysteines in CRISP-1 does not support hypothetical covalent attachment to a sperm receptor, and physical association with sperm appears to be loose (Rankin *et al.*, 1992; Eberspaecher *et al.*, 1995). However, Cuasnicú and co-workers have recently found that DE exists on the sperm surface in at least two different populations. One of these is readily dissociated from the cells with hypertonic salt whereas the second population is dissociated only by using detergents or chaotropic agents (Cuasnicú *et al.*, 1996). Interestingly, this latter DE population constitutes the DE which relocalizes to the equatorial segment following the AR. Recently, a human epididymal secretory protein, AEG-Related Protein, was named a potential functional counterpart of CRISP-1/DE based on its localization to the postacrosomal region of the sperm head and on its conserved CRISP family features (Hayashi *et al.*, 1996). However, a role in fertilization has not yet been established and has been challenged recently (Krätzschmar *et al.*, 1996). Thus, studies of homologs of DE have so far lent little support to a targetted role in sperm–egg fusion. Rather, the presence of CRISP-1/DE in the salivary glands and the discovery of its close relatives suggest a more general function in secretion.

2. Fertilin

The heterodimeric glycoprotein now known as fertilin was initially identified in the guinea pig when a monoclonal antibody, PH-30, was found to inhibit fertilization (Primakoff *et al.*, 1987). This antibody was subsequently shown to recognize a protein with two polypeptides, 60 and 44 kDa on reducing SDS–PAGE, and the antigen was localized to the posterior head surface of the sperm (Primakoff *et al.*, 1987). The postacrosomal localization of fertilin is one potential weakness in its candidacy as a mediator of

sperm–egg fusion. However, the region of the sperm plasma membrane that initiates fusion with the egg is currently debated, and fertilin should not be discounted on this basis. Several other lines of evidence strongly support the idea that fertilin participates in gamete membrane fusion. For instance, PH-30 antibody binding is first detected on sperm from the proximal cauda epididymis, where fertilization competence is believed to be acquired (Horan and Bedford, 1972; Dyson and Orgebin-Crist, 1973). Detection of binding occurs concomitantly with the final of several proteolytic processing steps that convert the larger precursor subunits to their mature counterparts (Blobel *et al.,* 1990).

The cDNA and deduced amino acid sequences of the mature and precursor polypeptides reveal that fertilin is composed of two subunits, α and β (Fig. 9 and Table III). These subunits are both type I integral membrane glycoproteins and similar in sequence. Sequence information permitted the identification of a viral fusion peptide domain in the mature α subunit and a disintegrin domain in the mature β subunit (Blobel *et al.,* 1992). A disintegrin domain was subsequently found in the precursor α subunit and, in addition, both precursor subunits contain metalloprotease domains (Wolfsberg *et al.,* 1993). Mouse (Evans *et al.,* 1995a; Wolfsberg *et al.,* 1995b) and monkey (Perry *et al.,* 1995; Ramarao *et al.,* 1996) fertilins also share this organization. The discovery of these highly conserved functional domains has important implications for fertilin in sperm–egg interaction.

Viral membrane fusion has long been studied as a model for cell–cell fusion (White, 1992). Viral fusion proteins contain a "fusion peptide" that is (i) located in a membrane-anchored subunit, (ii) relatively hydrophobic, and (iii) able to adopt an alpha-helical structure (Blobel and White, 1992). Because viral fusion proteins are responsible for membrane binding as well as fusion, the identification of a viral fusion peptide in the α subunit of fertilin supports its role in binding as well as in fusion with the egg plasma membrane. Further evidence is contributed by the ability of synthetic peptides corresponding to the putative fusion sequence to interact with phospholipid bilayers and induce intervesicular fusion (Muga *et al.,* 1994).

Disintegrins are a family of soluble, integrin-binding proteins initially isolated from snake venom. Since integrins have a widespread role in cell–cell and cell–matrix interaction, the identification of a disintegrin sequence in the β subunit of fertilin, a membrane-bound protein, raises the possibility of a role in binding to the egg plasma membrane. Two important characteristics that many disintegrins share are (i) consensus integrin-binding tripeptides, such as RGD (although some contain an alternative tripeptide sequence in their disintegrin loop) (Blobel and White, 1992), and (ii) dependence on divalent cations for binding.

Prior to the identification of a disintegrin domain in fertilin, a role for integrins in fertilization had been proposed. Bronson and Fusi examined

the effect of RGD-containing peptides on fertilization and found an inhibition of sperm–egg binding and binding of eggs to RGD-coated immunobeads in hamsters (Bronson and Fusi, 1990). This has also been shown, more recently, in humans (Fusi *et al.*, 1992) and in mice (Evans *et al.*, 1995a). The RGD sequence is not the universal tripeptide found at the disintegrin loop and RGD-containing peptides can inhibit the binding of non-RGD-containing disintegrins to integrins (Hynes, 1992; Rosales *et al.*, 1995). Instead of RGD, guinea pig fertilin β has a TDE tripeptide at the disintegrin loop, and mouse fertilin β has a QDE tripeptide. TDE-containing peptides are thus able to inhibit guinea pig sperm–egg fusion, presumably by inhibiting binding (Myles *et al.*, 1994), and QDE-containing peptides similarly inhibit mouse sperm–egg binding and fusion (Evans *et al.*, 1995a). In the monkey *Macaca fascicularis,* the disintegrin loop of fertilin β is FDE (Perry *et al.*, 1995); the corresponding loop in the human is FEE (Gupta *et al.*, 1996a). The divalent cation dependence characteristic of disintegrins has also been demonstrated in mice; binding requires the presence of calcium, magnesium, or manganese, whereas fusion requires calcium (Evans *et al.*, 1995a).

The identification of metalloprotease domains in the precursor regions of both fertilin subunits suggests that fertilin may have another, earlier, role in sperm development. Pro-α and pro-β fertilin are present in testicular spermatogenic cells, although sequence information suggests that only pro-α is catalytically active (Wolfsberg *et al.*, 1993). Metalloprotease activity may be involved in spermatogenesis, where proteolysis has many potential roles, from facilitating the migration of the developing germ cell to processing the β subunit or other molecules to their mature, fertilization-competent forms (Wolfsberg *et al.*, 1993).

The identification of the disintegrin and metalloprotease domains also made fertilin α and β the founding members of a novel family of proteins that contain "a disintegrin and metalloprotease domain," the ADAMs (Wolfsberg *et al.*, 1995a). Twelve members of this family have now been identified in both reproductive and nonreproductive tissues (Wolfsberg *et al.*, 1995a; Cho *et al.*, 1996). Although all members possess potential disintegrin and metalloprotease domains, these domains may or may not be active. In addition, several of the members (such as fertilin α) possess potential viral fusion peptide domains. Six have been found in spermatogenic cells of various species: fertilin α and β, mouse cyritestin (Heinlein *et al.*, 1994), and ADAMs 4–6 (Wolfsberg *et al.*, 1995a,b). As determined by RT–PCR, however, only fertilin β and mouse cyritestin are testis-specific. Another interesting family member, Epididymal Apical Protein-1, is synthesized by and located along the epithelium of the caput epididymis, adjacent to the epididymal region in which fertilization competence is acquired (Perry *et al.*, 1992). Finally, meltrin-α, an ADAM which contains a viral fusion peptide

domain, has been recently shown to be required for myoblast fusion (Yagami-Hiromasa *et al.*, 1995).

3. Summary

Overall, the multiple activities of the fertilin heterodimer suggest multiple roles in gametogenesis and fertilization. The hypothesis that fertilin β is binding the egg plasma membrane via its disintegrin domain with fertilin α mediating fusion via its viral fusion peptide domain is an attractive one that is finding increasing support. However, the growing ADAM family, with many members represented in reproductive tissues, suggests a bigger story. Though complicated by interspecies variation, a picture is emerging of a large family whose members share features important for cell–cell interaction but retain critical differences in expression, localization, and processing. It is likely that, in addition to fertilin, other ADAM family members as well as other molecules, perhaps some related to protein DE, work in concert to bring about the union of the sperm and the egg.

IV. Concluding Remarks

Complex and dynamic cellular interactions between sperm and egg regulate the entire process of fertilization, from the first binding of gametes to the fusion of their membranes. These interactions include adhesion and binding events which in turn induce signaling cascades that trigger profound cellular consequences, such as exocytosis and reorganization of cell membrane components. Detailed study of fertilization in a variety of mammalian organisms has already identified molecular components involved in gamete interaction. These components participate in binding and signaling processes that closely parallel processes in other cells. Integration of data from these studies with the information already amassed on their somatic cell counterparts has greatly enhanced our understanding of the molecular and cellular events of fertilization.

The molecular identification and biological analysis of the zp proteins and their sperm binding partners have defined two phases of sperm–zp interaction, primary and secondary binding. These phases appear to involve distinct zp proteins as well as distinct sets of sperm receptor candidates that have been summarized here. Sperm–egg plasma membrane interactions can be separated into binding and fusion components. Egg integrin $\alpha_6\beta_1$ and the sperm proteins fertilin and DE/CRISP-1 have been proposed to participate in these events; however, their precise roles have not been firmly established. Although a variety of gamete proteins have been suggested to

participate in fertilization, we have discussed only those which we feel are the strongest candidates. It is readily apparent, however, that considerable additional research is needed to more firmly establish the roles of the proteins discussed and to identify additional molecules that participate in gamete interactions.

Until recently, studies of the molecular components of mammalian fertilization were limited because, for many species, obtaining gametes in large quantities is cumbersome and costly. Molecular cloning of the proteins involved in gamete interaction will enable the next decade of fertilization research to transcend this limitation. Detailed analysis can be achieved due to the expression of adequate quantities of the necessary proteins; this knowledge will help to build a picture of fertilization in individual species and define common themes among them. The results of these investigations will likely indicate that the coordination of many systems is necessary for sperm and egg to interact in stepwise progress to conception. Experimentation in the coming years will determine the validity of these predictions.

Acknowledgments

The authors are grateful to our lab members for their patience and support during the writing of this review. We also thank the many colleagues who supplied prepublication results and pertinent discussion of their work, in particular, J. D. Bleil, P. Bork, R. Bronson, J. Dean, H. M. Florman, D. L. Garbers, J. D. Harris, K. Koyama, D. M. Phillips, B. D. Shur, and J. M. White, and apologize that not all valuable information could be cited. We are indebted to K. Burridge and J. Kiefer for improving our manuscript. Work from the authors' laboratory was funded by the NIH and The Andrew W. Mellon Foundation. S.B.M.gratefully acknowledges a Pre-doctoral Fellowship from the NSF; R.R.W. thanks the HHMI for support.

References

Aarons, D. J., Boettger-Tong, H. L., Holt, G., and Poirier, G. R. (1991). Acrosome reaction induced by immunoaggregation of a proteinase inhibitor bound to the murine sperm head. *Mol. Reprod. Dev.* **30,** 258–264.

Aitken, R. J., Ross, A., and Lees, M. M. (1983). Analysis of sperm function in Kartagener's syndrome. *Fertil. Steril.* **40,** 696–698.

Allen, C. A., and Green, D. P. (1995). Monoclonal antibodies which recognize equatorial segment epitopes presented de novo following the A23187-induced acrosome reaction of guinea pig sperm. *J. Cell Sci.* **108,** 767–777.

Almeida, E. A. C., Chen, M. S., Huovila, A. P., Shaw, L. M., Mercurio, A. M., Primakoff, P., Myles, D. G., and White, J. M. (1995a). Alternate affinity states of the integrin $\alpha_6\beta_1$ show preferential binding to laminin or sperm. *Mol. Biol. Cell* **6,** 8a.

Almeida, E. A. C., Huovila, A. P., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G., and White, J. M. (1995b). Mouse egg integrin $\alpha_6\beta_1$ functions as a sperm receptor. *Cell* **81,** 1095–1104.

Alves, M. S., Martins, M. S., and Pena, S. D. (1995). Monoclonal antibody against a 52 K sperm surface protein inhibits sperm-zona pellucida interactions in the rat. *J. Exp. Zool.* **272,** 123–133.

Arts, E. G. J. M., Kuiken, J., Jager, S., and Hoekstra, D. (1993). Fusion of artificial membranes with mammalian spermatozoa—Specific involvement of the equatorial segment after acrosome reaction. *Eur. J. Biochem.* **217,** 1001–1009.

Austin, C. R. (1951). Observations on the penetration of the sperm into the mammalian egg. *Aust. J. Sci. Res. Ser. B* **4,** 581.

Austin, C. R. (1961). "The Mammalian Egg." Thomas, Springfield, IL.

Austin, C. R., and Braden, A.W.H. (1956). Early reaction of the rodent egg to sperm penetration. *J. Exp. Biol.* **33,** 358–365.

Baba, T., Kashiwabara, S., Watanabe, K., Itoh, H., Michikawa, Y., Kimura, K., Takada, M., Fukamizu, A., and Arai, Y. (1989a). Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. *J. Biol. Chem.* **264,** 11920–11927.

Baba, T., Watanabe, K., Kashiwabara, S., and Arai, Y. (1989b). Primary structure of human proacrosin deduced from its cDNA sequence. *FEBS Lett.* **244,** 296–300.

Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994). Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J. Biol. Chem.* **269,** 31845–31849.

Bagavant, H., Yurewicz, E. C., Sacco, A. G., Talwar, G. P., and Gupta, S. K. (1993a). Delineation of epitopes on porcine zona pellucida relevant for binding of sperm to oocyte using monoclonal antibodies. *J. Reprod. Immunol.* **23,** 265–279.

Bagavant, H., Yurewicz, E. C., Sacco, A. G., Talwar, G. P., and Gupta, S. K. (1993b). Block in porcine gamete interaction by polyclonal antibodies to a pig ZP3β fragment having partial sequence homology to human ZP3. *J. Reprod. Immunol.* **25,** 277–283.

Bairoch, A. (1991). PROSITE: A dictionary of sites and patterns in proteins. *Nucleic Acids Res.* **19(Suppl.),** 2241–2245.

Baldi, E., Krausz, C., Luconi, M., Bonaccorsi, L., Maggi, M., and Forti, G. (1995). Actions of progesterone on human sperm: A model of non-genomic effects of steroids. *J. Steroid Biochem. Mol. Biol.* **53,** 199–203.

Barratt, C. L. R., and Hornby, D. P. (1995). Induction of the human acrosome reaction by rhuZP3. *In* "The Human Sperm Acrosome Reaction" (P. Fenichel and J. Parinaud, eds.), pp. 105–122. John Libbey Eurotext, Montrouge, France.

Barros, C., and Yanagimachi, R. (1971). Induction of zona reaction in golden hamster eggs by cortical granule material. *Nature* **233,** 268–269.

Barros, C., and Yanagimachi, R. (1972). Polyspermy-preventing mechanisms in the golden hamster egg. *J. Exp. Zool.* **180,** 251–265.

Bedford, J. M. (1983). Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* **28,** 108–120.

Bedford, J. M. (1994). The contraceptive potential of fertilization: A physiological perspective. *Hum. Reprod.* **9,** 842–858.

Bedford, J. M., Moore, H. D. M., and Franklin, L. E. (1979). Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. *Exp. Cell Res.* **119,** 119–126.

Beebe, S. J., Leyton, L., Burks, D., Ishikawa, M., Fuerst, T., Dean, J., and Saling, P. (1992). Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. *Dev. Biol.* **151,** 48–54.

Benoff, S. (1993). Preliminaries to fertilization. The role of cholesterol during capacitation of human spermatozoa. *Hum. Reprod.* **8,** 2001–2006.

Berruti, G. (1991). Evidence for Ca^{2+}-mediated F-actin/phospholipid binding of human sperm calpactin II. *Cell Biol. Int. Rep.* **15,** 917–927.

Bleil, J. D., and Wassarman, P. M. (1980). Mammalian sperm–egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20,** 873–882.

Bleil, J. D., and Wassarman, P. M. (1983). Sperm–egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* **95,** 317–324.

Bleil, J. D., and Wassarman, P. M. (1986). Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J. Cell Biol.* **102,** 1363–1371.

Bleil, J. D., and Wassarman, P. M. (1988). Galactose at the nonreducing terminus of *O*-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proc. Natl. Acad. Sci. USA* **85,** 6778–6782.

Bleil, J. D., and Wassarman, P. M. (1990). Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. *Proc. Natl. Acad. Sci. USA* **87,** 5563–5567.

Bleil, J. D., Greve, J. M., and Wassarman, P. M. (1988). Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reacted sperm to eggs. *Dev. Biol.* **128,** 376–385.

Blobel, C. P., and White, J. M. (1992). Structure, function and evolutionary relationship of proteins containing a disintegrin domain. *Curr. Opin. Cell Biol.* **4,** 760–765.

Blobel, C. P., Myles, D. G., Primakoff, P., and White, J. M. (1990). Proteolytic processing of a protein involved in sperm–egg fusion correlates with acquisition of fertilization competence. *J. Cell Biol.* **111,** 69–78.

Blobel, C. P., Wolfsberg, T. G., Turck, C. W., Myles, D. G., Primakoff, P., and White, J. M. (1992). A potential fusion peptide and an integrin ligand domain in a protein active in sperm–egg fusion. *Nature* **356,** 248–252.

Boldt, J., Howe, A. M., and Preble, J. (1988). Enzymatic alteration of the ability of mouse egg plasma membrane to interact with sperm. *Biol. Reprod.* **39,** 19–27.

Boldt, J., Gunter, L. E., and Howe, A. M. (1989a). Characterization of cell surface polypeptides of unfertilized, fertilized, and protease-treated zona-free mouse eggs. *Gamete Res.* **23,** 91–101.

Boldt, J., Howe, A. M., Parkerson, J. B., Gunter, L. E., and Kuehn, E. (1989b). Carbohydrate involvement in sperm–egg fusion in mice. *Biol. Reprod.* **40,** 887–896.

Bookbinder, L. H., Cheng, A., and Bleil, J. D. (1995). Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein. *Science* **269,** 86–89.

Bork, P., and Sander, C. (1992). A large domain common to sperm receptors (Zp2 and Zp3) and TGF-β type III receptor. *FEBS Lett.* **300,** 237–240.

Bronson, R. A., and Fusi, F. (1990). Evidence that an Arg–Gly–Asp adhesion sequence plays a role in mammalian fertilization. *Biol. Reprod.* **43,** 1019–1025.

Brooks, D. E. (1987). Developmental expression and androgenic regulation of the mRNA for major secretory proteins of the rat epididymis. *Mol. Cell. Endocrinol.* **53,** 59–66.

Brooks, D. E., Means, A. R., Wright, E. J., Singh, S. P., and Tiver, K. K. (1986). Molecular cloning of the cDNA for androgen-dependent sperm-coating glycoproteins secreted by the rat epididymis. *Eur. J. Biochem.* **161,** 13–18.

Brucker, C., Sandow, B. A., Blackmore, P. F., Lipford, G. B., and Hodgen, G. D. (1992). Monoclonal antibody AG7 inhibits fertilization post sperm-zona binding. *Mol. Reprod. Dev.* **33,** 451–462.

Burkman, L. J., Coddington, C. C., Franken, D. R., Krugen, T. F., Rosenwaks, Z., and Hodgen, G. D. (1988). The hemizona assay (HZA): Development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil. Steril.* **49,** 688–697.

Burks, D. J., Carballada, M. R., Moore, H. D. M., and Saling, P. M. (1995). Interaction of a tyrosine kinase from human sperm with the zona pellucida at fertilization. *Science* **269,** 83–86.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility and signaling. *Annu. Rev. Cell Dev. Biol.* **12,** 463–518.

Calvete, J. J., Sanz, L., Dostàlovà, Z., and Töpfer-Petersen, E. (1993a). Characterization of AWN-1 glycosylated isoforms helps define the zona pellucida and serine proteinase inhibitor-binding region on boar spermadhesins. *FEBS Lett.* **334,** 37–40.

Calvete, J. J., Solís, D., Sanz, L., Díaz-Mauriño, T., Schäfer, W., Mann, K., and Töpfer-Petersen, E. (1993b). Characterization of two glycosylated boar spermadhesins. *Eur. J. Biochem.* **218,** 719–725.

Calvete, J. J., Solis, D., Sanz, L., Diaz-Maurino, T., and Töpfer-Petersen, E. (1994). Glycosylated boar spermadhesin AWN-1 isoforms. Biological origin, structural characterization by lectin mapping, localization of O-glycosylation sites, and effect of glycosylation on ligand binding. *Biol. Chem. Hoppe-Seyler* **375,** 667–673.

Camaioni, A., Salustri, A., Yanagishita, M., and Hascall, V. C. (1996). Proteoglycans and proteins in the extracellular matrix of mouse cumulus cell–oocyte complexes. *Arch. Biochem. Biophys.* **325,** 190–198.

Cardullo, R. A., and Wolf, D. E. (1995). Distribution and dynamics of mouse sperm surface galactosyltransferase: Implications for mammalian fertilization. *Biochemistry* **34,** 10027–10035.

Chamberlin, M. E., and Dean, J. (1990). Human homolog of the mouse sperm receptor. *Proc. Natl. Acad. Sci. USA* **87,** 6014–6018.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into fallopian tubes. *Nature* **168,** 697–698.

Chang, Y. S., Wang, S. C., Tsao, C. C., and Huang, F. L. (1996). Molecular cloning, structural analysis, and expression of carp *Zp3* gene. *Mol. Reprod. Dev.* **44,** 295–304.

Cheng, A., Le, T., Palacios, M., Bookbinder, L. H., Wassarman, P. M., Suzuki, F., and Bleil, J. D. (1994). Sperm–egg recognition in the mouse: Characterization of sp56, a sperm protein having specific affinity for ZP3. *J. Cell Biol.* **125,** 867–878.

Cho, C. H., Primakoff, P., White, J. M., and Myles, D. G. (1996). Chromosomal assignment of four testis-expressed mouse genes from a new family of transmembrane proteins (ADAMs) involved in cell–cell adhesion and fusion. *Genomics* **34,** 413–417.

Cohen, D. J., Munuce, M. J., and Cuasnicú, P. S. (1996). Mammalian sperm–egg fusion—The development of rat oolemma fusibility during oogenesis involves the appearance of binding sites for sperm protein DE. *Biol. Reprod.* **55,** 200–206.

Cornwall, G. A., Tulsiani, D. R., and Orgebin-Crist, M. C. (1991). Inhibition of the mouse sperm surface alpha-D-mannosidase inhibits sperm–egg binding *in vitro*. *Biol. Reprod.* **44,** 913–921.

Cuasnicú, P. S., Echeverria, F. G., Piazza, A., Pineiro, L., and Blaquier, J. A. (1984a). Epididymal proteins mimic the androgenic effect on zona pellucida recognition by immature hamster spermatozoa. *J. Reprod. Fertil.* **71,** 427–431.

Cuasnicú, P. S., Gonzalez Echeverria, F., Piazza, A. D., Cameo, M. S., and Blaquier, J. A. (1984b). Antibodies against epididymal glycoproteins block fertilizing ability in rat. *J. Reprod. Fertil.* **72,** 467–471.

Cuasnicú, P. S., Conesa, D., and Rochwerger, L. (1990). Potential contraceptive use of an epididymal protein that participates in fertilization. *In* "Gamete Interaction: Prospects for Immunocontraception" (N. J. Alexander, D. Griffin, J. M. Speiler, and G. M. H. Waites, eds.), pp. 143–153. Wiley–Liss, New York.

Cuasnicú, P. S., Cohen, D. J., Ellerman, D. A., and Rochwerger, L. (1996). Relationship between the association of an epididymal protein to the sperm surface and its behavior and function. *Biol. Reprod.* **54,** 140.

De, A., Brown, D. G., Gorman, M. A., Carr, M., Sanderson, M. R., and Freemont, P. S. (1994). Crystal structure of a disulfide-linked "trefoil" motif found in a large family of putative growth factors. *Proc. Natl. Acad. Sci. USA* **91,** 1084–1088.

Díaz-Pérez, E., and Meizel, S. (1992). Importance of mammalian sperm metalloendoprotease activity during the acrosome reaction to subsequent sperm–egg fusion: Inhibitor studies with human sperm and zona-free hamster eggs. *Mol. Reprod. Dev.* **31,** 122–130.

Dostàlovà, Z., Calvete, J. J., Sanz, L., and Töpfer-Petersen, E. (1995). Boar spermadhesin AWN-1. Oligosaccharide and zona pellucida binding characteristics. *Eur. J. Biochem.* **230,** 329–336.

Drickamer, K., and Taylor, M. E. (1993). Biology of animal lectins. *Annu. Rev. Cell Biol.* **9,** 237–264.

Dunbar, B. S., and Wolgemuth, D. J. (1984). Structure and function of the mammalian zona pellucida, a unique extracellular matrix. *Mod. Cell Biol.* **3,** 77–111.

Dyson, A. L., and Orgebin-Crist, M. C. (1973). Effect of hypophysectomy, castration and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. *Endocrinology* **93,** 391–402.

Eberspaecher, U., Roosterman, D., Krätzschmar, J., Haendler, B., Habenicht, U. F., Becker, A., Quensel, C., Petri, T., Schleuning, W. D., and Donner, P. (1995). Mouse androgen-dependent epididymal glycoprotein CRISP-1 (DE/AEG): Isolation, biochemical characterization, and expression in recombinant form. *Mol. Reprod. Dev.* **42,** 157–172.

Eddy, E. M., and O'Brien, D. A. (1994). The spermatozoon. *In* "The Physiology of Reproduction" (E. Knobil and J. D. Neill, eds.), 2nd Ed., pp. 29–77. Raven Press, New York.

Epifano, O., and Dean, J. (1994). Biology and structure of the zona pellucida: A target for immunocontraception. *Reprod. Fertil. Dev.* **6,** 319–330.

Epifano, O., Liang, L. F., and Dean, J. (1995a). Mouse Zp1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish. *J. Biol. Chem.* **270,** 27254–27258.

Epifano, O., Liang, L. F., Familari, M., Moos, M. C., Jr., and Dean, J. (1995b). Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* **121,** 1947–1956.

Eppig, J. J., Obrien, M., and Wigglesworth, K. (1996). Mammalian oocyte growth, and development *in vitro*. *Mol. Reprod. Dev.* **44,** 260–273.

Evans, J. P., Schultz, R. M., and Kopf, G. S. (1995a). Mouse sperm–egg plasma membrane interactions: Analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) beta. *J. Cell Sci.* **108,** 3267–3278.

Evans, S. C., Youakim, A., and Shur, B. D. (1995b). Biological consequences of targeting beta 1,4-galactosyltransferase to two different subcellular compartments. *BioEssays* **17,** 261–268.

Faye, J. C., Duguet, L., Mazzuca, M., and Bayard, F. (1980). Purification, radioimmunoassay, and immunohistochemical localization of a glycoprotein produced by the rat epididymis. *Biol. Reprod.* **23,** 423–432.

Florman, H. M., and Babcock, D. F. (1991). Progress toward understanding the molecular basis of capacitation. *In* "Elements of Mammalian Fertilization" (P. M. Wassarman, ed.), Vol. 1, pp. 105–132. CRC Press, Boca Raton, FL.

Florman, H. M., and First, N. L. (1988). The regulation of acrosomal exocytosis. I. Sperm capacitation is required for the induction of acrosome reactions by the bovine zona pellucida *in vitro*. *Dev. Biol.* **128,** 453–463.

Florman, H. M., and Wassarman, P. M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* **41,** 313–324.

Florman, H. M., Bechtol, K. B., and Wassarman, P. M. (1984). Enzymatic dissection of the functions of the mouse egg's receptor for sperm. *Dev. Biol.* **106,** 243–255.

Foster, J. A., and Gerton, G. L. (1996). Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human Tpx1 and is a member of the cysteine-rich secretory protein (CRISP) family. *Mol. Reprod. Dev.* **44,** 221–229.

Fraser, L. R. (1995). Cellular biology of capacitation and the acrosome reaction. *Hum. Reprod.* **10,** 22–30.

Fusi, F. M., Vignali, M., Busacca, M., and Bronson, R. A. (1992). Evidence for the presence of an integrin cell adhesion receptor on the oolemma of unfertilized human oocytes. *Mol. Reprod. Dev.* **31,** 215–222.

Fusi, F. M., Vignali, M., Gailit, J., and Bronson, R. A. (1993). Mammalian oocytes exhibit specific recognition of the RGD (Arg–Gly–Asp) tripeptide and express oolemmal integrins. *Mol. Reprod. Dev.* **36,** 212–219.

Gajhede, M., Petersen, T. N., Henriksen, A., Petersen, J. F. W., Dauter, Z., Wilson, K. S., and Thim, L. (1993). Pancreatic spasmolytic polypeptide: First three-dimensional structure of a member of the mammalian trefoil family of proteins. *Structure* **1,** 253–262.

Garberi, J. C., Kohane, A. C., Cameo, M. S., and Blaquier, J. A. (1979). Isolation and characterization of specific rat epididymal proteins. *Mol. Cell. Endocrinol.* **13,** 73–82.

Gong, X. H., Dubois, D. H., Miller, D. J., and Shur, B. D. (1995). Activation of a G protein complex by aggregation of β-1,4-galactosyltransferase on the surface of sperm. *Science* **269,** 1718–1721.

Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R., and Earp, H. S. (1994). Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. *Cell Growth Differ.* **5,** 647–657.

Greve, J. M., and Wassarman, P. M. (1985). Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. *J. Mol. Biol.* **181,** 253–264.

Grootenhuis, A. J., Philipsen, H. L. A., de Breet-Grijsbach, J. T. M., and Van Duin, M. (1996). Immunocytochemical localization of ZP3 in primordial follicles of rabbit, marmoset, rhesus monkey and human ovaries using antibodies against human ZP3. *J. Reprod. Fertil. Suppl.* **50,** 43–54.

Gupta, S. K., Alves, K., Palladino, L. O., Mark, G. E., and Hollis, G. F. (1996a). Molecular cloning of the human fertilin β subunit. *Biochem. Biophys. Res. Commun.* **224,** 318–326.

Gupta, S. K., Chadha, K., Harris, J. D., Yurewicz, E. C., Sacco, A. G., Kolluri, S. K., and Afzalpurkar, A. (1996b). Mapping of epitopes on porcine zona pellucida-3-alpha by monoclonal antibodies inhibiting oocyte–sperm interaction. *Biol. Reprod.* **55,** 410–415.

Haendler, B., Krätzschmar, J., Theuring, F., and Schleuning, W.-D. (1993). Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. *Endocrinology* **133,** 192–198.

Hardy, D. M., and Garbers, D. L. (1995). A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. *J. Biol. Chem.* **270,** 26025–26028.

Harris, J. D., Hibler, D. W., Fontenot, G. K., Hsu, K. T., Yurewicz, E. C., and Sacco, A. G. (1994). Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: The ZPA, ZPB and ZPC gene families. *DNA Seq.* **4,** 361–393.

Hatanaka, Y., Nagai, T., Tobita, T., and Nakano, M. (1992). Changes in the properties and composition of zona pellucida of pigs during fertilization *in vitro*. *J. Reprod. Fertil.* **95,** 431–440.

Hayashi, M., Fujimoto, S., Takano, H., Ushiki, T., Abe, K., Ishikura, H., Yoshida, M. C., Kirchhoff, C., Ishibashi, T., and Kasahara, M. (1996). Characterization of a human glycoprotein with a potential role in sperm–egg fusion—cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEGL1). *Genomics* **32,** 367–374.

Hedrick, J. L., Wardrip, N. J., and Berger, T. (1987). Differences in the macromolecular composition of the zona pellucida isolated from pig oocytes, eggs, and zygotes. *J. Exp. Zool.* **241,** 257–262.

Heinlein, U. A. O., Wallat, S., Senftleben, A., and Lemaire, L. (1994). Male germ cell-expressed mouse gene *TAZ83* encodes a putative, cysteine-rich transmembrane protein (*cyritestin*) sharing homologies with snake toxins and sperm–egg fusion proteins. *Dev. Growth Differ.* **36,** 49–58.

Hessing, M. (1991). The interaction between complement component C4b-binding protein and the vitamin K-dependent protein S forms a link between blood coagulation and the complement system. *Biochem. J.* **277,** 581-592.

Hirao, Y., and Yanagimachi, R. (1978). Temperature dependence of sperm–egg fusion and post-fusion events in hamster fertilization. *J. Exp. Zool.* **205,** 433–437.

Hoffman, W., and Hauser, F. (1996). The P-domain or trefoil motif: A role in renewal and pathology of mucous epithelia. *Trends Biochem. Sci.* **18,** 239–243.

Hogervorst, F., Kuikman, I., von dem Borne, A. E., and Sonnenberg, A. (1990). Cloning and sequence analysis of β-4 cDNA: An integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J.* **9,** 765–770.

Horan, A. H., and Bedford, J. M. (1972). Development of the fertilizing ability of spermatozoa in the epididymis of the Syrian hamster. *J. Reprod. Fertil.* **30,** 417–423.

Horvath, P. M., Kellom, T., Caulfield, J., and Boldt, J. (1993). Mechanistic studies of the plasma membrane block to polyspermy in mouse eggs. *Mol. Reprod. Dev.* **34,** 65–72.

Hunnicutt, G. R., Primakoff, P., and Myles, D. G. (1996). Sperm surface protein PH-20 is bifunctional—One activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. *Biol. Reprod.* **55,** 80–86.

Hynes, R. O. (1991). The complexity of platelet adhesion to extracellular matrices. *Thromb. Haemostasis* **66,** 40–43.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11-25.

Jansen, S., Quigley, M., Reik, W., and Jones, R. (1995). Analysis of polysulfate-binding domains in porcine proacrosin, a putative zona adhesion protein from mammalian spermatozoa. *Int. J. Dev. Biol.* **39,** 501-510.

Johnson, M. H., Eager, D., Muggleton-Harris, A., and Grave, H. M. (1975). Mosaicism in organisation concanavalin A receptors on surface membrane of mouse egg. *Nature* **257,** 321-322.

Jones, R. (1990). Identification and functions of mammalian sperm–egg recognition molecules during fertilization. *J. Reprod. Fertil. Suppl.* **42,** 89–105.

Jones, R., and Brown, C. R. (1987). Identification of a zona-binding protein from boar spermatozoa as proacrosin. *Exp. Cell Res.* **171,** 503–508.

Kadam, A. L., Fateh, M., and Naz, R. K. (1995). Fertilization antigen (FA-1) completely blocks human sperm binding to human zona pellucida: FA-1 antigen may be a sperm receptor for zona pellucida in humans. *J. Reprod. Immunol.* **29,** 19–30.

Kalab, P., Visconti, P., Leclerc, P., and Kopf, G. S. (1994). p95, the major phosphotyrosine-containing protein in mouse spermatozoa, is a hexokinase with unique properties. *J. Biol. Chem.* **269,** 3810-3817.

Kasahara, M., Gutknecht, J., Brew, K., Spurr, N., and Goodfellow, P. N. (1989). Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics* **5,** 527–534.

Kaul, R., Afzalpurkar, A., and Gupta, S. K. (1996). Strategies for designing an immunocontraceptive vaccine based on zona pellucida synthetic peptides and recombinant antigen. *J. Reprod. Fertil. Suppl.* **50,** 127–134.

Kellom, T., Vick, A., and Boldt, J. (1992). Recovery of penetration ability in protease-treated zona-free mouse eggs occurs coincident with recovery of a cell surface 94 kD protein. *Mol. Reprod. Dev.* **33,** 46–52.

Kinloch, R. A., Ruiz-Seiler, B., and Wassarman, P. M. (1990). Genomic organization and polypeptide primary structure of zona pellucida glycoprotein hZP3, the hamster sperm receptor. *Dev. Biol.* **142,** 414–421.

Kinloch, R. A., Mortillo, S., Stewart, C. L., and Wassarman, P. M. (1991). Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylate similar polypeptides and secrete active mouse sperm receptor. *J. Cell Biol.* **115,** 655–664.

Kinloch, R. A., Sakai, Y., and Wassarman, P. M. (1995). Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis. *Proc. Natl. Acad. Sci. USA* **92,** 263–267.

Kipersztok, S., Osawa, G. A., Liang, L. F., Modi, W. S., and Dean, J. (1995). POM-ZP3, a bipartite transcript derived from human ZP3 and a POM121 homologue. *Genomics* **25,** 354–359.

Kohane, A. C., Gonzalez Echeverria, F., Pineiro, L., and Blaquier, J. A. (1980). Interaction of proteins of epididymal origin with spermatozoa. *Biol. Reprod.* **23,** 737–742.

Kölle, S., Sinowatz, F., Boie, G., Totzauer, I., Amselgruber, W., and Plendl, J. (1996). Localization of the mRNA encoding the zona protein ZP3α in the porcine ovary, oocyte and embryo by non-radioactive *in situ* hybridization. *Histochem. J.* **28,** 441–447.

Kolluri, S. K., Kaul, R., Banerjee, K., and Gupta, S. K. (1995). Nucleotide sequence of cdna encoding bonnet monkey (macaca radiata) zona pellucida glycoprotein-zp3. *Reprod. Fertil. Dev.* **7,** 1209–1212, 2232.

Kong, M., Richardson, R. T., Widgren, E. E., and O'Rand, M. G. (1995). Sequence and localization of the mouse sperm autoantigenic protein, Sp17. *Biol. Reprod.* **53,** 579–590.

Krätzschmar, J., Haendler, B., Eberspaecher, U., Roosterman, D., Donner, P., and Schleuning, W. D. (1996). The human cysteine-rich secretory protein (CRISP) family primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. *Eur. J. Biochem.* **236,** 827–836.

Lathrop, W. F., Carmichael, E. P., Myles, D. G., and Primakoff, P. (1990). cDNA cloning reveals the structure of a sperm surface protein, PH-20, involved in sperm–egg adhesion and the wide distribution of its gene among mammals. *J. Cell Biol.* **111,** 2939–2949.

Lea, O. A., and French, F. S. (1981). Characterization of an acidic glycoprotein secreted by principal cells of the rat epididymis. *Biochim. Biophys. Acta* **668,** 370–376.

Lea, I. A., Richardson, R. T., Widgren, E. E., and O'Rand, M. G. (1996). Cloning and sequencing of cDNAs encoding the human sperm protein, Sp17. *Biochim. Biophys. Acta Gene Struct. Expression* **1307,** 263–266.

Lee, V. H., and Dunbar, B. S. (1993). Developmental expression of the rabbit 55-kDa zona pellucida protein and messenger RNA in ovarian follicles. *Dev. Biol.* **155,** 371–382.

Lee, V. H., Schwoebel, E., Prasad, S., Cheung, P., Timmons, T. M., Cook, R., and Dunbar, B. S. (1993). Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. *J. Biol. Chem.* **268,** 12412–12417.

Leyton, L., and Saling, P. (1989a). Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *J. Cell Biol.* **108,** 2163–2168.

Leyton, L., and Saling, P. (1989b). 95 kd Sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. *Cell* **57,** 1123–1130.

Leyton, L., LeGuen, P., Bunch, D., and Saling, P. M. (1992). Regulation of mouse gamete interaction by a sperm tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **89,** 11692–11695.

Leyton, L., Tomes, C. N., and Saling, P. M. (1995). LL95 monoclonal antibody mimics functional effects of ZP3 on mouse sperm: Evidence that the antigen recognized is not hexokinase. *Mol. Reprod. Dev.* **42,** 347–358.

Liang, L.-F., and Dean, J. (1993). Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes. *Dev. Biol.* **156,** 399–408.

Liang, L. F., Chamow, S. M., and Dean, J. (1990). Oocyte-specific expression of mouse Zp-2: Developmental regulation of the zona pellucida genes. *Mol. Cell. Biol.* **10,** 1507–1515.

Lin, Y., Kimmel, L. H., Myles, D. G., and Primakoff, P. (1993). Molecular cloning of the human and monkey sperm surface protein PH-20. *Proc. Natl. Acad. Sci. USA* **90,** 10071–10075.

Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G., and Primakoff, P. (1994). A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J. Cell Biol.* **125,** 1157–1163.

Litscher, E. S., and Wassarman, P. M. (1996). Characterization of a mouse ZP3-derived glycopeptide, gp55, that exhibits sperm receptor and acrosome reaction-inducing activity *in vitro*. *Biochemistry* **35,** 3980–3985.

Litscher, E. S., Juntunen, K., Seppo, A., Penttila, L., Niemela, R., Renkonen, O., and Wassarman, P. M. (1995). Oligosaccharide constructs with defined structures that inhibit binding of mouse sperm to unfertilized eggs *in vitro*. *Biochemistry* **34,** 4662–4669.

Liu, C. Y., Litscher, E. S., Mortillo, S., Sakai, Y., Kinloch, R. A., Stewart, C. L., and Wassarman, P. M. (1996). Targeted disruption of the mzp3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proc. Natl. Acad. Sci. USA* **93,** 5431–5436.

Longo, F. J., and Yanagimachi, R. (1993). Detection of sperm–egg fusion. *Methods Enzymol.* **221,** 249–260.

Lopata, A., and Leung, P. C. (1988). The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. NY Acad. Sci.* **541,** 324–336.

Lopez, L. C., and Shur, B. D. (1987). Redistribution of mouse sperm surface galactosyltransferase after the acrosome reaction. *J. Cell Biol.* **105,** 1663–1670.

Lyons, C. E., Payette, K. L., Price, J. L., and Huang, R. C. C. (1993). Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene. *J. Biol. Chem.* **268,** 21351–21358.

Macek, M. B., Lopez, L. C., and Shur, B. D. (1991). Aggregation of β-1,4-galactosyltransferase on mouse sperm induces the acrosome reaction. *Dev. Biol.* **147,** 440–444.

Mahi, C. A., and Yanagimachi, R. (1976). Maturation and sperm penetration of canine ovarian oocytes *in vitro*. *J. Exp. Zool.* **196,** 189–196.

Martinez, M. L., Fontenot, G. K., and Harris, J. D. (1996). The expression and localization of the zona pellucida glycoproteins and mRNA in cynomolgus monkeys (*Macaca fascicularis*). *J. Reprod. Fertil. Suppl.* **50,** 35–41.

Miller, D. J., Macek, M. B., and Shur, B. D. (1992). Complementarity between sperm surface β-1,4-galactosyl-transferase and egg-coat ZP3 mediates sperm–egg binding. *Nature* **357,** 589–593.

Miller, D. J., Gong, X., Decker, G., and Shur, B. D. (1993). Egg cortical granule *N*-acetylglucosaminidase is required for the mouse zona block to polyspermy. *J. Cell Biol.* **123,** 1431–1440.

Mintz, B. (1962). Experimental study of the developing egg after removal of the zona pellucida. *Science* **135,** 594–595.

Mizuki, N., and Kasahara, M. (1992). Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. *Mol. Cell. Endocrinol.* **89,** 25–32.

Mizuki, N., Sarapata, D. E., Garcia-Sanz, J. A., and Kasahara, M. (1992). The mouse male germ cell-specific gene Tpx-1: Molecular structure, mode of expression in spermatogenesis, and sequence similarity to two non-mammalian genes. *Mamm. Genome* **3,** 274–280.

Modlinski, J. A. (1970). The role of the zona pellucida in the development of mouse eggs *in vivo*. *J. Embryol. Exp. Morphol.* **23,** 539–547.

Moller, C. C., and Wassarman, P. M. (1989). Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. *Dev. Biol.* **132,** 103–112.

Moller, C. C., Bleil, J. D., Kinloch, R. A., and Wassarman, P. M. (1990). Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Dev. Biol.* **137,** 276–286.

Monroy, A. (1985). Processes controlling sperm–egg fusion. *Eur. J. Biochem.* **152,** 51–56.

Moore, H. D. M., and Bedford, J. M. (1978). Ultrastructure of the equatorial segment of hamster spermatozoa during penetration of oocytes. *J. Ultrastruct. Res.* **62,** 110–117.

Moore, H. D. M., Hartman, T. D., Bye, A. P., Lutjen, P., De Witt, M., and Trounson, A. O. (1987). Monoclonal antibody against a sperm antigen Mr 95,000 inhibits attachment of human spermatozoa to the zona pellucida. *J. Reprod. Immunol.* **11,** 157–166.

Moos, J., Faundes, D., Kopf, G. S., and Schultz, R. M. (1995). Composition of the human zona pellucida and modifications following fertilization. *Hum. Reprod.* **10,** 2467–2471.

Mori, E., Kashiwabara, S., Baba, T., Inagaki, Y., and Mori, T. (1995). Amino acid sequences of porcine Sp38 and proacrosin required for binding to the zona pellucida. *Dev. Biol.* **168,** 575–583.

Mortillo, S., and Wassarman, P. M. (1991). Differential binding of gold-labeled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. *Development* **113,** 141–149.

Muga, A., Neugebauer, W., Hirama, T., and Surewicz, W. K. (1994). Membrane interaction and conformational properties of the putative fusion peptide of PH-30, a protein active in sperm–egg fusion. *Biochemistry* **33,** 4444–4448.

Murata, K., Sasaki, T., Yasumasu, S., Iuchi, I., Enami, J., Yasumasu, I., and Yamagami, K. (1995). Cloning of cDNAs for the precursor protein of a low-molecular-weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*. *Dev. Biol.* **167,** 9–17.

Myles, D. G. (1993). Molecular mechanisms of sperm–egg membrane binding and fusion in mammals. *Dev. Biol.* **158,** 35–45.

Myles, D. G., Hyatt, H., and Primakoff, P. (1987). Binding of both acrosome-intact and acrosome-reacted guinea pig sperm to the zona pellucida during *in vitro* fertilization. *Dev. Biol.* **121,** 559–567.

Myles, D. G., Kimmel, L. H., Blobel, C. P., White, J. M., and Primakoff, P. (1994). Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion. *Proc. Natl. Acad. Sci. USA* **91,** 4195–4198.

Nakano, M., Yonezawa, N., Hatanaka, Y., and Noguchi, S. (1996). Structure and function of the *N*-linked carbohydrate chains of pig zona pellucida glycoproteins. *J. Reprod. Fertil. Suppl.* **50,** 25–34.

Naz, R. K., and Ahmad, K. (1994). Molecular identities of human sperm proteins that bind human zona pellucida: Nature of sperm–zona interaction, tyrosine kinase activity, and involvement of FA-1. *Mol. Reprod. Dev.* **39,** 397–408.

Niwa, K., and Chang, M. C. (1975). Fertilization of rat eggs *in vitro* at various times before and after ovulation with special reference to fertilization of ovarian oocytes matured in culture. *J. Reprod. Fertil.* **43,** 435–451.

Okabe, M., Yagasaki, M., Oda, H., Matzno, S., Kohama, Y., and Mimura, T. (1988). Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs. *J. Reprod. Immunol.* **13,** 211–219.

Okabe, M., Yagasaki, M., Matzno, S., Nagira, M., Kohama, Y., and Mimura, T. (1989). Glucosamine enhanced sperm–egg binding but inhibited sperm–egg fusion in mouse. *Experientia* **45,** 193–194.

Okabe, M., Nagira, M., Kawai, Y., Matzno, S., Mimura, T., and Mayumi, T. (1990). A human sperm antigen possibly involved in binding and/or fusion with zona-free hamster eggs. *Fertil. Steril.* **54,** 1121–1126.

O'Rand, M. G. (1988). Sperm–egg recognition and barriers to interspecies fertilization. *Gamete Res.* **19,** 315–328.

Oura, C., and Toshimori, K. (1990). Ultrastructural studies on the fertilization of mammalian gametes. *Int. Rev. Cytol.* **122,** 105–151.

Oyen, O., Myklebust, F., Scott, J. D., Hansson, V., and Jahnsen, T. (1989). Human testis cDNA for the regulatory subunit RIIα of cyclic AMP-dependent protein kinase encodes an alternate amino-terminal region. *FEBS Lett.* **246,** 57–64.

Pan, Y. C., and Li, S. S. (1982). Structure of secretory protein IV from rat seminal vesicles. *Int. J. Pept. Protein Res.* **20,** 177–187.

Parrish, J. J., Susko-Parrish, J. L., Handrow, R. R., Ax, R. L., and First, N. L. (1989). Effect of sulfated glycoconjugates on capacitation and the acrosome reaction of bovine and hamster spermatozoa. *Gamete Res.* **24,** 403–413.

Perez Martinez, S., Conesa, D., and Cuasnicú, P. S. (1995). Potential contraceptive use of epididymal proteins: Evidence for the participation of specific antibodies against rat epididymal protein DE in male and female fertility inhibition. *J. Reprod. Immunol.* **29,** 31–45.

Perry, A. C. F., Jones, R., Barker, P. J., and Hall, L. (1992). A mammalian epididymal protein with remarkable sequence similarity to snake venom haemorrhagic peptides. *Biochem. J.* **286,** 671–675.

Perry, A. C. F., Gichuhi, P. M., Jones, R., and Hall, L. (1995). Cloning and analysis of monkey fertilin reveals novel α subunit isoforms. *Biochem. J.* **307,** 843–850.

Phelps, B. M., Primakoff, P., Koppel, D. E., Low, M. G., and Myles, D. G. (1988). Restricted lateral diffusion of PH-20,a PI-anchored sperm membrane protein. *Science* **240,** 1780–1782.

Phillips, D. M., and Shalgi, R. (1980). Surface architecture of the mouse and hamster zona pellucida and oocyte. *J. Ultrastruct. Res.* **72,** 1–12.

Phillips, D. M., and Shalgi, R. (1982). Sperm penetration into rat ova fertilized *in vivo. J. Exp. Zool.* **221,** 373–378.

Polge, E., and Dziuk, P. J. (1965). Recovery of immature eggs penetrated by spermatozoa following induced ovulation in the pig. *J. Reprod. Fertil.* **9,** 357–358.

Prasad, S. V., Mujtaba, S., Lee, V. H., and Dunbar, B. S. (1995). Immunogenicity enhancement of recombinant rabbit 55-kilodalton zona pellucida protein expressed using the baculovirus expression system. *Biol. Reprod.* **52,** 1167–1178.

Prasad, S. V., Wilkins, B., Skinner, S. M., and Dunbar, B. S. (1996). Evaluating zona pellucida structure and function using antibodies to rabbit 55 kda zp protein expressed in baculovirus expression system. *Mol. Reprod. Dev.* **43,** 519–529.

Primakoff, P. (1994). Sperm proteins being studied for use in a contraceptive vaccine. *Am. J. Reprod. Immunol.* **31,** 208–210.

Primakoff, P., Hyatt, H., and Myles, D. G. (1985). A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J. Cell Biol.* **101,** 2239–2244.

Primakoff, P., Hyatt, H., and Tredick-Kline, J. (1987). Identification and purification of a sperm surface protein with a potential role in sperm–egg membrane fusion. *J. Cell Biol.* **104,** 141–149.

Primakoff, P., Lathrop, W., Woolman, L., Cowan, A., and Myles, D. (1988). Fully effective contraception in male and female guinea pigs immunized with the sperm protein. *Nature* **335,** 543–546.

Ramarao, C. S., Myles, D. G., White, J. M., and Primakoff, P. (1996). Initial evaluation of fertilin as an immunocontraceptive antigen and molecular cloning of the cynomolgus monkey fertilin β subunit. *Mol. Reprod. Dev.* **43,** 70–75.

Rankin, T., Familari, M., Lee, E., Ginsberg, A., Dwyer, N., Blanchettemackie, J., Drago, J., Westphal, H., and Dean, J. (1996). Mice homozygous for an insertional mutation in the *Zp3* gene lack a zona pellucida and are infertile. *Development* **122,** 2903–2910.

Rankin, T. L., Tsuruta, K. J., Holland, M. K., Griswold, M. D., and Orgebin-Crist, M.-C. (1992). Isolation, immunolocalization, and sperm-association of three proteins of 18, 25, and 29 kilodaltons secreted by the mouse epididymis. *Biol. Reprod.* **46,** 747–766.

Reid, K. B. M., and Day, A. J. (1989). Structure–function relationships of the complement components. *Immunol. Today* **10,** 177–180.

Repin, V. S., and Akimova, I. M. (1976). A study of the protein composition of the zona pellucida of mammalian oocytes and zygotes by a method of microelectrophoresis in polyacrylamide gel. *Biokhimiia* **41,** 50–57.

Richardson, R. T., and O'Rand, M. G. (1996). Site-directed mutagenesis of rabbit proacrosin: Identification of residues involved in zona pellucida binding. *J. Biol. Chem.* **271,** 24069–24074.

Richardson, R. T., Yamasaki, N., and O'Rand, M. G. (1994). Sequence of a rabbit sperm zona pellucida binding protein and localization during the acrosome reaction. *Dev. Biol.* **165,** 688–701.

Ringuette, M. J., Chamberlin, M. E., Baur, A. W., Sobieski, D. A., and Dean, J. (1988). Molecular analysis of cDNA coding for ZP3, a sperm binding protein of the mouse zona pellucida. *Dev. Biol.* **127,** 287–295.

Robinson, R., Richardson, R., Hinds, K., Clayton, D., and Poirier, G. R. (1987). Features of a seminal proteinase inhibitor–zona pellucida-binding component on murine spermatozoa. *Gamete Res.* **16,** 217–228.

Rochwerger, L., and Cuasnicú, P. S. (1992). Redistribution of a rat sperm epididymal glycoprotein after *in vitro* and *in vivo* capacitation. *Mol. Reprod. Dev.* **31,** 34–41.

Rochwerger, L., Cohen, D. J., and Cuasnicú, P. S. (1992). Mammalian sperm–egg fusion: The rat egg has complementary sites for a sperm protein that mediates gamete fusion. *Dev. Biol.* **153,** 83–90.

Rosales, C., O'Brien, V., Kornberg, L., and Juliano, R. (1995). Signal transduction by cell adhesion receptors. *Biochim. Biophys. Acta* **1242,** 77–98.

Rosiere, T. K., and Wassarman, P. M. (1992). Identification of a region of mouse zona pellucida glycoprotein mZP3 that possesses sperm receptor activity. *Dev. Biol.* **154,** 309–317.

Ruoslahti, E. (1996a). Integrin signaling and matrix assembly. *Tumour Biol.* **17,** 117–124.

Ruoslahti, E. (1996b). RGD and other recognition sequences for integrins. *Annu. Rev. Cell Dev. Biol.* **12,** 697–715.

Sacco, A. G., Yurewicz, E. C., Subramanian, M. G., and Matzat, P. D. (1989). Porcine zona pellucida: Association of sperm receptor activity with the α-glycoprotein component of the M_r = 55,000 family. *Biol. Reprod.* **41,** 523–532.

Saling, P. M. (1989). Mammalian sperm interaction with extracellular matrices of the egg. *Oxf. Rev. Reprod. Biol.* **11,** 339–388.

Saling, P. M. (1996). Gamete interactions leading to fertilization in mammals: Principles, paradigms, and paradoxes. *In* "Reproductive Endocrinology, Surgery, and Technology" (E. Y. Adashi, J. A. Rock, and Z. Rosenwaks, eds.), pp. 403–421. Raven Press, New York.

Saling, P. M., Irons, G., and Waibel, R. (1985). Mouse sperm antigens that participate in fertilization. I. Inhibition of sperm fusion with the egg plasma membrane using monoclonal antibodies. *Biol. Reprod.* **335,** 515–526.

Saling, P. M., Burks, D. J., and Tomes, C. N. (1996). Sperm–zona pellucida interaction: A model for ZRK-mediated signalling. *In* "Signal Transduction in Testicular Cells" (V. Hansson, F. D. Levy, and K. Tasken, eds.), pp. 247–270. Springer-Verlag, Berlin.

Sanjurjo, C., Dawidowsky, A. R., Cameo, M. S., Gonzalez Echeverria, F., and Blaquier, J. A. (1990). Participation of human epididymal sperm coating antigens in fertilization. *J. Androl.* **11,** 476–483.

Santella, L., Alikani, M., Talansky, B. E., Cohen, J., and Dale, B. (1992). Is the human oocyte plasma membrane polarized? *Hum. Reprod.* **7,** 999–1003.

Sanz, L., Calvete, J. J., Mann, K., Schäfer, W., Schmid, E. R., and Töpfer-Petersen, E. (1991). The amino acid sequence of AQN-3, a carbohydrate-binding protein isolated from boar sperm. Location of disulphide bridges. *FEBS Lett.* **291,** 33–36.

Sanz, L., Calvete, J. J., Mann, K., Schäfer, W., Schmid, E. R., Amselgruber, W., Sinowatz, F., Ehrhard, M., and Töpfer-Petersen, E. (1992a). The complete primary structure of the spermadhesin AWN, a zona pellucida-binding protein isolated from boar spermatozoa. *FEBS Lett.* **300,** 213–218.

Sanz, L., Calvete, J. J., Mann, K., Schäfer, W., Schmid, E. R., and Töpfer-Petersen, E. (1992b). The complete primary structure of the boar spermadhesin AQN-1, a carbohydrate-binding protein involved in fertilization. *Eur. J. Biochem.* **205,** 645–652.

Schollmeyer, J. E. (1986). Identification of calpain II in porcine sperm. *Biol. Reprod.* **34,** 721–731.

Schwartz, M. A., Ingber, D. E., Lawrence, M., Springer, T. A., and Lechene, C. (1991). Multiple integrins share the ability to induce elevation of intracellular pH. *Exp. Cell Res.* **195,** 533–535.

Schwoebel, E., Prasad, S., Timmons, T. M., Cook, R., Kimura, H., Niu, E., Cheung, P., Skinner, S., Avery, S., Wilkins, B., and Dunbar, B. S. (1991). Isolation and characterization of a full length cDNA encoding the 55 kDa rabbit zona pellucida protein. *J. Biol. Chem.* **266,** 7214–7219.

Sengoku, K., Tamate, K., Horikawa, M., Takaoka, Y., Ishikawa, M., and Dukelow, W. R. (1995). Plasma membrane block to polyspermy in human oocytes and preimplantation embryos. *J. Reprod. Fertil.* **105,** 85–90.

Shabanowitz, R. B., and O'Rand, M. G. (1988). Characterization of the human zona pellucida from fertilized and unfertilized eggs. *J. Reprod. Fertil.* **82,** 151–161.

Shalgi, R., and Phillips, D. (1980a). Mechanics of *in vitro* fertilization in the hamster. *Biol. Reprod.* **23,** 433–444.

Shalgi, R., and Phillips, D. (1980b). Mechanics of sperm entry in cycling hamsters. *J. Ultrastruct. Res.* **71,** 154–161.

Shaper, N. L., Hollis, G. F., Douglas, J. G., Kirsch, I. R., and Shaper, J. H. (1988). Characterization of the full length cDNA for murine β-1,4-galactosyltransferase. *J. Biol. Chem.* **263,** 10420–10428.

Shaper, N. L., Wright, W. W., and Shaper, J. H. (1990). Murine β1,4-galactosyltransferase: Both the amount and the structure of the mRNA are regulated during spermatogenesis. *Proc. Natl. Acad. Sci. USA* **87,** 791–795.

Shur, B. D., and Bennett, D. (1979). A specific defect in galactosyltransferase regulation on sperm bearing mutant alleles of the T/t locus. *Dev. Biol.* **71,** 243–259.

Shur, B. D., and Hall, N. G. (1982). A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J. Cell Biol.* **95,** 574–579.

Shur, B. D., and Neely, C. A. (1988). Plasma membrane association, purification, and partial characterization of mouse sperm β1,4-galactosyltransferase. *J. Biol. Chem.* **263,** 17706–17714.

Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., and Cantley, L. C. (1994). Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* **14,** 2777–2785.

Stauss, C. R., Votta, T. J., and Suarez, S. S. (1995). Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol. Reprod.* **53,** 1280–1285.

Stephens, L. R., Jackson, T. R., and Hawkins, P. T. (1993). Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: A new intracellular signalling system. *Biochim. Biophys. Acta.* **1179,** 27–75.

Sutherland, A. E., Calarco, P. G., and Damsky, C. H. (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119,** 1175–1186.

Suzuki, S., and Naitoh, Y. (1990). Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.* **9,** 757–763.

Talansky, B. E., Malter, H. E., and Cohen, J. (1991). A preferential site for sperm–egg fusion in mammals. *Mol. Reprod. Dev.* **28,** 183–188.

Taya, T., Yamasaki, N., Tsubamoto, H., Hasegawa, A., and Koyama, K. (1995). Cloning of a cDNA coding for porcine zona pellucida glycoprotein ZP1 and its genomic organization. *Biochem. Biophys. Res. Commun.* **207,** 790–799.

Taylor, S., Buechler, J. A., and Yonemoto, W. (1990). cAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* **59,** 971–1005.

Tesarik, J., Drahorad, J., Testart, J., and Mendoza, C. (1990). Acrosin activation follows its surface exposure and precedes membrane fusion in human sperm acrosome reaction. *Development* **110,** 391–400.

Tezon, J. G., Ramella, E., Cameo, M. S., Vazquez, M. H., and Blaquier, J. A. (1985). Immunochemical localization of secretory antigens in the human epididymis and their association with spermatozoa. *Biol. Reprod.* **32,** 591–597.

Thaler, C. D., and Cardullo, R. A. (1996). The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. *J. Biol. Chem.* **271,** 23289–23297.

Thillai-Koothan, P., Van Duin, M., and Aitken, R. J. (1993). Cloning, sequencing and oocyte-specific expression of the marmoset sperm receptor protein, ZP3. *Zygote* **1,** 93–101.

Thim, L. (1989). A new family of growth factor-like peptides. "Trefoil" disulphide loop structures as a common feature in breast cancer-associated peptide (p52), pancreatic spasmolytic peptide (PSP), and frog skin peptides (spasmolysins). *FEBS Lett.* **250,** 85–90.

Tomes, C. N., McMaster, C., and Saling, P. M. (1996). Activation of mouse PIP_2-PLC by zona pellucida is modulated by tyrosine phosphorylation. *Mol. Reprod. Dev.* **43,** 196–204.

Töpfer-Petersen, E. (1996). Molecular mechanism of fertilization in the pig. *Reprod. Domest. Anim.* **31,** 93–100.

Töpfer-Petersen, E., and Calvete, J. J. (1995). Molecular mechanisms of the interaction between sperm and the zona pellucida in mammals—Studies on the pig. *Int. J. Androl.* **18** (2), 20–26, 1275.

Töpfer-Petersen, E., and Cechová, D. (1990). Zona pellucida induces conversion of proacrosin to acrosin. *Int. J. Androl.* **13,** 190–196.

Töpfer-Petersen, E., and Henschen, A. (1987). Acrosin shows zona and fucose binding, novel properties for a serine proteinase. *FEBS Lett.* **226,** 38–42.

Töpfer-Petersen, E., and Henschen, A. (1988). Zona pellucida-binding and fucose-binding of boar sperm acrosin is not correlated with proteolytic activity. *Biol. Chem. Hoppe-Seyler* **369,** 69–76.

Töpfer-Petersen, E., Steinberger, M., von Eschenbach, C. E., and Zucker, A. (1990). Zona pellucida-binding of boar sperm acrosin is associated with the N-terminal peptide of the acrosin B-chain (heavy chain). *FEBS Lett.* **265,** 51–54.

Töpfer-Petersen, E., Mann, K., and Calvete, J. J. (1993). Identification of porcine oocyte 55 kDa α and β proteins within the zona pellucida glycoprotein families indicates that oocyte sperm receptor activity is associated with different zona pellucida proteins in different mammalian species. *Biol. Chem. Hoppe-Seyler* **374,** 411–417.

Töpfer-Petersen, E., Calvete, J. J., Sanz, L., and Sinowatz, F. (1995). Carbohydrate- and heparin-binding proteins in mammalian fertilization. *Andrologia* **27,** 303–324.

Tsubamoto, H., Hasegawa, A., Inoue, M., Yamasaki, N., and Koyama, K. (1996). Binding of recombinant pig zona pellucida protein 1 (zp1) to acrosome-reacted spermatozoa. *J. Reprod. Fertil.* **50,** 63–67.

Urch, U. A., and Patel, H. (1991). The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides. *Development* **111,** 1165–1172.

Van Duin, M., Polman, J. E. M., Suikerbuijk, R. F., Geurts van Kessel, A. H. M., and Olijve, W. (1993). The human gene for the zona pellucida glycoprotein ZP3 and a second polymorphic locus are located on chromosome 7. *Cytogenet. Cell Genet.* **63,** 111–113.

Van Duin, M., Polman, J. E., De Breet, I. T., van Ginneken, K., Bunschoten, H., Grootenhuis, A., Brindle, J., and Aitken, R. J. (1994). Recombinant human zona pellucida protein ZP3 produced by chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm–egg fusion. *Biol. Reprod.* **51,** 607–617.

Varticovski, L., Harrison-Findik, D., Keeler, M. L., and Susa, M. (1994). Role of PI 3-kinase in mitogenesis. *Biochim. Biophys. Acta* **1226,** 1–11.

Vigil, P. (1989). Gamete membrane fusion in hamster spermatozoa with reacted equatorial segment. *Gamete Res.* **23,** 203–213.

Wassarman, P. M. (1988). Zona pellucida glycoproteins. *Annu. Rev. Biochem.* **57,** 415–442.

Wassarman, P. M., and Albertini, D. F. (1994). The mammalian ovum. *In* "The Physiology of Reproduction" (E. Knobil and J. D. Neill, eds.), 2nd Ed., pp. 79–122. Raven Press, New York.

Wassarman, P. M., and Litscher, E. S. (1995). Sperm–egg recognition mechanisms in mammals. *Curr. Top. Dev. Biol.* **30,** 1–19.

White, J. M. (1992). Membrane fusion. *Science* **258,** 917–924.

Wolf, D. E., and Ziomek, C. A. (1983). Regionalization and lateral diffusion of membrane proteins in unfertilized and fertilized mouse eggs. *J. Cell Biol.* **96,** 1786–1790.

Wolf, D. P. (1977). Involvement of a trypsin-like activity in sperm penetration of zona-free mouse ova. *J. Exp. Zool.* **199,** 149–156.

Wolf, D. P. (1978). The block to sperm penetration in zonal-free mouse eggs. *Dev. Biol.* **64,** 1–10.

Wolfsberg, T. G., Bazan, J. F., Blobel, C. P., Myles, D. G., Primakoff, P., and White, J. M. (1993). The precursor region of a protein active in sperm–egg fusion contains a metalloprotease and a disintegrin domain: Structural, functional, and evolutionary implications. *Proc. Natl. Acad. Sci. USA* **90,** 10783–10787.

Wolfsberg, T. G., Primakoff, P., Myles, D. G., and White, J. M. (1995a). ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain— Multipotential functions in cell–cell and cell–matrix interactions. *J. Cell Biol.* **131,** 275–278.

Wolfsberg, T. G., Straight, P. D., Gerena, R. L., Huovila, A. P., Primakoff, P., Myles, D. G., and White, J. M. (1995b). ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin and metalloprotease domain. *Dev. Biol.* **169,** 378–383.

Wong, P. Y., and Tsang, A. Y. (1982). Studies on the binding of a 32K rat epididymal protein to rat epididymal spermatozoa. *Biol. Reprod.* **27,** 1239–1246.

Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y., and Fujisawa-Sehara, A. (1995). A metalloprotease–disintegrin participating in myoblast fusion. *Nature* **377,** 652–656.

Yamada, K. M., and Miyamoto, S. (1995). Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* **7,** 681–689.

Yamasaki, N., Richardson, R. T., and O'Rand, M. G. (1995). Expression of the rabbit sperm protein Sp17 in COS cells and interaction of recombinant Sp17 with the rabbit zona pellucida. *Mol. Reprod. Dev.* **40,** 48–55.

Yamasaki, N., Tsubamoto, H., Hasegawa, A., Inoue, M., and Koyama, K. (1996). Genomic organization of the gene for pig zona pellucida glycoprotein zp1 and its expression in mammalian cells. *J. Reprod. Fertil.* **50,** 19–23.

Yanagimachi, R. (1978). Calcium requirement for sperm–egg fusion in mammals. *Biol. Reprod.* **19,** 949–958.

Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. *In* "Fertilization and Embryonic Development *in Vitro*" (L. Mastroianni and J. D. Biggers, eds.), pp. 81–187. Plenum, New York.

Yanagimachi, R. (1994). Mammalian fertilization. *In* "The Physiology of Reproduction" (E. Knobil and J. D. Neill, eds.), 2nd Ed., pp. 189–317. Raven Press, New York.

Yanagimachi, R., and Noda, Y. D. (1970). Physiological changes in the post-nuclear cap region of mammalian spermatozoa: A necessary preliminary to the membrane fusion between sperm and egg cells. *J. Ultrastruct. Res.* **31,** 486–493.

Yanagimachi, R., Miyashiro, L. R., and Yanagimachi, H. (1980). Reversible inhibition of sperm–egg fusion in the hamster by low pH. *Dev. Growth Differ.* **22,** 281–288.

Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1993). Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* **268,** 25846–25856.

Yonezawa, N., Aoki, H., Hatanaka, Y., and Nakano, M. (1995). Involvement of *N*-linked carbohydrate chains of pig zona pellucida in sperm–egg binding. *Eur. J. Biochem.* **233,** 35–41.

Youakim, A., Hathaway, H. J., Miller, D. J., Gong, X. H., and Shur, B. D. (1994). Overexpressing sperm surface β1,4-galactosyltransferase in transgenic mice affects multiple aspects of sperm–egg interaction. *J. Cell Biol.* **126,** 1573–1583.

Yurewicz, E. C., Sacco, A. G., and Subramanian, M. G. (1983). Isolation and preliminary characterization of a purified pig zona antigen (PPZA) from porcine oocytes. *Biol. Reprod.* **29,** 511–523.
Yurewicz, E. C., Sacco, A. C., and Subramanian, M. G. (1987). Structural characterization of the M_r = 55 000 antigen (ZP3) of porcine oocyte zona pellucida:Purification and characterization of α- and β-glycoproteins following digestion of lactosaminoglycan with endo-β-galactosidase. *J. Biol. Chem.* **262,** 564–571.
Yurewicz, E. C., Hibler, D., Fontenot, G. K., Sacco, A. G., and Harris, J. (1993a). Nucleotide sequence of cDNA encoding ZP3α, a sperm-binding glycoprotein from zona pellucida of pig oocyte. *Biochim. Biophys. Acta* **1174,** 211–214.
Yurewicz, E. C., Pack, B. A., Armant, D. R., and Sacco, A. G. (1993b). Porcine zona pellucida ZP3α glycoprotein mediates binding of the biotin-labeled M_r 55,000 family (ZP3) to boar sperm membrane vesicles. *Mol. Reprod. Dev.* **36,** 382–389.
Yurewicz, E. C., Zhang, S., and Sacco, A. G. (1993c). Generation and characterization of site-directed antisera against an amino-terminal segment of a 55 kDa sperm adhesive glycoprotein from zona pellucida of pig oocytes. *J. Reprod. Fertil.* **98,** 147–152.
Zuccotti, M., Yanagimachi, R., and Yanagimachi, H. (1991). The ability of hamster oolemma to fuse with spermatozoa: Its acquisition during oogenesis and loss after fertilization. *Development* **112,** 143–152.

This Page Intentionally Left Blank

Coordination of Nuclear and Chloroplast Gene Expression in Plant Cells

Michel Goldschmidt-Clermont
Department of Molecular Biology and Department of Plant Biology, University of Geneva, Sciences II, CH - 1211 Geneva 4, Switzerland

Plastid proteins are encoded in two genomes, one in the nucleus and the other in the organelle. The expression of genes in these two compartments is coordinated during development and in response to environmental parameters such as light. Two converging approaches reveal features of this coordination: the biochemical analysis of proteins involved in gene expression, and the genetic analysis of mutants affected in plastid function or development. Because the majority of proteins implicated in plastid gene expression are encoded in the nucleus, regulatory processes in the nucleus and in the cytoplasm control plastid gene expression, in particular during development. Many nucleus-encoded factors involved in transcriptional and posttranscriptional steps of plastid gene expression have been characterized. We are also beginning to understand whether and how certain developmental or environmental signals perceived in one compartment may be transduced to the other.
KEY WORDS: Plastids, Transcription, RNA splicing, RNA processing, RNA degradation, Protein degradation, Signal transduction, Plastid signal.

I. Introduction

Two genomes directly govern the biogenesis of the plastid: most genes are located in the nuclear chromosomes, but a separate set is maintained in the plastid. According to the endosymbiotic theory of organelle evolution, plastids are descendants of a prokaryotic photosynthetic organism related to cyanobacteria that established a symbiotic relationship with an early eukaryotic host (Gray, 1988, 1991; Palmer, 1992). This situation then

0074-7696/98 $25.00
Copyright © 1998 by Academic Press.
All rights of reproduction in any form reserved.

evolved as the original endosymbiotic ancestor of the plastid gradually lost genetic information that was partly relocated in the nucleus. The evolutionary flow of genetic information from the plastid to the nucleus is compensated, for the development and maintenance of plastids, by the converse flow of information carried by the many polypeptides that are encoded in the nucleus, translated in the cytoplasm, and imported into the organelle (Fig. 1). A similar situation is found with mitochondria which also maintain a separate genome. Plastid genomes of land plants and green algae contain approximately 120 genes, but the original endosymbiotic cyanobacterium probably contained a good order of magnitude more. Part of the genes which were not retained in the plastid may have been lost, some of them functionally replaced by genes in the nucleus, but others have been transferred from the organelle to the nucleus (Palmer, 1991; Thorsness and Weber, 1996). It is also likely that new functions have emerged to ensure the coordinate expression of the two genomes and to promote the differentiation of plastids into some of their specialized forms.

Plastids are extraordinarily flexible in their developmental capacity (Kirk and Tilney-Bassett, 1978). They can differentiate to form specialized organelles, of which the familiar chloroplasts have been studied most extensively. The meristematic tissues contain undifferentiated proplastids which can develop to become the photosynthetic chloroplasts with their abundant network of photosynthetic membrane vesicles, the thylakoids. But, for example, proplastids can also develop into the starch-rich amyloplasts found in roots. In the dark, leaf plastids can differentiate to form etioplasts, which are deficient in thylakoid membranes but contain arrays of prolamellar bodies arranged in quasicrystalline lattices. Upon transfer to light, etioplasts can mature and become fully developed chloroplasts, with a concomitant large increase in the complement of thylakoid membranes and photosynthetic proteins. In plants with the C4 pathway of carbon fixation, chloroplasts further specialize in the bundle sheath cells and in the mesophyll cells, and correspondingly the chloroplast genes are differentially expressed. For example, in maize and sorghum the plastid genes for photosystem II subunits are expressed more in the mesophyll chloroplasts, but the plastid gene for the large subunit of Rubisco (*rbcL*) is expressed to a greater level in the bundle sheath (Kubicki *et al.,* 1994). Plastids can also differentiate to become the colorful carotenoid-containing chromoplasts of some flowers and fruits. Contemplation of a vegetable stand at the market is thus a wonder to this reviewer, pondering the long evolutionary way from an endosymbiotic cyanobacterium to a bell-pepper chromoplast. Literally spectacular changes also occur in the plastids of senescing leaves. All these specializations require different complements of proteins and thus call for differential gene expression in the plastid according to the developmental pathway and the developmental stage on one hand and environmental clues

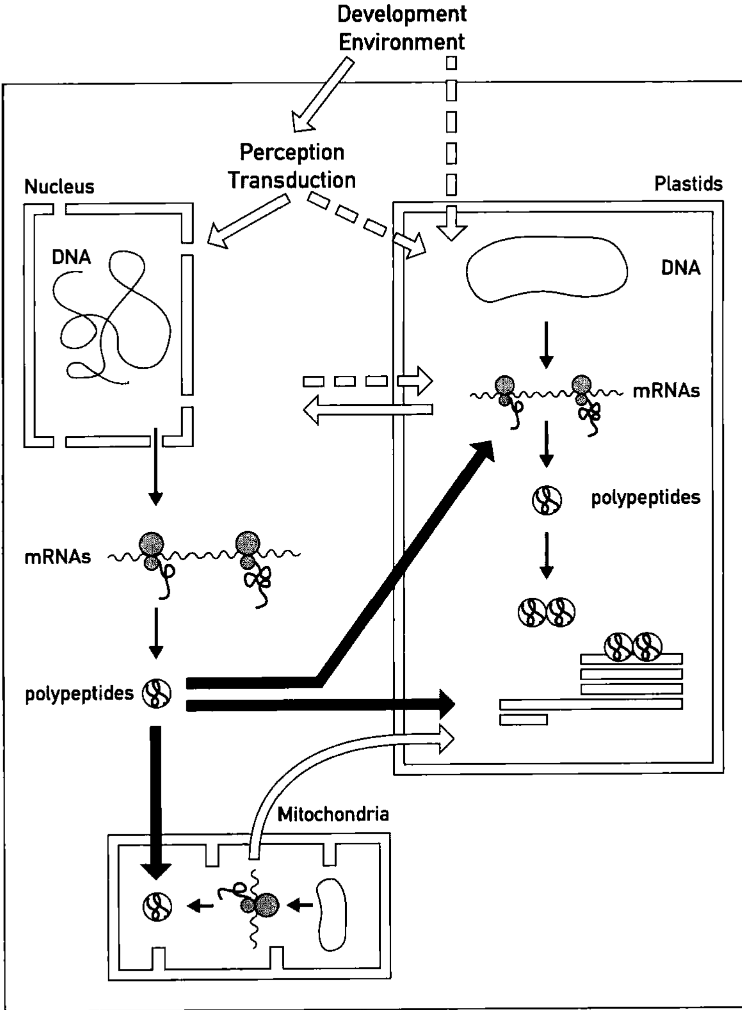


FIG. 1 Coordination of gene expression in plant cells. Plant cells contain three genetic systems, in the plastid (right), in the mitochondria (bottom), and in the nucleus and cytosol (left). Organellar genes are expressed to produce RNAs and proteins. In addition, many polypeptides are encoded in the nucleus, translated in the cytosol, and imported into the organelles (thick black arrows). Some of the polypeptides that are imported into the plastids are structural or enzymatic components, while others are involved in plastid gene expression. Environmental or developmental signals are perceived and transduced in the nucleocytosolic compartment (thick white arrows). It is not known whether such signals are directly perceived in the plastids or whether signals are transduced from the cytoplasm to the organelles (dotted white arrows). There is evidence for the control of nuclear gene expression by a signal(s) emanating from the plastid. Plastid development is dependent on mitochondrial function.

such as light on the other hand. Gene expression is also modulated by circadian rhythms. All these regulated changes are obviously paralleled in the nucleocytosolic compartment. The tight integration of the activity of the two genetic systems implies a regulatory network to perceive and transduce the developmental and environmental clues in the two compartments.

There are only a few copies of the nuclear genome in a cell (depending on its ploidy) but there are many copies of the chloroplast genome in every chloroplast, and often many chloroplasts per cell, so that there can be thousands of copies of the plastid genome in a cell (Bendich, 1987). This number also varies during development, with few copies in meristematic cells and a large increase during leaf development. The coordinate expression of the two genomes must compensate for this variation in gene dosage.

The biogenesis of plastids requires the coordinate expression of genes in the nucleus and in the chloroplast, and probably also in the mitochondria. Schematically, coordinate expression can be achieved in two general modes. In the first mode, the different compartments independently perceive developmental or environmental cues, transduce the signals, and respond (Fig. 1). Coordination will result from the independent implementation of appropriate responses to the developmental and environmental parameters in the separate compartments. Increased understanding of gene regulation in different compartments contributes to our comprehension of this type of coordination by comparing the responses to a given stimulus. There have been considerable advances in dissecting gene expression and its regulation in the nucleocytosol (Gallie, 1993; Bowler and Chua, 1994; Terzaghi and Cashmore, 1995) and in the plastids (Rochaix, 1992, 1996; Mullet, 1993; Gruissem and Tonkyn, 1993; Mayfield *et al.*, 1995).

In the second mode, a signal is perceived in one compartment which then regulates its own response but also governs the response in the other. In the interactions between the nucleus and the plastid, this type of coordination is in part a corollary of the fact that many plastid proteins involved in gene expression are actually encoded in the nucleus and imported, so that in this sense the plastids are largely controlled by the nucleocytosolic compartment (Fig. 1). Even though these imported factors may be acting entirely within the plastid, their synthesis is placed under regulatory circuits of the nucleus and cytoplasm. But it is also possible that a signal from a transduction chain or a metabolic pathway is transmitted from one compartment to the other. It is mainly this second mode of control which will be the subject of this review, focused on the molecular genetics of nuclear mutants that affect plastid gene expression and on the biochemistry of the nucleus-encoded factors that are involved.

A. Why Is There a Genetic System in Plastids?

Although plastids of land plants and green algae have very similar gene sets, there is more variation when more distant algae are compared (Palmer, 1991; Reith, 1995; Reardon and Price, 1995). The selective pressure for the maintenance of an active genetic system in the chloroplast is not understood, and the rules that govern which genes are retained in the organelle genome and which ones become established in the nucleus are not known. An obscuring factor in this respect is that serendipity may have influenced the process to an unknown extent. The plastid genome of land plants is apparently mainly dedicated to the expression of components of the photosynthetic apparatus. Plant cells with plastids that have lost all their ribosomes, and thus cannot express the plastid genome, are bleached for lack of pigment-containing proteins and are nonphotosynthetic, but are nevertheless viable if they are fed by other parts of the plants such as seed reserves in seedlings, green sectors in striped plants, or by the medium in cell or tissue culture. The plastidic compartment is still maintained, implying that its propagation does not require plastid gene expression.

The unicellular alga *Chlamydomonas reinhardtii* is a facultative phototroph that can also be maintained on acetate in the dark. Thus many mutants of *C. reinhardtii* that completely abolish photosynthesis have been recovered (see below), but mutations that affect the expression of all chloroplast genes, for example, mutations in ribosomal protein genes, always have leaky phenotypes and allow some reduced level of expression. Attempts to inactivate chloroplast RNA polymerase genes by site-directed mutagenesis have also indicated that chloroplast transcription is essential for the viability of this alga (Fischer *et al.*, 1996; Rochaix, 1995). The basis for this difference with plant cells which do not strictly require plastid gene expression has not been established, but it could be that some of the genes that are present on the algal chloroplast genome but not in the plant plastid genome are essential for the survival of the cell. Another explanation could be that in both cases the plastids encode an enzyme that is involved in producing a metabolite that the plant cells can import but not *C. reinhardtii.*

In other more distantly related algae, the chloroplast genome contains more genes (Reith, 1995; Reardon and Price, 1995). They encode polypeptides with other functions, for example, enzymes of lipid and amino acid metabolism, or chaperonins involved in protein folding. Some of the "extra" plastid genes encode certain subunits of the photosynthetic complexes that are products of nuclear genes in vascular plants. Here again, it is unclear how much selective pressure (functional constraints) and stochastic events have contributed to these differences.

An example which illustrates the selective pressure that is exerted to maintain a functional genetic system in the organelle is provided by the

analysis of the plastid genome in the nongreen parasitic plant *Epifagus virginiana* (Wolfe *et al.*, 1992). In this heterotrophic plant, the photosynthetic genes have been lost by mutation and deletion but many genes for components of the plastid translation machinery have been retained in functional form. Some function(s), other than photosynthesis, is apparently encoded at least in part in the plastid and seems essential for viability.

While the requirement for the maintenance of a plastid organelle might be understood in terms of compartmentalization of biochemical pathways, the requirement for a separate plastid genetic system is still unclear: why have all genes not been transferred to the nucleus (Bogorad, 1975)? One explanation could be that there are polypeptides that cannot be imported across the plastid envelope and therefore have to be encoded and expressed in the organelle. This hypothesis has been tested for the large subunit of ribulose–bisphosphate carboxylase oxygenase (LS of Rubisco) which is encoded in the plastid genome by the *rbcL* gene. The enzyme is composed of 8 small subunits (SS), which are encoded by a gene family in the nucleus (*rbcS*) and imported into the chloroplast, and 8 large subunits to form a complex of 16 subunits (Rubisco form I, LS_8SS_8). A site-directed chloroplast mutant of tobacco was first obtained with a knock-out of the *rbcL* gene (Kanevski and Maliga, 1994). This pale, nonphotosynthetic mutant was transformed in the nucleus with a construct to express the *rbcL* gene with a transit peptide for chloroplast targeting (allotypic expression; Nagley and Devenish, 1989). The transformed lines were green in culture and accumulated Rubisco, albeit to reduced levels, and some were capable of slow photosynthetic growth in the greenhouse. So it appears that the Rubisco large subunit can be imported across the envelope into the organelle, as had been suggested by the fact that it can be imported into yeast mitochondria (Hurt *et al.*, 1986), and it remains unclear why the *rbcL* gene is retained in the plastid while the *rbcS* genes are nuclear. In Dinoflagellates, a different type of Rubisco with only a large subunit (L_2 or form II) is encoded in the nuclear genome and imported into the plastid (Morse *et al.*, 1995; Rowan *et al.*, 1996).

A variation of the previous hypothesis proposes that the polypeptides than cannot be imported are those which have too many hydrophobic transmembrane helices (more than three) (Popot *et al.*, 1994). An example could be the D1 polypeptide of PSII which is imbedded in the thylakoid membrane with five transmembrane segments and is always encoded in the plastid genome by the *psbA* gene. Allotypic expression in the nucleus of an *Amaranthus psbA* gene with a mutation for atrazine resistance confers tolerance to this herbicide in tobacco, but only transiently (Cheung *et al.*, 1988). Low levels of the modified gene product can be detected immunologically in thylakoid membranes. It is not clear whether these low levels of expression reflect problems with protein import, defects in other aspects

of transgene expression, or competition with the endogenous D1 because the resident *psbA* gene is still present in the transformed plants. Although this experiment shows that the five transmembrane helices do not completely preclude translocation, it might be interpreted to indicate that the helices still significantly interfere with the import process.

It has also been suggested that it is for regulatory reasons that some genes are located in the plastid. Sensing of the redox state in the organelle and regulation of the expression of relevant genes might best function if maintained within the same cellular compartment, thus minimizing the signal transduction chain (Allen, 1993). Another possibility is that the persistence of a plastid genetic system could just be an evolutionary happenstance, that we are contemplating snapshots of a continuing process. This is a question that remains unresolved.

B. Technical Developments in Molecular Genetics

In recent years, several technical developments have enhanced our approaches to unravel nuclear–chloroplast interactions. Stable transformation of the plastid genome has become possible using particle-gun bombardment with micron-sized projectiles carrying transforming DNA. This technique, first used in *C. reinhardtii* (Boynton *et al.*, 1988), has also been applied to tobacco (Svab *et al.*, 1990). The high level of homologous recombination in the plastids and the development of dominant selectable markers allow the site-directed mutagenesis of the plastid genome or the introduction of chimeric reporter genes to study cis-acting elements involved in chloroplast gene expression.

Other advances have come from the use of insertional mutagenesis as a tool for the molecular cloning of nuclear genetic loci. If a mutation is caused by the insertion of a known DNA element for which a molecular probe is available, then the genetic locus is tagged and can be subjected to molecular analysis and recovered by cloning (Walbot, 1992). This has been used to clone nuclear genes involved in chloroplast function, which will discussed in this review. One approach uses endogenous transposable elements, such as Robertson's mutator (Mu) or Ac in maize (Martienssen *et al.*, 1989; Han *et al.*, 1992; Schultes *et al.*, 1996; Voelker *et al.*, 1997) or Tam3 in *Anthirrinum* (Chatterjee *et al.*, 1996). The Ac/Ds transposons from maize have also been used to mutate and clone genes in other plants, as illustrated by the cloning of *DAG* in tomato (Keddie *et al.*, 1996). Another approach is to use transformation as the insertional mutagen: T-DNA tagged mutants can thus be obtained and cloned in *Arabidopsis thaliana* (Koncz *et al.*, 1990; Deng *et al.*, 1992; Ahmad and Cashmore, 1993; Castle and Meinke, 1994; Reiter *et al.*, 1994; Wei *et al.*, 1994; Grevelding *et al.*, 1996; Szekeres *et al.*, 1996). A

similar strategy is possible in *C. reinhardtii* where transforming DNA also apparently integrates at many sites in the nuclear genome, generating mutations with a variety of phenotypes including defects in chloroplast gene expression (Gumpel and Purton, 1994). The use of mapped molecular markers has also made it possible to obtain nuclear genes of *Arabidopsis* by positional cloning (Pepper *et al.,* 1994; Li *et al.,* 1996).

II. Classical Genetics of Nuclear–Plastid Interactions

Early studies with mutants in many plant species have established the basic principles that govern plastid inheritance and interactions between genomes of organelles and of the nucleus (Kirk and Tilney-Bassett, 1978; Gillham 1978, 1994). More recent investigations at the molecular level have generated new insight which will be highlighted here by a few selected examples.

A. Plastid Inheritance

While the nuclear genome is inherited according to Mendelian rules, transmission of the organelle genomes deviates. The study of this "cytoplasmic" inheritance founded the genetics of plastids and mitochondria (Gillham, 1978, 1994; Gillham *et al.,* 1991; Boynton *et al.,* 1992; Hagemann, 1992). However, the use of the term "cytoplasmic" will be avoided in this review to prevent any confusion with the nucleocytosolic compartment where the nuclear genes are expressed. In many plants, the plastid genome is inherited principally or exclusively from one parent (uniparental inheritance, often maternal but also paternal), but in some plants from both (biparental inheritance). The basis of plastid inheritance has been the subject of several reviews (Kirk and Tilney-Bassett, 1978; Sears, 1980; Gillham *et al.,* 1991; Hagemann, 1992). In some cases, plastids are excluded from gametes; in others they are inactivated or lost at a later stage. An extreme case is found in the green alga *C. reinhardtii:* chloroplasts of the two gametes fuse after zygote formation, but the plastid genomes from the mating-type minus parent are first selectively degraded. In a small fraction of zygotes, biparental inheritance and recombination of parental markers are observed so that genetic maps can be derived. The inheritance of the plastid genome is governed by the nuclear mating-type locus, and genes that play a role in this mechanism have recently been identified (Armbrust *et al.,* 1993; Goodenough *et al.,* 1995).

B. Nuclear–Plastid Compatibility

A classical example of nuclear–plastid interaction was provided by studies on *Oenothera* (Stubbe, 1989). Some crosses between different species result in variegated plants with deficient plastids. This depends on the particular combination of nuclear and plastid genomes. A given plastid genome will give rise to normal green chloroplasts in a compatible nuclear background, but to deficient plastids in a different nuclear background. In the latter background, however, another compatible plastid genome will give rise to normal chloroplasts. When crossed from an incompatible background back into a compatible one, the plastid again gives rise to normal chloroplasts, showing that no genetic change is induced in the incompatible combination. *Oenothera* have particular genetic features that have allowed these observations: plastids are transmitted biparentally, but principally from the mother, giving rise to variegated plants that differ in reciprocal crosses (hence the name of hybrid variegation given to this phenomenon of incompatibility). Furthermore, the nuclear chromosomes do not assort independently in meiosis. Because of a series of translocations they form rings at meiosis I in heterozygous plants, and the chromosomes from one parent then segregate together, forming defined haploid sets which are transmitted as such (Kirk and Tilney-Bassett, 1978). Three types of haploid nuclear genomes (six diploid combinations) and five types of plastid genomes were identified in the subgenus *Euoenothera,* depending on their compatibilities and the degree of the resulting phenotype and its developmental features (Stubbe, 1989). Mutations in the chloroplast and nuclear genomes must have evolved in concert to maintain viability, highlighting the requirement for a close functional integration of the two genomes.

Beyond the interesting formal concepts that these incompatibilities reveal comes the question of the molecular basis for the different genotypes and their interactions. What are the relevant changes in one genome, and how are they paralleled in the other genome? Molecular analysis of plastid DNA shows many restriction-fragment-length polymorphisms between the five different plastid types, in correlation with the genetic assignments and thus confirming them (Gordon *et al.,* 1982). Sequencing of selected fragments reveals changes such as point mutations, short duplications, and variation in the length of A-tracts within intergenic regions, but the coding regions are conserved (Wolfson *et al.,* 1991; Johnson and Sears, 1990b). Although the CP47 polypeptide of photosystem II has a different electrophoretic mobility in different plastid types, the coding sequences are identical, implying that this electrophoretic variation is due to posttranslational modification (Offermann-Steinhard and Herrmann, 1990). However, a size difference in one large open reading frame (ORF2280) is due to variation in the number of repeats in a tandem array (Glick and Sears, 1993). Differences

in a variable spacer between the 16S rDNA and *trnI* are mostly due to insertions and duplications (Hornung *et al.,* 1996). The characterization of these differences between the different plastid types at the molecular level bring to light a major hurdle to understand nuclear–plastid compatibility in *Oenothera:* the relevant, causative differences between plastid types will be difficult to identify in a background of polymorphic, probably neutral sequence variation.

The compatibility or incompatibility of certain combinations of nuclear genomes and organelles is also observed in many experiments with cybrids (Perl *et al.,* 1991). An example is provided by those obtained in protoplast fusions of *Atropa belladona* and *Nicotiana tabacum* (Kushnir *et al.,* 1991). The *Nicotiana* plastids in the *Atropa* background are chlorophyll deficient, in contrast with the reciprocal combination where the *Atropa* plastids are green in the *Nicotiana* background. In subsequent fusions, the deficient cybrids give rise to green combinations when the *Nicotiana* plastids are returned to the *Nicotiana* background or when *Atropa* plastids are reintroduced in the *Atropa* context, showing that their competence for a compatible interaction has not been lost in the procedure. Experiments of this type illustrate the constraints for genetic cooperation of the nucleus and the organelles (plastids and mitochondria), but here again it would be difficult to pinpoint the relevant differences among the many silent genetic changes that have accumulated between the parental strains.

During evolution, the appearance of certain mutations in one compartment must have required corresponding changes in the other compartment so as to maintain a functional interaction. This relation is reminiscent of the mutations and their suppressors, some of them allele specific, that have been valuable genetic tools for studying interacting proteins. Such suppressors sometimes have a defective phenotype of their own in the absence of the initial mutation. Similarly, the coordinate changes in the nucleus and the chloroplast genome can be expected to eventually lead to an incompatibility with the original genotypes. The plastid genome evolves relatively slowly (Palmer, 1992), and compatibility can be retained for some relatively close species, but incompatibility becomes more probable with increasing phylogenetic distance.

C. Interactions with Mitochondria

Some mutants that have variegated or striped phenotypes with pale, yellow, or white sectors have revealed an interesting interaction between mitochondria and plastids. Well-characterized examples are provided by some of the maternally inherited NCS (nonchromosomal stripe) mutants of maize. The NCS2 mutant exhibits reduced growth, pale-green leaf stripes, and

sectors of aborted kernels on the ears. In this mutant, a rearrangement in the mitochondrial genome cosegregates with the defective phenotype, suggesting that the primary mutation is actually in the mitochondrion. In the affected sectors both the mitochondria and the plastids are abnormal by a number of morphological and functional criteria (Roussell *et al.*, 1991). A similar interaction seems to prevail in the NCS5 and NCS6 mutants which also have reduced height, yellow stripes on the leaves with abnormal plastids, and sectors of aborted kernels. In stripe tissue from NCS5 and NCS6, segments of the cytochrome oxidase subunit 2 gene (*Cox2*) are deleted in the mitochondrial genome (Newton *et al.*, 1990; Gu *et al.*, 1993).

The nuclear *chm* (chloroplast mutator) mutants of *Arabidopsis* have pale or white sectors with deficient plastids. The variegation is induced at high frequency in the homozygous *chm* background and is subsequently inherited maternally. Cells with mixed populations of plastids having different ultrastructural defects are observed (Mourad and White, 1992). The plastid phenotype becomes stable when wild-type *CHM* function is restored by crossing, and plants with morphologically homogeneous populations of plastids (homoplastidic) can then be derived. However, it is in the mitochondrial genome of variegated *chm* plants that specific new restriction fragments have been observed, indicative of DNA rearrangements (Martinez-Zapater *et al.*, 1992). These new mitochondrial DNA restriction fragments are inherited maternally and cosegregate with the variegated phenotype. The same novel restriction fragments are observed with different *chm* alleles. The MDL (maternal distorted leaf) mutant was derived from a cross of *chm* to wild type and exhibits poor growth, distorted leaves, and aborted floral organs (Sakamoto *et al.*, 1996). In MDL, the mitochondria have abnormal ultrastructure and the mitochondrial DNA contains rearrangements. The rearranged genomes can be detected in low amounts in wild type, but are predominant in the mutant, suggesting that the *CHM* locus could be involved in the maintenance of the wild-type master mitochondrial genome. It is thus possible that the primary effects of mutations at this nuclear locus are with the mitochondrial genome and that the defects in plastid development may be an indirect consequence.

Although it is difficult to completely rule that there could also be a change in the plastid genomes of these mutants, the cosegregation of the phenotype with the mitochondrial marker would imply that mutant chloroplasts and mitochondria somehow always cosegregate, and wild-type chloroplast and mitochondria likewise. It seems more likely that plastid development is dependent on normal mitochondrial function, so that the plastids become abnormal as a secondary consequence of impaired mitochondrial function. Either alternative is obviously interesting, and the physiological and molecular basis for this relationship remains to be investigated. Experiments with *C. reinhardtii* mitochondrial mutants resistant to mixothiazol

have shown that mitochondrial respiration can influence the electrochemical gradient across the chloroplast thylakoid membrane and the redox state of the plastoquinone pool (Bennoun 1994). It may be that metabolic interactions of this kind play an essential role at some stages of plastid development and that the defective mitochondria in the mutant sectors of the striped plants cannot fulfill this requirement.

These observations also raise the possibility that some of the incompatible interactions between nuclear and plastid genomes (described above) are actually due to a primary defect in the interaction with mitochondria and that this secondarily affects the plastids.

D. Genetic Analysis

In order to avoid the problems of the numerous polymorphic genetic differences that have accumulated in the different species that were used in the studies of nuclear–plastid compatibility, it is necessary to isolate mutants in a defined genetic background. A direct genetic approach to identify mutations affecting nuclear–plastid interactions has been taken in a number of plants and algae. Because of the central role of plastids in plant metabolism, these mutants are often not viable or fertile, but can be maintained as heterozygotes. Homozygous seedlings showing the mutant phenotype can be analyzed while they temporarily strive on seed reserves or grow on synthetic media. Mutant tissues can also be propagated in culture with the appropriate medium. In some cases the mutations are expressed in sectors or in chimeras with the normal tissue feeding the mutant one. The green alga *C. reinhardtii* is a facultative phototroph that can be maintained on acetate medium in the dark, in the absence of photosynthesis.

1. Nuclear Mutants with Altered Plastids

Many nuclear mutations that alter plastid pigmentation have been studied. Some of these mutants have defects in pigment biosynthesis (von Wettstein *et al.*, 1995; Bartley *et al.*, 1994) and will not be discussed here except for one aspect, the fact that pigment deficiency can influence plastid or nuclear gene expression (see section V). In many cases, impaired expression of plastid genes, or of nuclear genes implicated with the plastids, can indirectly cause the lack of pigment accumulation. Thus altered pigmentation is an easily scored, but also sensitive, monitor of plastid function.

The plastome mutator (*pm*) of *Oenothera* is a recessive nuclear mutation that induces a high frequency of mutations in the chloroplast genome which appear as chlorotic sectors (Sears and Sokalski, 1991). The plastid mutations are subsequently inherited in non-Mendelian fashion, independent of the

pm mutation. They show a variety of phenotypes (Johnson and Sears, 1990a,b; Johnson *et al.*, 1991). The *pm* mutation also induces DNA polymorphisms, due to deletions in specific regions of the genome, at least one of which contains a variable number of short direct repeats (Chiu *et al.*, 1990; Blasko *et al.*, 1988). In the nuclear *pm* mutant a defect in chloroplast DNA replication or recombination could thus explain the appearance of both silent DNA polymorphisms and chlorotic plastid mutations.

A different situation seems to prevail in some nuclear mutants like *iojap* in maize and *albostrians* in barley, which have defective plastids that appear in sectors or stripes of tissue (Börner and Sears, 1986). Unlike the *pm* mutant of *Oenothera*, these mutants only induce one type of plastid phenotype. The deficient plastids can be maternally transmitted in crosses to yield some completely white seedlings, even when the wild-type function of the nuclear gene is restored in the progeny (from the paternal parent). The affected plastids have abnormal, undifferentiated morphology and contain no detectable ribosomes (Taylor, 1989). In *iojap* plastids many transcripts are affected in their processing and abundance, and many polypeptides are missing (Han *et al.*, 1993). This does not seem to occur as a result of mutations in the plastid genome induced by a nuclear "mutator," the *iojap* sectors are not randomly distributed, the patterns cannot be explained by clonal cell lineages passing from leaf to leaf, and they are influenced by developmental parameters (position in the leaf) and genetic background (Han *et al.*, 1992). This contrast between the maternal inheritance of the deficiency and the appearance of nonclonal stripes is explained by the following hypothesis. The defect may be due to a sort of vicious circle: once established, a general defect in chloroplast gene expression cannot be reversed (Walbot and Coe, 1979; Börner and Sears, 1986). This is because the plastid genome encodes the rRNAs, tRNAs, some of the ribosomal proteins, and subunits of RNA polymerase which are essential for expression of the plastid genome. As a consequence, if a block leads to an arrest of chloroplast gene expression, this cannot be restored even when the block is removed. Such a block could be caused by a defective nuclear gene involved in plastid gene expression and be influenced by developmental parameters. Han *et al.* (1992) point out that cells of the leaf margins, which are more subject to striping, also divide and expand faster and thus could depend more stringently on the proper expression of the *Iojap* product for plastid development. An *iojap* allele tagged with the transposable element *Mu1* (Robertson's mutator) has been cloned, and the wild-type *Iojap* DNA sequence has been determined (Han *et al.*, 1992). The deduced polypeptide sequence (25 kDa) does not have homology to known proteins. By using antibodies against Iojap protein produced in *Escherichia coli*, Han and Martienssen have found that the Iojap polypeptide is located in the chloroplast, apparently associated with the 50S ribosomal subunit (personal communication). This is in agree-

ment with the hypothesis that the nuclear *Iojap* gene encodes a factor required for chloroplast gene expression.

2. Photosynthetic Mutants

Photosynthetic deficiency is another phenotype that has been extensively investigated. Defects in photosynthetic electron transport often result in altered chlorophyll fluorescence, a phenotype that can readily be screened nondestructively. High-chlorophyll-fluorescence mutants (*hcf*) have been studied in many plants, most prominently maize (Miles, 1994; Barkan *et al.,* 1995) and *Arabidopsis,* and in algae such as *C. reinhardtii* (Bennoun and Delepelaire, 1982). In *C. reinhardtii,* which can be maintained heterotrophically on acetate, conditional mutants that are acetate-dependent also present a broad range of photosynthetic defects. The mutants include those affected in photosynthetic electron transport, but also in other parts of the photosynthetic apparatus such as the chloroplast ATP synthase or the enzymes of the Calvin cycle. Some mutations alter genes for components of the photosynthetic machinery directly. More pertinent to this review, there are also many mutations that affect different aspects of plastid gene expression. These mutations will be discussed in later sections bearing on the different steps of gene expression that are altered.

III. Plastid Development

The differentiation of proplastids into specialized forms, and in particular the development of chloroplasts, is coordinated with the differentiation of the cells and the tissues in which they reside. This process appears to be largely governed by the nucleocytosolic compartment. For instance, proplastids will form chloroplasts in some leaf cells, but not in the vascular tissue. Conversely, cell development and morphogenesis are not grossly affected in plants with deficient plastids, for example, in albino seedlings or following photooxidative destruction (see section V). However, the phenotypes of some novel mutants raise the possibility that chloroplast development is required for the proper differentiation of cells in the palisade layer of the leaf.

A. Plastid Differentiation

Chloroplast development seems to be arrested at a very early stage in the *dcl* (defective chloroplasts and leaves) mutant of tomato (Keddie *et al.,*

1996) and in the *dag* (differentiation and greening) mutant of *Antirrhinum* (Chatterjee *et al.*, 1996). Both mutations are due to transposable element insertions and are unstable, giving variegated plants. In the mutant sectors, the plastids do not differentiate to form chloroplasts but retain the size and morphology of proplastids. If the *dag* mutant is grown in the dark, etioplast development is likewise impaired. The effect of the mutations is cell autonomous, since single cells can revert to wild type. Both mutants also have a defect in palisade cell development in the leaf: this distinct layer of elongated, columnar cells is not formed in the mutant sectors. This dual phenotype suggests that chloroplast development may be necessary at an early stage for differentiation of palisade cells. The mRNA for *rpoB* (which encodes a subunit of plastid RNA polymerase) is expressed very early in development in the wild type. It is accumulated to higher than wild-type levels in the *dcl* mutant, but is not detectable in *dag* mutant cells. The *DAG* and *DCL* genes have been isolated and found to encode two different, novel polypeptides. Both have predicted transit peptides and can be imported *in vitro* into isolated chloroplasts. The difference in *rpoB* gene expression and the lack of sequence similarity between the two genes suggest that *DAG* and *DCL* are involved in different aspects of early chloroplast development.

In the *pac* (*pale cress*) mutants of *Arabidopsis,* proplastids or etioplasts undergo only the initial stages of differentiation but are then arrested and form only few thylakoid lamellae (Reiter *et al.*, 1994; Grevelding *et al.*, 1996). The *pac* mutations also affect leaf development: the palisade layer does not differentiate. Thus the phenotype of *pac* is reminiscent of *dag* or *dcl,* but arrest occurs at a later stage. This later arrest could explain the fact that the expression of the nuclear *Cab* genes, which requires a chloroplast signal (see below), is blocked in *dag* and *dcl* but not in *pac.* The *pac-2* phenotype can be rescued by cytokinins, which induce greening of mutant plants. It is interesting that cytokinins can also promote plastid development and deetiolation in dark-grown seedlings (Chory *et al.*, 1994; see below).

It is intriguing that the palisade layer is specifically affected in these mutants. The development of the palisade layer is also affected in the mitochondrial mutants NCV of tomato (nonchromosomal variegation) and A-1b of tobacco (Bonnema *et al.*, 1995; Bonnett *et al.*, 1993). Both of these were obtained as cybrids, show pale green variegation, and contain rearranged mitochondrial genomes. In the mutant sectors the mitochondria have abnormal morphology and the palisade layer fails to differentiate. The *dag* and *dcl* mutants have apparently normal mitochondria, so it seems that palisade cell differentiation is particularly dependent on both mitochondrial and plastid function.

In the *cue1* (*CAB underexpressed*) mutant of *Arabidopsis,* there is a defect in the expression of light-regulated nuclear genes involved in photo-

synthesis, as well as of the plastid genes *psbA* and *rbcL* (encoding the D1 subunit of PSII and the large subunit of Rubisco; Li *et al.*, 1995). The mesophyll cells are specifically affected in this mutant, and they are also smaller and contain smaller chloroplasts than the wild type. Gene expression and morphology are normal in the bundle-sheath cells surrounding the veins, giving rise to a reticulate pattern of greener tissue. The *CUE1* gene is apparently involved in cell-type-specific, light-regulated gene expression during development.

Another class of *Arabidopsis* mutants reveals nuclear genes that are involved in chloroplast division. The *arc mutants* (*accumulation and replication of chloroplasts*) belong to five loci and have altered numbers of chloroplasts per cell (Pyke and Leech, 1992, 1994; Pyke *et al.*, 1994). The changes in chloroplast number are compensated by changes in chloroplast size: in *arc1* there are more chloroplasts but they are smaller, while in *arc2, arc3, arc5,* and *arc6* there are fewer but larger chloroplasts. In the most extreme mutant, *arc6,* there are on average 2 chloroplasts instead of 90 per mesophyll cell, and these are 20 times larger than normal. Proplastids in meristematic tissue and plastids in the roots are also affected, and the differentiation of plastids and amyloplasts is altered in *arc6* (Robertson *et al.*, 1995). The *arc* mutant plants grow essentially normally and are fertile, although leaves of *arc6* have altered morphology. It seems likely that the *arc* mutations affect nuclear genes involved in plastid division or its control. They raise interesting questions on the relation between plastid division and plastid differentiation.

B. Photomorphogenesis

Light has profound effects on plant growth and development (Kendrick and Kronenberg, 1993). Plants sense and respond to the intensity, the spectral properties, the direction, and the daily time distribution of light. The response of plants to light is a vital adaptation to their environment, since light is their source of energy. Not surprisingly, plastids are profoundly involved in the process. Some of these responses are strikingly apparent in developing seedlings: in the light, proplastids differentiate into chloroplasts which have abundant thylakoid membranes, partly stacked to form the grana, and are active in photosynthesis. In dark-grown seedlings, a different developmental pathway leads to etioplasts which have few thylakoid membranes, but accumulate paracrystalline arrays, the prolamellar bodies. If they are then exposed to light, etioplasts rapidly differentiate into chloroplasts.

When germinating seedlings are exposed only to far-red light, plastid development is arrested, etioplasts are not formed, and subsequent greening

upon transfer to white light does not occur (Runge *et al.*, 1996; Barnes *et al.*, 1996). This block, which is dependent on the phytochrome A signaling pathway, is also accompanied by the lack of the major etioplast protein protochlorophyllide oxidoreductase (PorA) and of its mRNA. PorA accumulates in etioplasts as a complex with its substrates (protochlorophyllide and NADPH), and upon illumination catalyzes chlorophyllide formation but is rapidly degraded (Reinbothe and Reinbothe, 1996; Reinbothe *et al.*, 1996a). PorB is thought to ensure chlorophyll synthesis in green plants. The correlation between lack of PorA and failure to green is shown to actually represent a causal relationship by overexpressing PorA in transgenic *Arabidopsis*. In spite of the far-red light treatment, the PorA-overexpressing seedlings largely regain the ability to green in white light (Runge *et al.*, 1996). These experiments indicate an essential role for PorA during greening, probably to allow rapid chlorophyll synthesis and prevent light-induced photodamage by protochlorophyllide (Reinbothe et al., 1996b).

In dark-grown seedlings of dicots like *Arabidopsis*, the overall morphology of the plantlet is also different: the hypocotyl is elongated and forms an apical hook, and the cotyledons are small and unexpanded. Conversely, in the light, the features of photomorphogenesis include restricted hypocotyl elongation, unfolding of the apical hook, cotyledon expansion, and development of proplastids into chloroplasts (McNellis and Deng, 1995). Genetic screens in *Arabidopsis* based on such morphological differences have led to the identification of two types of mutants: those that develop in the light with some phenotypic properties of dark-grown seedlings (*hy*, long hypocotyl; *fre* and *fhy*, far-red elongated hypocotyl; *blu*, blue light uninhibited) and those that develop in the dark with aspects of light-grown seedlings (*det*, deetiolated; *cop*, constitutively photomorphogenic) (Chory, 1993; Deng, 1994; Bowler and Chua, 1994; Millar *et al.*, 1994; Short and Briggs, 1994; Quail, 1994; Quail *et al.*, 1995; Wei and Deng, 1996; Barnes *et al.*, 1997).

The *hy*, *fhy*, and *blu* mutants are apparently impaired in the perception of light and in the transduction of light signals. The *hy8*, *fhy2*, and *fre1* mutations affect the *PHYA* gene and the *hy3* mutations affect *PHYB*. These loci encode the apoproteins of phytochrome A and B, two members of the well-studied family of receptors for far-red and red light (Furuya, 1993; Quail, 1994; Quail *et al.*, 1995). Mutants of *HY1*, *HY2*, and *HY6* are defective in the synthesis of the phytochrome chromophore and are generally deficient in all types of phytochromes. The HY4 (*CRY1*) gene probably encodes a blue light receptor, *HY5* may be implicated in signal transduction from phytochromes, and the blue light receptor, *FHY1* and *FHY3*, in signaling from phytochrome A.

Mutants of the other type, *det* and *cop*, appear to undergo photomorphogenesis in the dark; some of them also accumulate high levels of anthocya-

nin, unlike dark-grown wild-type seedlings. Because these mutations are recessive, the wild-type *COP* and *DET* genes are probably required to repress photomorphogenesis in the dark. Some of the *fus* (fusca) mutants which have purple cotyledons (they accumulate anthocyanin) and reduced cell expansion also share other phenotypes of derepressed photomorphogenesis with the *det* and *cop* mutants (Miséra *et al.*, 1994; Castle and Meinke, 1994; Kwok *et al.*, 1996). Indeed, genetic analysis has shown that several of the *FUS* and *DET* or *COP* loci are identical (McNellis and Deng, 1995; Kwok *et al.*, 1996). Other dwarf mutants with reduced cell expansion such as *dim* (*diminuto*) and *cpd* (*constitutive photomorphogenesis and dwarfism*) also display aspects of photomorphogenesis in dark-grown seedlings (Takahashi *et al.*, 1995; Szekeres *et al.*, 1996).

Of particular interest for the topic of this review, in some of the *det/cop/fus* mutants the plastids do not develop as etioplasts in the dark, but more like chloroplasts with thylakoid membranes (light is required for chlorophyll synthesis, and chlorophyll is required for the assembly of the photosynthetic complexes, so it is not unexpected that differentiation is incomplete in the dark). This indicates that the *COP/DET/FUS* loci are controlling not only the morphology of the seedling, but also the developmental fate of plastids. There are 10 genes which belong to this pleitropic *COP/DET/FUS* class (Kwok *et al.*, 1996). Nuclear genes for chloroplast proteins are transcribed in the pleiotropic mutants grown in the dark, and the chloroplast *psbA* mRNA is accumulated, showing that at least this plastid gene is under *COP/DET/FUS* control. The control is probably indirect since there is so far no evidence of *COP/DET/FUS* gene products in the plastid. The products of several of these genes are nuclear and may be involved in transcriptional repression: the product of *DET1* is localized in the nucleus, the product of *COP1* is nuclear in the dark but cytoplasmic in the light, and the products of *COP9* and *FUS6/COP11* form part of a large nuclear protein complex (von Arnim and Deng, 1996; Wei and Deng, 1996).

Unlike those of the pleiotropic class, other mutants have a more restricted phenotype: *det2* seedlings accumulate nucleus-encoded mRNAs for chloroplast proteins and express chloroplast mRNAs in the dark; however, they develop normal etioplasts (Chory *et al.*, 1991). Although the dark-grown *det3* and *dim* mutant seedlings have deetiolated morphology, they contain normal etioplasts and show repressed expression of light-inducible nuclear and chloroplast mRNAs (Cabrera y Poch *et al.*, 1993; Takahashi *et al.*, 1995). Thus different aspects of photomorphogenesis may require different subsets of genes that belong to branched developmental pathways. These different types of mutants also show that plastid gene expression and plastid development are not necessarily always coupled. In the *cue1* mutant, light-regulated gene expression of nuclear and plastid genes is affected during development in a cell-specific pattern: the defect is apparent in mesophyll

but not in bundle-sheath cells (Li *et al.,* 1995; see section III.A). Another interesting effect of the pleitropic *det/cop/fus* mutations is that, in the light, chloroplasts rather than amyloplasts develop in the roots, mostly in the vascular regions. This implies that in the wild type *COP/DET/FUS* genes are involved in redirecting plastid development toward amyloplasts instead of chloroplasts, the latter presumably being the default pathway.

Similar mutants have also been identified in other plants. The *pew1* and *pew2* mutants (*partly etiolated in white light*) of *Nicotiana plumbaginifolia,* the *aurea* and *fri* (*far-red light insensitive*) mutants of tomato, and the *lh* (*long hypocotyl*) mutant of cucumber are defective in light perception by phytochrome (Kraepiel *et al.,* 1994; van Tuinen *et al.,* 1995; Sharma *et al.,* 1993; Lopez-Juez *et al.,* 1992). In pea, the *lip1* (*light-independent photomorphogenesis*) mutant seedlings have deetiolated morphology and contain partly developed chloroplasts when grown in the dark (Frances *et al.,* 1992).

Plant hormones also play a role in photomorphogenesis and the control of chloroplast development. For instance wild-type *Arabidopsis* seedlings grown in the dark in the presence of cytokinins have the same photomorphogenetic features as *det1* mutants; their plastids differentiate as chloroplasts and nucleocytosolic light-regulated mRNAs are expressed (Chory *et al.,* 1994). The *pac* mutants (*pale cress*) have a defect in plastid development resulting in white plants (see section III.A; Reiter *et al.,* 1994; Grevelding *et al.,* 1996), and the phenotype of *pac-2* can be rescued by treatment with cytokinins. Brassinosteroids can restore the defective hypocotyl elongation of *cpd, dim,* and *cop/fus/det* mutants in the dark (Szekeres *et al.,* 1996). The *DET2* gene probably encodes an enzyme involved in brassinosteroid synthesis (Li *et al.,* 1996). Thus these plant steroid hormones may play a role in the branch of the photomorphogenetic response requiring *DET2, DIM,* and *CPD,* which mainly affects seedling morphology and light-regulated nuclear gene expression rather than plastid development. The effects of the plant growth regulators, and some of the phenotypes like root chloroplast development in the *cop/det/fus* pleiotropic mutants, raise interesting questions about the role of genes in other control networks and of whether they are primarily or indirectly involved in light signal transduction (Millar *et al.,* 1994; Barnes *et al.,* 1997). The possible involvement of some *COP/DET/FUS* genes in other signal transduction pathways is also suggested by the finding that genes involved in other responses are derepressed in *det1, cop1,* and *cop9* plants (Mayer *et al.,* 1996).

Another fruitful approach to unravel signal transduction between light perception and chloroplast development is based on microinjection into cells of the *aurea* mutant of tomato (Neuhaus *et al.,* 1993; Bowler *et al.,* 1994; Bowler and Chua, 1994; Elich and Chory, 1994; Barnes *et al.,* 1997). Proplastid differentiation is blocked in the *aurea* mutant, but microinjection of oat phytochrome A rescues normal chloroplast development. The effect

of signaling intermediates, agonists, and inhibitors can also be monitored by injection in the *aurea* cells, or the effects of the drugs can be monitored by treatment of a photomixotrophic soybean cell culture. Two signaling pathways that affect nuclear gene expression have been identified: both involve heterotrimeric G proteins; one entails a calcium signal and calmodulin, while the other involves cGMP. The calcium/calmodulin branch is sufficient to induce the expression of genes required for PSII, ATP synthase, and Rubisco, but the expression of nuclear-encoded PSI and cytb_6f subunits and full chloroplast development require both branches.

These studies, like the genetic approaches, define components of the pathways that regulate plastid development. For some of the nuclear genes that are involved, transcriptional control has been demonstrated using chimeric reporter genes. How the expression of the plastid genome is regulated remains an open question. Although some small signaling molecules could enter the plastid and directly influence gene expression, it is also likely that imported products of nuclear genes are involved.

C. Chloroplast Differentiation in C4 Plants

In plants that fix carbon through the C4 pathway, two specialized cell types cooperate in photosynthesis: the bundle sheath cells and the mesophyll cells, which form two concentric layers around leaf veins (Nelson and Langdale, 1992; Furbank and Taylor, 1995). These two types of cells, as well as the plastids they contain, are morphologically and biochemically different. The mesophyll cells carry out initial CO_2 fixation to form C4 compounds. These are then transported to the bundle sheath cells, where they are decarboxylated to deliver CO_2 which is fixed by Rubisco. The C4 pathway functions as a CO_2 concentrating mechanism and improves photosynthetic efficiency. During leaf development, positional information and light direct the differentiation of the two cell types (Langdale and Nelson, 1991). This involves the specific expression of certain genes, both in the nucleus and in the plastid (Furbank and Taylor, 1995). A prominent example is the *rbcL* gene which encodes the large subunit of Rubisco and is expressed predominantly in the bundle sheath chloroplast. In amaranth, the control seems to be at the level of mRNA stability: although the *rbcL* gene is transcribed in both cell types as measured in a run-on transcription assay, the mRNA only accumulates in the bundle sheath chloroplasts (Boinski *et al.*, 1993). In maize and sorghum, transcriptional and posttranscriptional controls lead to the preferential accumulation of *rbcL* mRNA in the bundle sheath, and of *psbA* mRNA in the mesophyll plastids (Kubicki *et al.*, 1994).

In maize, the *bundle sheath defective* (*bsd1* and *bsd2*) mutations preferentially affect the differentiation of plastids in one of the two cell types. The *bsd1-m1* mutation, an unstable allele generated by transposon mutagenesis, causes abnormal chloroplast differentiation in the bundle sheath cells, but not in mesophyll cells of light-grown plants (Langdale and Kidner, 1994). Bundle sheath cells contain reduced levels of the plastid *rbcL* mRNA and protein, and also of nuclear gene products preferentially expressed in the bundle sheath. In dark-grown seedlings, bundle sheath etioplast morphology is selectively affected, but Rubisco protein is missing from both cell types (unlike the situation in the wild type where both cell types express Rubisco in a C3-like ground state in the dark). The function of the *Bsd1* gene is thus apparently required for plastid differentiation and for the proper expression of the plastid *rbcL* gene. The *bsd2-m1* mutation, also an unstable allele, leads to abnormal bundle sheath plastids while the mesophyll chloroplasts appear normal (Roth *et al.,* 1996). The Rubisco protein is missing, yet the *rbcL* mRNA nevertheless accumulates in the bundle sheath cells, but also ectopically in the mesophyll cells. These observations point to a requirement for the *Bsd2* gene in posttranscriptional steps of *rbcL* gene expression. It is interesting that both the molecular analysis of C4 differentiation and the analysis of the bundle sheath defective mutants converge to reveal controls of chloroplast gene expression at posttranscriptional steps. In the *leaf permease 1* mutant (*lpe1-m1*), the bundle sheath chloroplasts are specifically affected under moderate light (Schultes *et al.,* 1996). This selective effect may be indirect since the *Lpe1* gene encodes a polypeptide related to microbial purine and pyrimidine transporters.

IV. Nuclear Control of Plastid Gene Expression

The expression of plastid genes is largely dependent on nucleus-encoded factors since many components of the plastid genetic system are imported. These may be constitutive components, for example, ribosomal proteins, but also regulatory factors. It is thus difficult to separate the nuclear control of plastid gene expression from the more general topic of plastid gene expression and regulation. This review will try to focus (perhaps somewhat arbitrarily at times) on biochemical and genetic investigations that reveal nuclear factors and their roles in plastid gene expression (Taylor, 1989; Mayfield, 1990). The broader topics of plastid gene expression and regulation are extensively discussed in many excellent reviews (Rochaix, 1992, 1996; Herrmann *et al.,* 1992; Mullet, 1993; Gruissem and Tonkyn, 1993; Mayfield *et al.,* 1995).

A. Transcription

The transcriptional apparatus of plastids has been the subject of thorough reviews (Igloi and Kössel, 1992; Bogorad, 1991), and we will only consider here a few aspects that are relevant for interactions of plastids with the nucleus. Regulation of chloroplast transcription has been investigated in many organisms (Bogorad, 1991; Hermann *et al.*, 1992; Gruissem and Tonkyn, 1993; Mullet, 1993). Different plastid genes are transcribed at very different rates that vary with a range of several hundredfold. This activity is modulated during development, as the overall transcriptional activity rises and then decreases. Superimposed on these general changes, there are differences in the relative transcription of certain genes during the course of development. Some genes involved in chloroplast gene expression (encoding RNA polymerase subunits, tRNAs, rRNAs, and ribosomal proteins) are differentially transcribed at early stages. Furthermore, the transcription of some genes is regulated by environmental factors such as light, and also by a circadian clock.

1. Chloroplast RNA Polymerases

Purified preparations of chloroplast RNA polymerase contain eight to a dozen or more polypeptides, some of which are immunologically related to subunits of bacterial RNA polymerase (Bogorad, 1991; Igloi and Kössel, 1992). For the maize enzyme, N-terminal sequencing has established that four subunits ($\alpha, \beta, \beta', \beta''$) are the products of chloroplast genes, *rpoA, rpoB, rpoC1*, and *rpoC2* (Hu and Bogorad, 1990; Hu *et al.*, 1991). These chloroplast genes are homologous to the bacterial genes encoding the subunits of core RNA polymerase (where *rpoC1* and *rpoC2* correspond to two parts of bacterial *rpoC*). Immunological studies indicate that another subunit (64 kDa) may be related to the sigma subunit of a cyanobacterial RNA polymerase (Troxler *et al.*, 1994). Sequencing of two other polypeptides has shown that they are contaminants of the purified fraction. The remaining polypeptide from the maize enzyme (85 kDa) as well as the extra ones in preparations from other sources have not yet been identified (with the exception of the 110-kDa polypeptide of the spinach enzyme described below). They could be *bona fide* subunits of the enzyme, associated transcription factors, or contaminants. The genes encoding them, as well as the sigma-related subunit, have not been described but most or all of them are probably located in the nucleus.

The sigma subunits of bacterial polymerases enhance the binding of the core enzyme to specific promoter sequences. Using a functional assay with *E. coli* core RNA polymerase and total chloroplast DNA, two sigma-like activities have been identified in *C. reinhardtii*, which also stimulate RNA

polymerase activity of the homologous plastid enzyme (Surzycki and Shellenbarger, 1976). Similarly, with *E. coli* RNA polymerase and plastid promoter sequences, three sigma-like factors (SLF) have been purified from mustard (Bülow and Link, 1988; Tiller *et al.,* 1991), and one has been identified in spinach (Lerbs *et al.,* 1988). In mustard, some aspects of the holoenzyme differ between etioplasts and chloroplasts: the specificity for different plastid promoters, the DNA-binding properties, and the phosphorylation state. *In vitro* treatment of the chloroplast holoenzyme with protein kinase converts it to an etioplast type, and conversely treatment of the etioplast enzyme with phosphatase converts it to a chloroplast-like form. In these experiments the DNA-binding properties depend on the phosphorylation status of the SLF subunits (Tiller and Link, 1993). Thus light-induced transcriptional regulation of plastid genes such as *psbA* could involve phosphorylation–dephosphorylation modifications of sigma-like transcription factors. The SLFs themselves, whose genes have not yet been described, as well as the phosphatase and kinase activities, are most likely encoded by nuclear genes: this would place regulation of plastid transcription by the plastid-encoded RNA polymerase under nuclear control. In the unicellular rhodophyte *Cyanidium caldarium,* a nuclear gene encodes a plastid sigma factor. The cytoplasmic mRNA for this sigma factor is not detectable in dark-grown cells, but accumulates upon illumination. This sigma factor may thus be involved in light-regulated transcription of plastid genes (Liu and Troxler, 1996).

Transcription can also be modulated by factors that directly bind to DNA. Evidence for such a factor involved in *psbD* transcription has been obtained in barley (Kim and Mullet, 1995). The *psbD* gene is part of a complex operon transcribed from at least three different promoters, one of which is activated by blue light (Berends-Sexton *et al.,* 1990; Christopher *et al.,* 1992). A DNA-binding factor, AGF, binds sequences upstream of this promoter that are essential for transcription activity *in vitro.* Mutations that abolish binding of the AGF factor also affect transcription, indicating that AGF is probably required for the activity of the blue-light-responsive *psbD* promoter. Whether this factor is directly involved in light regulation remains to be established. The light-responsive element of the tobacco *psbD* promoter has been mapped *in vivo* using chimeric reporter genes to transform tobacco chloroplasts (Allison and Maliga, 1995), and factors that bind this regulatory element can be detected in plastid extracts. Whether they play a role in light regulation was not determined. In wheat, transcription *in vitro* from the light-responsive *psbD* promoter is higher in extracts from light-treated plastids than that in extracts from dark-grown seedlings (Wada *et al.,* 1994). A crude fraction from a plastid extract of light-treated plants enhances transcription by a dark-grown plastid extract at this promoter. This fraction is in itself devoid of RNA polymerase activity and is

inferred to differ from the sigma-like factors (the latter but not the former bind heparin agarose).

2. Nucleus-Encoded RNA Polymerases

Besides the bacterial-like RNA polymerase, at least partly encoded by the chloroplast *rpo* genes, there is evidence for at least one additional plastid RNA polymerase encoded entirely in the nucleus. In the nonphotosynthetic parasitic plant *E. virginiana,* the genes encoding the photosynthetic apparatus or the putative respiratory chain (*ndh* genes) have been deleted during evolution from the small residual plastid genome or only remain as nonfunctional pseudogenes (dePamphilis and Palmer, 1990; Morden *et al.,* 1991; Wolfe *et al.,* 1992). The preservation and sequence conservation of genes involved in translation (rRNA, ribosomal protein, and tRNA genes) and of some large open reading frames provide evidence that the retained plastid genes are expressed, and plastid transcripts have indeed been found in *E. virginiana.* However, the plastid RNA polymerase genes have been deleted (*rpoB, rpoC1, rpoC2*) or inactivated (truncated *pseudo rpoA*). The retained and presumably functional genes must therefore be transcribed by an imported RNA polymerase, encoded in the nucleus. This may well be a different enzyme, also encoded in the nucleus of other plants as will be further discussed below. But it has not been excluded that in this case the *rpo* genes might have been relocated from the plastid to the nucleus, as appears to be the case for some tRNA genes which are also missing in the plastid genome of *E. virginiana.*

Another line of evidence for an exclusively nuclear-encoded RNA polymerase is provided by observations with the white seedlings of the homozygous *albostrians* (*as*) mutant of barley or white leaves of heat-bleached rye (Hess *et al.,* 1993). These white plastids are devoid of detectable ribosomes and thus cannot translate chloroplast mRNAs, and in particular mRNAs for the RNA polymerase subunits encoded by the plastid *rpo* genes. Transcripts for some plastid genes nevertheless accumulate in these ribosome-deficient plastids, implying the activity of a nuclear-encoded imported RNA polymerase. While some genes involved in plastid gene expression are actively transcribed and some mRNAs accumulate to high levels in the white tissue (*rpoB/C1/C2* and *rps15*), mRNAs for proteins involved in photosynthesis are not detectable. This pattern of gene expression is reminiscent of the pattern in early stages of plastid development, suggesting that the nuclear-encoded RNA polymerase may be involved in this differential developmental regulation (Mullet, 1993). In heat-bleached plastids of barley, which are also deficient in plastid ribosomes, run-on assays of transcriptional activity show that most genes are as actively transcribed as in control plants and that transcription of rDNA is enhanced (Falk *et al.,* 1993). This

again points to the presence of an additional RNA polymerase expressed even when plastid translation is deficient. Because the corresponding mRNAs only accumulate to low levels, posttranscriptional degradation is probably involved.

Chloroplast transformation allows the site-directed inactivation of plastid genes and has been applied to the *rpo* genes in *C. reinhardtii* and in tobacco (Rochaix, 1995; Allison *et al.*, 1996). Inactivation of the *rpoB* gene in tobacco leads to photosynthetic deficiency, but the mutant plants are viable on sugar-containing medium. Transcripts of some photosynthetic genes are much less abundant, while 16S rRNA is only little affected and ribosomal subunit *rpl16* transcripts are somewhat elevated. Thus another RNA polymerase activity, independent of *rpoB,* must be present in the mutant plastids. In rDNA, it uses a novel promoter, different from the canonical promoters of the *E. coli* sigma70 type (see below). In *C. reinhardtii,* inactivations of the *rpo* genes by insertion of a selectable marker remain heteroplasmic: among the 80 or so copies of the plastid genome, some carry the inactivated gene with the selectable marker, but some wild-type copies of the gene are also retained (Rochaix, 1995; Fischer *et al.*, 1996). This indicates that the *rpo* genes have an essential function and that their complete inactivation would make cells inviable. In this case the *rpo*-encoded RNA polymerase must transcribe plastid genes which are essential for the survival of the alga, unlike the photosynthetic genes which are dispensable. It remains unclear whether *C. reinhardtii* also contains an additional nuclear-encoded RNA polymerase activity.

A single-subunit RNA polymerase has been identified in purified preparations from spinach (Lerbs-Mache, 1993). This 110-kDa polypeptide has DNA-dependent RNA polymerase activity on its own and does not initiate from the promoters recognized by the bacterial-like RNA polymerase. The 110-kDa polypeptide is also found in purified fractions of the plastid enzyme containing the chloroplast *rpo*-encoded subunits (see above). This separate RNA polymerase activity is only prevalent in extracts from very young leaves, again providing a correlation with the differential expression of some plastid genes in early development. Whether this enzyme represents the nuclear-encoded RNA polymerase active in ribosome-deficient plastids or in *Epifagus* is not yet known. In the spinach ribosomal RNA (*rrn*) operon there are two promoters which were defined *in vitro* and have the consensus sequences recognized by *E. coli* RNA polymerase (P1 and P2). However, the 5′ end of the *rrn* transcripts *in vivo* does not correspond to either of these promoters, so a third promoter(P_c) may operate *in vivo* (Baeza et al, 1991) and may be the promoter used by the 110-kDa RNA polymerase. This promoter lacks homology to the canonical consensus sequences at −10 and −35 of prokaryotic-like promoters of the *E. coli* sigma70 type (Hanley-Bowdoin and Chua, 1987; Bogorad, 1991). A sequence-specific

DNA-binding factor, CDF2, inhibits *in vitro* transcription by the chloroplast *rpo*-encoded polymerase from the P1 and P2 promoters (Iratni *et al.*, 1994). Thus the *rrn* genes may be transcribed by the 110-kDa RNA polymerase not only early during development, but also at later stages when the *rpo*-encoded polymerase is present but prevented from transcribing the *rrn* operon by the CDF2 factor. The sequences in the *rrn* promoter region are highly conserved in plants (Baeza *et al.*, 1991; Vera and Sugiura, 1995). However, in tobacco, two different promoters were mapped for the *rrn* operon (Vera and Sugiura, 1995): one, P1, has the classical consensus sequences and corresponds to the P2 promoter described in spinach. The second promoter (P2 in tobacco) is found downstream of the first and is not canonical, but it differs from the P_c promoter in spinach, which is upstream. Steady-state levels of the transcript from the noncanonical promoter in tobacco are more prevalent in proplastids of cultured cells than in leaf chloroplasts. This novel promoter is also used in the tobacco mutant with the inactivation of the *rpoB* gene described above (Allison *et al.*, 1996), suggesting that in tobacco, the two types of *rrn* promoters are recognized by different RNA polymerases.

Two different RNA polymerases have been isolated from mustard seedlings (Pfannschmidt and Link, 1994). These two large-molecular-weight complexes differ in size and polypeptide composition. One apparently contains the chloroplast-encoded *rpo* subunits and is the main form in etioplasts of dark-grown seedlings, while the other is prevalent in green chloroplasts.

These findings, demonstrating different types of RNA polymerases, sigma-like transcription factors, and DNA-binding proteins in the plastids have interesting implications for plastid gene regulation. Some subunits of the bacterial-like RNA polymerase, most subunits of the other polymerases, and the other factors are probably encoded in the nucleus where their expression may be under developmental control. In turn, their activity and promoter specificity are expected to influence gene expression in plastids. As pointed out above, a nucleus-encoded RNA polymerase may be playing a particular role early in development for the transcription of the plastid loci involved in gene expression (Mullet, 1993). The expression of these genes, and in particular of the plastid-encoded RNA polymerase genes (*rpo*), would then allow the transcription of plastid genes required for subsequent development of the plastid and of the photosynthetic apparatus. The promoter specificity of these different enzymes and associated transcription factors is not yet clearly understood. In addition to promoters resembling canonical *E. coli* sigma70 promoters, there is evidence for additional types of promoters in plastid genes (Igloi and Kössel, 1992, see examples discussed above). Using plastid transformation, *in vivo* promoter analysis has also unveiled different kinds of promoters in *C. reinhardtii* (Klein *et al.*, 1992). Such studies have also revealed *cis*-acting elements that

affect the activity and the light regulation of the *psbD* promoter in tobacco (Allison and Maliga, 1995) and shown an enhancing element within the coding sequence of the *rbcL* gene in *C. reinhardtii* (Klein *et al.,* 1994). It will be of interest to unravel further how the different promoters are recognized by the different RNA polymerases and how *cis*-acting elements mediate transcriptional regulation.

3. Transcription Mutants

Besides the *rpoB* disruption mutants discussed above, there is only one well-characterized mutant which may have a specific defect in plastid transcription. This is a nuclear mutant of *C. reinhardtii,* 76-5EN (Hong and Spreitzer, 1994), which is deficient in the chloroplast mRNA for the large subunit of ribulose–bisphosphate carboxylase oxygenase (Rubisco). Only low steady-state levels of the *rbcL* mRNA accumulate in this mutant, and correspondingly low levels of incorporation into *rbcL* transcripts are measured by pulse-labeling with [^{32}P]phosphate. This is interpreted as a defect in mRNA transcription, although very rapid RNA degradation is difficult to rule out and remains a possibility. Like many mutants that affect chloroplast gene expression in *C. reinhardtii,* 76-5EN seems to be gene-specific and to only affect *rbcL.*

The scarcity of nuclear mutations affecting plastid transcription contrasts with the many mutations that cause posttranscriptional defects (see below). This may be taken to indicate that control of specific chloroplast genes is mostly exerted posttranscriptionally. However, an alternative may be that the same transcription factors are shared by many genes, for instance, if these genes are part of the same operon or if they share the same control network. Such mutations would have complex, pleiotropic phenotypes and in some organisms like *C. reinhardtii* they might be inviable (like the *rpo* disruption mutants) and would not be recovered.

B. RNA Maturation and Degradation

Plastid transcripts are subject to processing events such as 5′ or 3′ cleavage, trimming, splicing, and editing (Sugiura, 1991; Herrmann *et al.,* 1992; Gruissem and Tonkyn, 1993). Many genes are part of polycistronic units that are transcribed as precursors. These are then matured to produce the individual mRNAs through a series of partially processed intermediates. The maturation steps often do not follow an ordered pathway, so that a complex set of transcripts accumulate in the plastid. Transcription from multiple promoters and splicing of intervening sequences can also contribute to the complexity of RNA species that are derived from a gene cluster.

Plastid transcripts are eventually degraded, a controlled process that is also important for the regulation of gene expression. The structure of the mRNA influences its translation, and conversely translation can influence the stability of the mRNA positively or negatively. These various aspects of mRNA biogenesis and destruction are clearly important for gene expression in the plastid and for its regulation, and we shall review here what is known about their control by the nucleus. Some of the steps have been analyzed *in vitro* and factors that are involved have been identified biochemically. There are also mutations that reveal nuclear factors required for these processes.

1. RNA Processing and Stability

In vitro extracts that can process RNA precursors have been used to analyze 3′end formation. In mustard chloroplasts, a protein that can bind in the 3′ flanking regions of the *trnK* and *rps16* transcripts has been identified and purified by virtue of its dual binding and processing activities (Nickelsen and Link, 1991, 1993). The purified 54-kDa polypeptide has endonuclease activity and can faithfully process downstream of *trnK,* but not of *rps16* where ancillary factors are probably required.

Many plastid RNAs end with an inverted repeat near the 3′end, predicted to fold as a hairpin loop. *In vitro,* these structures are not efficient transcription terminators like some of their bacterial counterparts, but play a role in RNA processing and stabilization possibly by preventing exonucleolytic degradation (Stern and Gruissem, 1987; Stern *et al.,* 1989, 1991). In *C. reinhardtii* transformants with chloroplast 3′ ends inserted upstream of reporter genes, run-on assays (nascent chain elongation *in vitro*) indicate that the hairpin structures may cause only partial termination and allow readthrough transcription of the downstream reporter (Rott *et al.,* 1996). *In vivo* in *C. reinhardtii,* deletion of the *atpB* 3′ hairpin structure leads to length heterogeneity and reduced accumulation of transcripts (Stern *et al.,* 1991). A function of the hairpin structure may be to impede exonucleolytic degradation of the 3′ end of the RNA (Drager *et al.,* 1996). However, chimeric RNAs with chloroplast 3′ends (*rbcL* or *psaB*) fused to the *E.coli uidA* gene are not more stable than RNAs lacking the 3′ structure, although the latter are again heterogeneous in size (Blowers *et al.,* 1993). Thus *in vivo* the hairpin structures are required to form a precise 3′end, but may not always be involved in RNA stabilization.

Proteins that bind to these regions have been identified, for instance, a nuclear-encoded RNA-binding protein from spinach chloroplasts. This 28-kDa polypeptide (28RNP) can bind the precursor and the mature forms of several chloroplast mRNAs with little specificity and is required for correct 3′ processing *in vitro* in association with other polypeptides (Schuster and Gruissem, 1991; Lisitsky *et al.,* 1995). It has an acidic N-terminal

domain and two RNA-binding consensus sequences, RNP-CS1 and RNP-CS2 (Lisitsky *et al.,* 1994). These conserved domains are characteristic of a family of RNA-binding proteins including some that bind pre-mRNAs in the nuclei of animal cells and are involved in splicing. A direct search for members of this family in the chloroplast has allowed the identification of five proteins from *Nicotiana sylvestris* (Li and Sugiura, 1990; Ye *et al.,* 1991), two from *N. plumbaginifolia* (Mieszczak *et al.,* 1992), one from *Zea mays* (Cook and Walker, 1992), and three from *A. thaliana* (Ohta *et al.,* 1995). Three proteins from pea chloroplasts that bind single-stranded nucleic acids probably also belong to this family (Subbaiah and Tewari, 1993). All these proteins are of similar size (28–33 kDa) and, like the 28RNP polypeptide from spinach, have an acidic N-terminal. They can be classified into three groups based on sequence similarity. Their precise role in the chloroplast remains to be elucidated.

Spinach chloroplast proteins that bind to the *petD* 3′ end form a complex that contains three subunits (chloroplast stem–loop binding proteins or CSPs): CSP29, CSP41, and CSP55 (Chen *et al.,* 1995; Yang *et al.,* 1996). The complex shows specificity for the *petD* 3′end and recognizes the hairpin structure and a downstream sequence element. CSP29 probably corresponds to a protein previously identified by UV cross-linking which binds to the double-stranded stem of the *petD* 3′ end, formerly estimated as 33 kDa (Hsu-Ching and Stern, 1991a). A cDNA encoding CSP41 has been cloned and expressed in *E. coli,* showing that the CSP41 protein can bind RNA and is an endonuclease under certain conditions. These dual activities are reminiscent of those of the 54-kDa protein from mustard chloroplasts described above.

The biochemistry of chloroplast RNA maturation can be studied using choroplast extracts (Gruissem and Schuster, 1993). *In vitro* processing of the *atpB* RNA 3′ end in *C. reinhardtii* extracts is a two-step process involving an endonucleolytic cleavage followed by exonucleolytic trimming (Stern and Kindle, 1993). Processing of a *petD* RNA precursor at the 3′ end in a spinach chloroplast extract also involves endonucleolytic and exonucleolytic activities (Hsu-Ching and Stern, 1991b). Purification of the spinach processing extract has revealed a large-molecular-weight complex (550 kDa) containing different subunits, 100RNP, 67RNP, and 33RNP (Hayes *et al.,* 1996). The 33RNP can be UV cross-linked to the 3′ end of *petD* RNA (Hsu-Ching and Stern, 1991a; see above). The 100RNP can also be UV cross-linked to the *petD* 3′ end. A cDNA encoding the 100RNP shows homology to the polynucleotide phosphorylase of bacteria (PNPase), and the 100RNP does have 3′–5′ exonuclease activity like PNPase. In *E. coli,* PNPase forms part of a large complex in association with RNAseE, an endonuclease involved in RNA degradation (Carpousis *et al.,* 1994). Indeed, the 67RNP cross-reacts with antisera against bacterial RNaseE and has endonuclease

activity, cleaving upstream of the hairpin. This activity can be inhibited by RNP55, a loosely associated RNA-binding protein from the extract. The purified high-molecular-weight complex does not correctly process the *petD* precursor but rather degrades it; however, the addition of 28RNP partly restores processing. The 28RNP can be phosphorylated in its acidic N-terminal domain, and phosphorylation reduces its affinity for RNA (Lisitsky and Schuster, 1995). This modification of the 28RNP could be part of a regulatory mechanism that could modulate the two activities of the high-molecular-weight complex, 3′ processing and RNA degradation. While the 67RNP induces a cleavage 5′ to the hairpin that could lead to RNA degradation, the putative endonuclease involved in cleavage downstream of the hairpin for 3′ end formation is not yet identified.

These findings reveal an interesting connection between RNA processing and RNA stability or degradation which is echoed in the phenotype of some mutants described below. However, their analysis points to a role of the 5′ UTR (5′ untranslated region) in the control of mRNA degradation (see the next section). Cleavages occur in the 5′ UTR and in the coding region of *psbA* mRNA in chloroplast extracts from spinach leaves: degradation products of defined sizes indicate endonucleolytic cleavage sites that can also be observed *in vivo* (Klaff, 1995). These cleavages could be the initial steps in a *psbA* mRNA degradation pathway involving endo- and exonucleolytic digestion. Poly(A) or poly(A)-rich sequences that are apparently added posttranscriptionally can be detected in a small fraction of *psbA* RNA (Lisitsky *et al.*, 1996; Kudla *et al.*, 1996). They are found at the 3′ end, and at the positions of endonucleolytic cleavages observed *in vivo*. Polyadenylation is also found for *petD* RNA at the 3′ end and at internal sites that can be cleaved by the 67RNP. Transcripts with such poly(A) or poly(A)-rich sequences are unstable *in vitro* in chloroplast extracts. Polyadenylated RNAs are also degraded more rapidly than nonpolyadenylated RNAs by the 100RNP. These observations raise the possibility that in the plastid the poly(A)-rich sequences are added after endonucleolytic cleavage and, like in bacteria, target the RNA for subsequent degradation.

2. Nuclear Mutants Defective in mRNA Maturation and Stability

The stability of specific choroplast mRNAs is affected in some *C. reinhardtii* nuclear mutants. In these mutants the steady-state level of a chloroplast RNA is drastically reduced, but transcription is not affected as measured by pulse-labeling in living cells or run-on transcription assays in permeabilized cells. In the *nac2* mutant, *psbD* mRNA does not accumulate; in the *222E* and *GE2-10* mutants, *psbB* mRNA is missing; in 6.2z5, *psbC* mRNA is absent; in *nccl, atpA* mRNA levels are reduced 10-fold; in *thm24, atpB*

mRNA is absent; in MΦ11, *petA* mRNA is missing; and in MΦ37, *petB* mRNA is reduced 20-fold (Kuchka *et al.*, 1989; Monod *et al.*, 1992; Sieburth *et al.*, 1991; Drapier *et al.*, 1992; Gumpel *et al.*, 1995). The specificity of these mutations for individual chloroplast genes or transcription units is a striking property of *C. reinhardtii* nuclear-encoded factors involved in this and other steps of chloroplast gene expression.

Two of these mRNA stability mutants have been studied in more detail. The chloroplast *psbD* gene is located just upstream of the *trans*-spliced exon2 of *psaA* (see below), and both are part of a polycistronic transcription unit that extends downstream. In the nuclear *nac2* mutant, *psbD* mRNA fails to accumulate, but *psaA* mRNA levels are not affected (Kuchka *et al.*, 1989). However, in double mutants where *psaA* exon2 splicing is additionally blocked, both the *psbD* and the *psbD–psaA2* transcripts are unstable, suggesting that a sequence in the *psbD* moiety confers instability to the whole transcript in the *nac2* mutant. Transformation experiments with chimeric genes have indeed shown that the 5′ UTR of *psbD* is sufficient to confer instability to a reporter RNA in the *nac2* nuclear background (Nickelsen *et al.*, 1994). In the wild type, there are two 5′ ends for the *psbD* transcripts, suggesting that the longer 5′ UTR may undergo processing to yield the predominant mature form of *psbD* mRNA. *In vitro,* the longer 5′ UTR, but surprisingly not the shorter form, can bind a 47-kDa polypeptide which is absent in the *nac2* mutant. An interpretation could be that the product of *NAC2* is required prior to 5′ processing for an event that ensures subsequent stability of the processed *psbD* mRNA. The apparent association of the 47-kDa polypeptide with membranes led to the hypothesis that Nac2 could be involved in targeting of the *psbD* transcript to chloroplast membranes where its translation product, the D2 polypeptide of PSII, will be inserted. The recent cloning of the *NAC2* gene should help shed light on its mechanism of action (Nickelsen and Rochaix, personal communication).

In *C. reinhardtii,* the *psbB* gene is part of a polycistronic unit that includes *psbT* and probably *psbH* (Monod *et al.*, 1992; Johnson and Schmidt, 1993). In the *222E* nuclear mutant, the *psbB, psbB–psbT,* and *psbH* transcripts do not accumulate (Monod *et al.*, 1992). *In vivo,* the 5′ UTR of the *psbB* mRNA is sufficient to confer instability to chimeric reporter RNAs in the *222E* nuclear mutant (Vaistij, Monod, and Goldschmidt-Clermont, unpublished observations). Whether the absence of the *psbH* mRNA is due to its association with the unstable *psbB* 5′ sequence in a primary transcript or whether it is a separate effect of the *222E* mutation remains to be determined. The *222E* locus has also been cloned recently (Vaistij and Goldschmidt-Clermont, unpublished results).

Both of these mutations reveal an important role of the 5′ UTR in determining RNA stability in *C. reinhardtii* chloroplasts. An involvement of the 5′ UTR is also apparent in *C. reinhardtii* chloroplast transformants

harboring chimeras with the 5′ end of *rbcL* fused to a *uidA* reporter (Salvador *et al.,* 1993a). The *rbcL* 5′ UTR renders the transcript unstable in the light, but the effect is reversed by the inclusion of sequences from the coding part of *rbcL.*

Among the *hcf* (high chlorophyll fluorescence) nuclear mutants of maize and *Arabidopsis,* some have alterations in chloroplast RNA metabolism. In the *hcf7* mutant of maize, the amount of 16S rRNA is reduced, and the precursor accumulates (Barkan, 1993). This is indicative of a block in ribosomal RNA processing, as a cause or a consequence of deficient ribosome assembly, which would in turn explain the pleiotropic defects in translation of chloroplast proteins. In another maize mutant, *crp1* (*chloroplast RNA processing 1,* formerly *hcf136*), the monocistronic forms of *petB* and *petD* mRNA are missing (Barkan *et al.,* 1994). These genes are transcribed as part of the *psbB* gene cluster (*psbB–psbH–petB–petD*). However, polycistronic forms of the *petB* and *petD* RNAs do accumulate, indicating that *crp1* has a defect in the intricate processing of these transcripts. The incompletely matured *petD* RNAs are not translated, suggesting a model where processing is required to remove part of a secondary structure that otherwise blocks translation initiation.

In *hcf38,* a specific set of chloroplast transcripts encoding polypeptides of different photosynthetic complexes are missing (Barkan *et al.,* 1986). This could be due to a defect in RNA maturation or stability. A related phenotype occurs in *hcf42* where unprocessed or altered transcripts for a different set of chloroplast RNAs are observed (Miles, 1994). These pleiotropic phenotypes suggest that for maturation or stabilization, chloroplast mRNAs depend on nucleus-encoded factors that are shared by subsets of transcripts.

Not unlike these maize mutants, the *hcf109* mutant of *A. thaliana* has defects in several transcription units, but others remain unaffected (Meurer *et al.,* 1996). In three gene clusters, only some of the transcripts are missing: in the *psbB–psbT–psbH–petB–petD* cluster, only the *psbB–psbT* transcripts are absent. Similarly, *ycf9* (from *psbD–psbC–ycf9*) and *psaC* transcripts (from *ndhH–ndhA–ndhI–ndhG–ndhE–psaC–ndhD*) are most severely depleted in *hcf109.* But in the *ndhC–ndhK–ndhJ* cluster there are two major RNAs, both of which are missing in *hcf109.* Transcription of the affected genes is unaltered, but the steady-state amounts of the longer polycistronic precursors are reduced. The effect of the mutation thus seems to be on the processing or stability of specific transcripts in several different transcription units.

3. Splicing

Many plastid genes are interrupted by intervening sequences that belong to two conserved classes, group I and group II, which differ in their catalytic

mechanism and in their structure. Splicing of mitochondrial introns in yeast and *Neurospora* relies on nuclear-encoded splicing factors, even for introns that can self-splice *in vitro.* Little is known about the splicing machinery of plastid introns, but mutations that interfere with splicing have been characterized in *C. reinhardtii* and in maize.

In *C. reinhardtii,* there are group I introns in the 23S rDNA and in the *psbA* gene, and most of them can self-splice *in vitro* (Dürrenberger and Rochaix, 1991; Herrin *et al.,* 1990, 1991; Thompson and Herrin, 1991). In a nuclear mutant, *ac20,* unspliced precursors of these introns overaccumulate, indicating a direct or indirect role of the corresponding nuclear factor in chloroplast group I splicing (Herrin *et al.,* 1990, 1991).

In ribosome-deficient mutants of barley, only the splicing of the *rpl2* group II intron is affected, indicating that its removal is dependent on a factor or maturase encoded and translated in the plastid (Hess *et al.,* 1994). Other introns are spliced normally, so that any factors required for their maturation can be inferred to be nuclear-encoded. A similar situation is found in maize where splicing of a subset of the plastid introns is affected in ribosome-deficient plastids (Jenkins *et al.,* 1997). There are two nuclear mutants of maize that affect group II intron splicing, *crs1* and *crs2* (*chloroplast RNA splicing;* Jenkins *et al.,* 1997). The *crs1* mutation is specific, it blocks splicing of a single intron in *atpF,* while the *crs2* mutation interferes with the splicing of many chloroplast introns.

The *psaA* gene of *C. reinhardtii* and the *rps12* gene in vascular plants have unusual structures where exons are located at widely separate loci on the plastid genome. The mature mRNAs are assembled by splicing from separate precursor RNAs, a process called *trans*-splicing (Fukuzawa *et al.,* 1986; Torazawa *et al.,* 1986; Choquet *et al.,* 1988; Herrin and Schmidt, 1988; Bonen, 1993). In *C. reinhardtii,* the three exons of *psaA* are transcribed as separate precursors (Fig. 2). Exon 2 is part of a polycistronic unit and is located downstream of *psbD,* and exon 1 may be cotranscribed with *trnI.* The sequences that flank the three *psaA* exons have the conserved features of group II introns (Kück *et al.,* 1987). Splicing of exons 1 and 2 requires an additional small RNA, the product of the *tscA* gene (*trans*-splicing chloroplast; Roitgrund and Mets, 1990; Goldschmidt-Clermont *et al.,* 1991). The *tscA* RNA is thought to assemble with the exon 1 and exon 2 precursors to form the characteristic structure of group II introns, which in this case is assembled from at least three separate transcripts. Many mutants have been identified that are deficient in *psaA trans*-splicing. Some mutations affect the chloroplast locus encoding *tscA,* but the vast majority are nuclear. These nuclear *trans*-splicing mutants belong to at least 14 complementation groups (Goldschmidt-Clermont *et al.,* 1990). Some of them are highly specific and affect only one of the two *trans*-splicing steps: 7 complementation groups are defective in *trans*-splicing exons 1 and 2, while 5 other groups

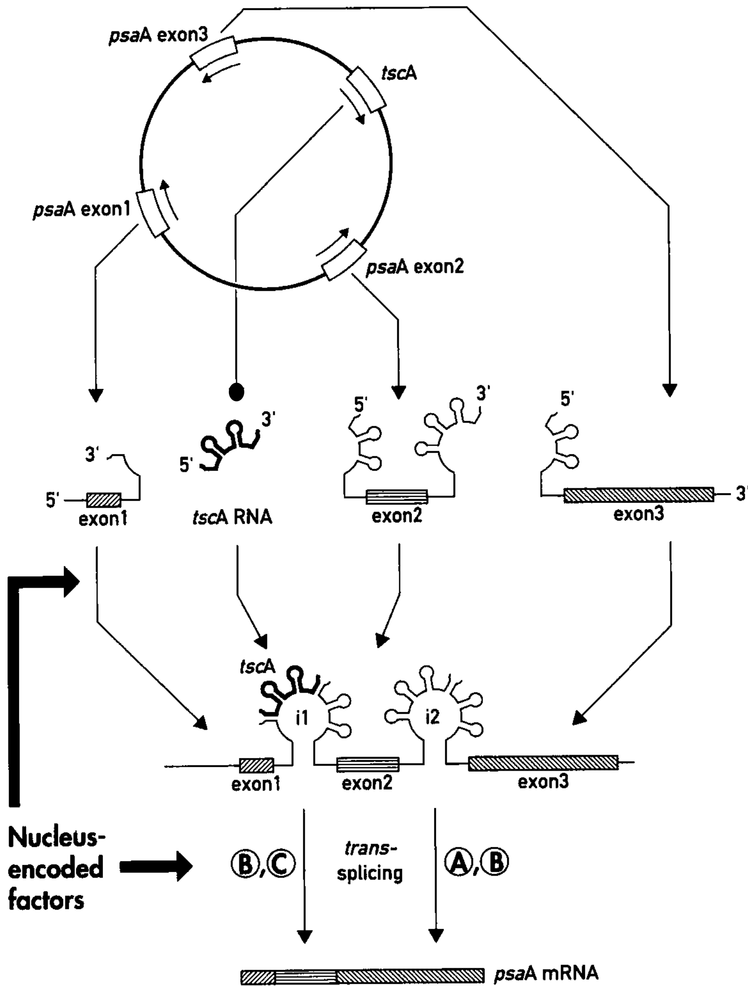


FIG. 2. Trans-splicing of psaA RNA. In *Chlamydomonas reinhardtii*, the *psaA* gene is composed of three exons located in widely separate loci of the chloroplast genome (not drawn to scale). The three exons and the *tscA* locus are transcribed independently to produce four transcript precursors. Intron 1 (i1) is thought to assemble from three separate transcripts (exon1 precursor, *tscA* RNA, and exon 2 precursor) and intron 2 from two transcripts (exon 2 precursor and exon 3 precursor). The six stem–loop domains characteristic of group II introns are represented schematically (not to scale). Two steps of *trans*-splicing, which can occur in either order, lead to the formation of mature *psaA* mRNA (bottom). Nucleus-encoded factors are required for *trans*-splicing of intron 1 (class C), of intron 2 (class A), or of both introns (class B).

are defective in *trans*-splicing exons 2 and 3. In 2 complementation groups, both *trans*-splicing steps are affected. Because most complementation groups are only represented by a single allele, it can be argued that many more nuclear loci than the 14 identified may be required for *psaA trans*-splicing. This major nuclear contribution to the expression of a single chloroplast gene, and even to the *trans*-splicing of individual introns, again raises questions about the number and specificity of the nuclear factors implicated in chloroplast gene expression. Cloning of two of these genes has recently been achieved, and the sequence derived for one of them predicts a novel polypeptide that is not yet represented in databases (Goldschmidt-Clermont, Perron and Rahire, unpublished observations).

4. RNA Editing

Editing of RNA is a posttranscriptional modification that alters the sequence of a transcript compared to its gene. In plastids substitutional RNA editing can change C residues to U at specific sites (Maier *et al.,* 1995). Similar but more extensive C-to-U editing also occurs in plant mitochondria (Hanson *et al.,* 1996). The mechanism and specificity of plastid RNA editing have not yet been elucidated. Because RNA editing is still observed in a ribosome-deficient mutant of barley (*albostrians*), it can be inferred that editing is independent of chloroplast translation and that any protein factors involved in the process must be nucleus-encoded (Zeltz *et al.,* 1993). Attempts at identifying chloroplast guide RNAs that could provide the sequence specificity of editing have not been successful (Bock and Maliga, 1995), suggesting that nuclear genes may provide both the specificity and the enzymatic activities of plastid RNA editing.

In the tobacco *psbL* and *ndhD* mRNAs, the AUG translation initiation codons are created by editing from ACG. Chimeric genes with the N-terminal part of *psbL* fused to bacterial drug resistance genes have been introduced into the tobacco chloroplast genome by transformation (Chaudhuri *et al.,* 1995; Chaudhuri and Maliga, 1996). In the chimeric mRNAs, the *psbL* or *ndhD* sites are edited and the drug resistance is hence expressed. However, the introduction of additional editing substrates leads to a decrease of editing at the corresponding site in endogenous mRNA, but not of editing at other sites. The competition indicates that at least one editing factor must be limiting in each case. The lack of competition between different substrates suggests that the limiting factors are site-specific. In contrast, introduction of an additional copy of edited sequences from ndhB has no effect on the editing of endogenous *ndhB* mRNA (Bock *et al.,* 1996).

5. Coupling of mRNA Metabolism and Translation

Translation of some chloroplast transcripts is affected by RNA processing events. Methyl jasmonate, which is known to evoke many responses of

nuclear genes, also has effects on plastid gene expression (Reinbothe *et al.*, 1993). In particular, processing of *rbcL* RNA is altered, so that after methyl jasmonate treatment its 5′ UTR is 35 bases longer. This leads to a selective inactivation of the translation of *rbcL* mRNA both in the plastids and *in vitro* in a heterologous extract. Another example is provided by the effect of the *crp1* mutation in maize on the expression of *petD*, where a block in RNA processing is thought to prevent translation (see section IV.B.2; Barkan *et al.*, 1994). In *C. reinhardtii* chloroplast transformants, a monocistronic *uidA* mRNA is translated to produce β-glucuronidase (GUS), but an unprocessed dicistronic transcript with *uidA* downstream of *petA* does not produce GUS (Sakamoto *et al.*, 1994b). Editing of some chloroplast transcripts is an obvious prerequisite for their translation when the AUG initiation codon is created by RNA editing (see section IV.B.4).

The accumulation of plastid mRNAs is in some cases affected, positively or negatively, by their translation and association with ribosomes (Gruissem and Schuster, 1993). For example, the levels of *rbcL* are reduced in maize nuclear mutants with defects in translation (Barkan, 1993). However, in tobacco plants transformed with antisense constructs against *rbcS* and which consequently show reduced translation and polysome association of *rbcL* mRNA (see below), the levels of *rbcL* mRNA are normal (Rodermel *et al.*, 1996). Similarly, in a *C. reinhardtii* mutant lacking the *rbcS* genes, *rbcL* translation is inhibited but mRNA levels are normal (Khrebtukova and Spreitzer, 1996). Inhibitor studies in spinach indicate that the *rbcL* and *psbA* mRNAs are less stable when they are associated with many ribosomes (chloramphenicol arrest of elongation) than when they are depleted of ribosomes (lincomycin treatment) (Klaff and Gruissem, 1991). The difference between these results could reflect differences in the control of *rbcL* stability in different plants, pleiotropic effects of maize mutations, or properties of the inhibitors used. For instance, lincomycin may stall a ribosome near the initiation site and thus mask a *cis*-acting element involved in mRNA degradation (Barkan, 1993).

In *C. reinhardtii*, a frameshift mutation in *psaB* is accompanied by an increase in the steady-state level of the *psaB* mRNA while its transcription is unaffected, indicating a change in RNA turnover (Xu *et al.*, 1993). In contrast, in a mutant of *psbD* (FUD47) with a small (46 bp) duplication causing a frameshift, *psbD* mRNA levels are reduced (Erickson *et al.*, 1986). Site-directed deletions of the *petD* 5′ leader that affect translation also cause reduced mRNA accumulation (Sakamoto *et al.*, 1993, 1994a). Similarly, site-directed mutations that alter the postulated ribosome-binding site of *psbA* mRNA also cause a strong reduction of mRNA levels (Mayfield *et al.*, 1994). Whether these are indirect effects of translation on mRNA stability or direct effects of the mutations on *cis*-acting RNA stability determinants is difficult to distinguish in these cases.

In nuclear mutants that affect the translation of a specific chloroplast mRNA, the sequence of the chloroplast transcript is unaltered, and changes in its accumulation are more clearly linked to its translation (unless the mutation is pleiotropic and separately affects both translation and RNA accumulation). In the nuclear *F54* mutant, synthesis of the α-subunit of ATP synthase is impaired, and the *atpA* mRNA accumulates to higher levels (Drapier *et al.*, 1992). In the *nac1* mutants which are probably defective in the translation of D2 or in the stabilization of the nascent polypeptide, the levels of *psbD* mRNA are similarly increased (Kuchka *et al.*, 1988; see section IV.D). However, in nuclear mutants that affect *psbA* (*F35*) or *psbC* translation (*F34, F64*), the respective mRNA levels remain unchanged (Girard-Bascou *et al.*, 1992; Rochaix *et al.*, 1989).

These indirect effects of translation or polysome assembly make it more difficult to determine whether changes in the accumulation of certain mRNAs are the primary defect in a mutant or are a consequence of an alteration of translation (section IV.B.1). This may, for example, apply to the *hcf2* mutant of *Arabidposis,* which has pleiotropic defects in many photosynthetic complexes. In *hcf2* the steady-state amount of *petA* mRNA is higher than that in the wild type (Dinkins *et al.*, 1994). It is not yet known whether this excess is due to a defect in processing or in translation of the *petA* mRNA.

C. Translation

The ribosomal RNAs, tRNAs, and some of the ribosomal proteins are encoded in the plastid genome, but many genes for ribosomal proteins, tRNA synthetases, and translation factors are located in the nucleus (Subramanian *et al.*, 1991; Subramanian, 1993; Gillham *et al.*, 1994; Harris *et al.*, 1994). Beside this general involvement of nuclear gene products in the basic translation machinery, nuclear mutations reveal factors that are required for translation of specific chloroplast mRNAs. Biochemical studies have focused on proteins that bind to the 5′ UTR of chloroplast mRNAs and which are thought to play a role in translation and its regulation.

1. RNA-Binding Proteins and Translation

In *C. reinhardtii* extracts, at least six proteins were identified that bind in vitro to the 5′ leaders of a series of chloroplast mRNAs encoding photosynthetic or ribosomal polypeptides (Hauser *et al.*, 1996). Some of these bind to the leaders of several different mRNAs, but others are more specific. There are changes in the spectrum of these RNA-binding proteins depending on the growth conditions (light and carbon source) or when overall

translation activity is altered (a condition which is known to favor translation of ribosomal protein mRNAs over photosynthetic protein mRNAs). A direct role for these proteins in translation has not yet been demonstrated.

A protein complex from *C. reinhardtii* binds to the 5′ leader of the *psbA* mRNA in a region that contains a hairpin-loop and is required for translation (Danon and Mayfield, 1991; Mayfield *et al.,* 1994). When light-grown versus dark-grown cells are compared, the increased *psbA* mRNA-binding activity *in vitro* parallels to a certain extent the highly active *psbA* translation *in vivo.* The abundance of a major component, the 47-kDa polypeptide, is only moderately elevated, but its slightly different electrophoretic properties suggest that it may undergo posttranslational modification. The *y-1* mutant of *C. reinhardtii* is yellow in the dark: it does not synthesize chlorophyll, is depleted of thylakoids, and accumulates *psbA* mRNA but does not translate it (Malnoe *et al.,* 1988). In dark-grown *y-1* cells, the amount of 47-kDa polypeptide is reduced, and the *psbA* mRNA-binding activity is not detected. These correlations between *psbA* mRNA translation and the binding activity of the complex are interpreted as an indication of a role in translational regulation. The mRNA-binding activity is inhibited by incubation with ADP, or less efficiently with ATP (Danon and Mayfield, 1994a). This is accompanied by phosphorylation of a 60-kDa component of the complex; however, the activity cannot be restored by treatment with phosphatases. The RNA-binding activity is also abolished by incubation with dithionitrobenzoic acid, an oxidizing agent, and is restored by treatment with dithiothreitol or reduced thioredoxin, but not β-mercaptoethanol, indicating that the inhibition may be due to the formation of a disulfide bond between vicinal thiols (Danon and Mayfield, 1994b). It is interesting that thioredoxins similarly regulate the activity of many chloroplast enzymes as a function of the reducing power produced by photosynthesis (Buchanan, 1991; Buchanan *et al.,* 1994). In dark-grown cells where translation of *psbA* mRNA is low, ADP levels in the chloroplast are expected to be high and reduced thioredoxin to be low: this corresponds to conditions where the *in vitro* binding activity is also low. The nuclear mutant *F35* is deficient in *psbA* translation and shows somewhat reduced levels of *psbA* mRNA (Girard-Bascou *et al.,* 1992). In *F35,* the *psbA* mRNA-binding activity and the amount of the 47-kDa polypeptide are lower, while the 60-kDa polypeptide is unchanged compared to wild type (Yohn *et al.,* 1996). Taken together, these results show changes in the *psbA* mRNA-binding activity that correlate qualitatively with the rate of translation of the *psbA* mRNA. A complication arises because there are two forms of the *psbA* RNA with different 5′ ends: a minor transcript containing the hairpin-loop with the binding site for the complex, and a much more abundant shorter form which lacks it (Rochaix, 1996). Whether the complex is

necessary for *psbA* mRNA translation and plays a direct role in translational regulation has not yet been demonstrated.

Translation of the Rubisco LS is modulated by the availability of the nuclear-encoded SS in tobacco and in *C. reinhardtii* (Rodermel *et al.*, 1996; Khrebtukova and Spreitzer, 1996). This is revealed in transgenic plants with antisense constructs directed against *rbcS* which have reduced levels of this mRNA. The antisense transformants accumulate coordinately reduced amounts of SS and LS. The decrease in LS is at least in part due to reduced translation of *rbcL* mRNA as measured by pulse-labeling and by polysome distribution (Rodermel *et al.*, 1996). Similarly, in a *C. reinhardtii* mutant with a deletion of the nuclear *rbcS* genes, there is a defect in the translation of the LS (Khrebtukova and Spreitzer, 1996). This highlights a control at the translational level in the plastid exerted, directly or indirectly, by the availability of the nuclear-encoded subunit. Degradative turnover of unassembled subunits also plays a role in the coordinate accumulation of SS and LS (see below).

A promising development for the study of translation regulation is the establishment of an *in vitro* translation assay from tobacco chloroplasts (Hirose and Sugiura, 1996). Using this assay, *cis*-acting elements have been identified in the *psbA* 5′ UTR, and an activity that binds one of them has been detected. Tobacco chloroplast transformation with chimeric genes has demonstrated the involvement of the *psbA* 5′ UTR in light regulation (Staub and Maliga, 1994). The conjunction of these *in vitro* and *in vivo* approaches should allow interesting developments in our understanding of chloroplast translation.

2. Nuclear Mutations That Affect Plastid Translation

There are many mutations in nuclear genes which have an effect on translation. Some of them interfere with chloroplast translation in general, because they affect components of the translation machinery, for example, ribosomal RNA or ribosomal proteins. Others are much more specific and affect a subset of chloroplast proteins or a single one.

The ribosome-deficient mutants *iojap* in maize and *albostrians* in barley have been described above (section II.D.1). Other less extreme mutants in maize which are light green and deficient in photosynthesis (*cps1, cps2, hcf7*) seem to have a general defect in chloroplast translation: they have reduced numbers of ribosomes associated with mRNA as deduced from polysome sedimentation in sucrose gradients (Barkan, 1993). One of these mutants has a defect in 16S ribosomal RNA processing (*hcf7;* see section IV.B.2).

There are a number of *C. reinhardtii* mutants with reduced numbers of ribosomes (Harris *et al.*, 1994). One of these mutants is impaired in splicing

of the 23S rRNA intron as well as other group I introns of *psbA* (*ac20;* see section IV.B.3; Herrin *et al.,* 1990, 1991). This general effect on splicing indicates that defective 23S RNA splicing may be the cause of the ribosome deficiency rather than a consequence. In another pair of allelic mutants, a nuclear-encoded protein of the chloroplast ribosome (L-29) is not synthesized (*cr-6* and *cr-7;* Myers *et al.,* 1984). A chloroplast-encoded protein, L-13, is also missing from the ribosomes but is synthesized normally. This suggests that L-29 is required for the stable assembly of L-13 into the 50S subunit.

In the *crp1* mutant of maize, there is a specific defect in the translation of two subunits of the cytochrome b_6f complex, encoded by *petA* and *petD* (Barkan *et al.,* 1994). While the *petD* deficiency seems due to a failure in processing the polycistronic precursor mRNA such that the translation initiation signals are masked by secondary structure (see section IV.B.2), the *petA* mRNA is apparently normal. The defect in *petA* translation may reflect a coupling between *petD* and *petA,* such that expression of one is dependent on the other. Alternatively, the *crp1* mutation may separately affect *petD* processing and *petA* translation.

In *C. reinhardtii,* there are several loci that may govern the translation of individual chloroplast genes. In these mutants, protein pulse-labeling experiments reveal the absence of a single polypeptide, even though its mRNA is present. Such results suggest that a mutation either affects translation or causes very rapid proteolytic degradation. A polypeptide would not be detected by pulse-labeling if its half-life is shorter than the pulse-labeling time, or if the nascent chains are degraded before translation is completed. In some cases, chloroplast transformation with chimeric genes shows that the effect is mediated by the 5′ untranslated leader of the relevant chloroplast mRNA and therefore that the defect is in translation initiation. The mutations *F34* and *F64* affect *psbC* translation (Rochaix *et al.,* 1989; Zerges and Rochaix, 1994); *F15* prevents translation of *psaB* (Girard *et al.,* 1980; see below) and *F54* blocks translation of *atpA* (Drapier *et al.,* 1992). The effect of *F35* on *psbA* (Girard-Bascou *et al.,* 1992) may be at the level of translation or of rapid degradation. Although the *psbD* defect in *ac115* and in two alleles of *nac1* (Kuchka *et al.,* 1988; Wu and Kuchka, 1995) may be due to very rapid degradation (see section IV.D), a defect in translation elongation is also possible.

The nuclear mutations that affect *psbC* translation have been studied in more detail. The effects of mutations *F34* and *F64* (in the *TBC1* and *TBC2* loci, respectively) are exerted through the 5′ untranslated leader of *psbC,* as shown *in vivo* by chloroplast transformation with chimeric mRNAs consisting of *psbC–aadA* fusions (Zerges and Rochaix, 1994). It is therefore the initiation of translation which is altered in these mutants. This is consistent with the finding that the nuclear *F34* mutant can be suppressed by a

chloroplast mutation in the *psbC* 5′ leader (*F34suI*). The suppressor mutation alters the stem of a large predicted stem–loop structure. The same putative element of secondary structure is altered by a chloroplast mutation (*FUD34*) that also blocks *psbC* translation (Rochaix *et al.*, 1989). A suppressor of the chloroplast *FUD34* mutation defines a third nuclear locus (*TBC3*) which is also involved in *psbC* translation (W. Zerges *et al.*, 1997). This dominant suppressor, *tbc3-rb1*, can also rescue the translation deficiency caused by deleting the stem-loop in the 5′UTR of *psbC*. Furthermore, *tbc3-rb1* suppresses the *tbc1-F34* mutation, suggesting a functional interaction between *TBC1*, *TBC3*, and the 5′ UTR. The *TBC* loci may be required to control *psbC* translation in a manner that allows proper integration of the PsbC polypeptide (P6, the 43-kDa subunit) into PSII.

The nuclear mutation *F15* (in the *TAB1* locus) affects *psaB* translation (Girard *et al.*, 1980). Chloroplast transformation with *psaB–aadA* chimeras shows that the effect of the mutation is mediated by the 5′ UTR, again indicating that translation initiation is probably affected (Stampacchia *et al.*, 1997). This is also indicated by a chloroplast suppressor of *F15* which has a mutation in the 5′ UTR of *psaB*. The suppressor mutation is predicted to destabilize a secondary structure that would otherwise mask the putative Shine–Dalgarno sequence near the translation initiation codon (Stampacchia *et al.*, 1997). The wild-type product of the *TAB1* locus may thus be directly or indirectly involved in making the 5′ UTR of *psaB* available for translation.

D. Assembly versus Degradation

Many chloroplast complexes are assembled from multiple subunits of nuclear and chloroplast origin, together with cofactors such as pigments and metal ions. There is considerable evidence that failure to assemble a polypeptide into a complex can lead to its degradation and that when one subunit of a complex is missing, the other subunits can become unstable (Rochaix, 1992, 1996; Gruissem and Tonkyn, 1993; Miles, 1994; Barkan *et al.*, 1995). This proteolytic control probably plays an important role in the coordinate accumulation of polypeptides originating from the two compartments. Lack of a cofactor or pigment can also lead to instability of the apoprotein. Mutations that prevent the synthesis of one subunit of a complex usually cause a strong reduction of other subunits, so that the primary defect is masked. The affected subunit can in some cases be distinguished from those that are subsequently degraded by pulse-labeling with radioactive precursors to assess their respective rates of synthesis. When the lack of synthesis of one subunit is due to a mutation that affects the accumulation of its mRNA, the primary defect can be revealed by hybridization with

appropriate gene probes. In these cases, the synthesis of other subunits proceeds normally and can be monitored by pulse-labeling because degradation is a subsequent process that occurs more slowly than synthesis. However, there are also mutations that reveal a much more rapid process which may involve the elongation step of translation or the very fast degradation of a nascent polypeptide (see below). Thus there are active controls at the posttranslational level in the plastids which are apparently tightly coupled with protein assembly.

The nuclear mutant *viridis-115* of barley is deficient in PSII, with a defect in the synthesis of D1 and CP47 that becomes apparent during the course of light-induced development (Gamble and Mullet, 1989). The levels of *psbA* mRNA (coding for D1) actually increase to greater than normal levels, and part of the mRNA is associated with ribosomes with the characteristic translational pauses that are also found in the wild type (Kim *et al.,* 1994a). However, when isolated mutant plastids are labeled *in vitro,* a lack of D1 synthesis is apparent, and translational intermediates are not observed. The lack of D1 accumulation may thus be due to a defect in the stabilization of the nascent D1 polypeptide. Chlorophyll-a is necessary for D1 apoprotein stabilization, and ribosome pausing may facilitate assembly of the pigments with the nascent polypeptide (Mullet *et al.,* 1990; Kim *et al.,* 1991, 1994b). It is proposed that the *vir-115* gene product may be required during the assembly of D1 into photosystem II, perhaps to assist cofactor binding or protect the nascent polypeptide lacking the cofactors (Kim *et al.,* 1994a). This is distinctly different from the light regulation of D1 translation initiation mediated by the 5′ UTR of the mRNA, as observed in transgenic tobacco plastids transformed with chimeric constructs (Staub and Maliga, 1994).

In the nuclear *nac1* and *ac-115* mutants of *C. reinhardtii,* synthesis of the D2 polypeptide, the product of the chloroplast *psbD* gene, is undetectable in short pulse-labeling experiments even though the mRNA is actually overaccumulated (Kuchka *et al.,* 1988; Wu and Kuchka, 1995). However, chloroplast transformation with chimeric genes shows that the 5′ leader of *psbD* is not sufficient to mediate the effect of the *nac1* mutations (Rochaix, 1996). Therefore, the defect must occur at a step later than translation initiation. This is also supported by the observation that in the *nac1* mutant, *psbD* mRNA is associated with ribosomes (H. Wu and M. Kuchka, personal communication). The lack of D2 synthesis in very short pulse-labeling protocols indicates either a block in translation elongation or very rapid turnover of D2. The wild-type products could be required for the assembly or folding of newly synthesized D2. It is possible that in their absence, nascent polypeptide chains could be degraded in these mutants. A dominant nuclear suppressor of *nac1-18* called *sup4b* can also suppress the other alleles, *nac1-11* and *ac-115* (Wu and Kuchka, 1995). This ability to suppress

mutations in different genes indicates that *sup4b* bypasses the requirements for *NAC1* and *AC115*. The *sup4b* mutation identifies a novel locus involved in the expression of D2.

Although little is known on the proteases involved in the degradation of unassembled or misassembled subunits of plastid complexes, there is an interesting correlation that relates to the ClpAP protease. In *E. coli,* this is a macromolecular complex composed of protease subunits (ClpP) and ATPase subunits (ClpA) which are required for substrate presentation to the protease (Goldberg, 1991). ClpA is part of a family of related ATPases (clpA/B/C), some of which have a function as molecular chaperones (Squires and Squires, 1992). Thus there is a close connection between protein folding and protein degradation, and the ATPase subunits could function to present polypeptides either for proper folding and assembly or to target them for proteolysis. The chloroplast genome harbors a *clpP* gene, which is essential for cell growth in *C. reinhardtii* (Huang *et al.,* 1994). In some algae the plastid genome also contains a *clpC* gene (Reith, 1995; Reardon and Price, 1995). In vascular plants, the nuclear genome contains the genes for ClpC-related polypeptides with chloroplast-targeting sequences (Gottesman *et al.,* 1990; Moore and Keegstra, 1993; Kiyosue *et al.,* 1993; Vierstra, 1993). The tomato nuclear genome also encodes a ClpP subunit with a putative chloroplast import sequence (Schaller and Ryan, 1995). The *clpP* and *clpC* gene products are localized in the stroma of *Arabidopsis* chloroplasts and are ubiquitously expressed in the plant (Shanklin *et al.,* 1995). It will be interesting to elucidate the possible role and substrate specificity of the Clp proteins in plastid protein assembly and degradation. It is tempting to speculate that rapid degradation of nascent polypeptide chains that cannot be assembled could be mediated by a chaperone that would present the polypeptides for membrane insertion or assembly, but also target them to a competing protease if assembly is delayed or fails.

E. Biogenesis of Cytochromes

In *C. reinhardtii,* there is a class of mutants with defects in the accumulation of the cytochrome b_6f complex as well as of cytochrome c_6. Cyt c_6 is a soluble lumenal protein that can substitute for the copper-protein plastocyanin in the transfer of electrons from the b_6f complex to PSI when copper ions are limiting. Three nuclear mutants (*F2D8, F18,* and *ac206*) belong to this class, as well as a chloroplast mutant (*B6*) affected in the *ccsA* gene (*c-type cytochrome synthesis;* Howe and Merchant, 1992; Xie and Merchant, 1996). In these mutants, pre-cyt c_6 is translated, imported to the chloroplast, and matured to apo-cyt c_6, but heme attachment does not occur and the

apoprotein is degraded. This class of mutants is characterized by the concomitant deficiency in the cytochrome b_6f complex which contains the other plastid c-type cytochrome (cyt f), but by the normal presence of cyt b_{559} in PSII and of cytochrome *c* in mitochondria. Thus the primary defect in these mutations is apparently not with the synthesis of heme, but may be with the accumulation of the c-type holocytochromes in the plastid. The mutants may be affected in genes involved in the delivery or the attachment of heme to the c-type apocytochromes: they could be deficient in chaperones, heme-binding proteins, heme transporters, or heme lyase. There are also two nuclear mutants of *C. reinhardtii* which may be defective in the insertion of the noncovalently bound hemes in cytochrome b_6 (Gumpel *et al.,* 1995).

There are also nuclear mutants of maize which are specifically lacking the cytochrome b_6f complex: two transposon-induced alleles of *pet2* and two of *pet3* (*photosynthetic electron transport*) as well as the chemically induced *hcf6* mutant which is an allele of *pet3* (Voelker and Barkan, 1995a). Cytochrome b_{559} of PSII is not affected in moderate light, indicating normal heme synthesis. In high light, the mutants are also defective in PSII, probably as a secondary effect of the b_6f deficiency. Pulse-labeling experiments show that in the *pet2* and *pet3* mutants, the core subunits of the complex are synthesized and processed normally, but they fail to accumulate. The mutants may, therefore, be deficient in a posttranslational step of cytochrome b_6f assembly, analogous to the *C. reinhardtii* mutants described above. Alternatively, a defect in the expression of another subunit of the complex may lead to its instability.

F. Protein Import and Targeting

Protein import into the plastid, and sorting of proteins (both nuclear and plastid encoded) to the many different compartments of the organelle, is beyond the subject of this review. However, these processes have an importance for the expression of many genes, since the products are usually not functional if they are not properly delivered. Furthermore, the lack of one subunit can prevent the assembly of a complex and cause the degradation of its components (see section IV.D). In this sense, import and targeting are relevant to nuclear and chloroplast gene expression, as highlighted by nuclear mutants that affect the targeting of proteins to the thylakoid.

Nuclear-encoded proteins that are destined to the thylakoid lumen have bipartite N-terminal targeting sequences, with a domain for import into the chloroplast stroma and a second domain for thylakoid targeting (Theg and Scott, 1993; Robinson and Klösgen, 1994; Voelker and Barkan, 1995b). Upon import into the chloroplast the first domain is removed to yield

intermediates which are lumenal targeting precursors. There are also chloroplast-encoded thylakoid proteins such as Cyt*f*, which is an integral membrane protein with a large lumenal domain. Cyt*f* is synthesized as a precursor with only a cleavable N-terminal lumenal targeting sequence. *In vitro* studies of targeting to the thylakoid membrane and the thylakoid lumen have revealed three pathways with different requirements for energy and cofactors and distinct substrate specificities. Thylakoid translocation of one group of polypeptides (e.g., plastocyanin and OEC33, the 33-kDa subunit of the oxygen evolving complex of PSII) is dependent on ATP and a stromal factor probably related to the bacterial SecA secretory protein. Import of another group of polypeptides is dependent on the membrane ΔpH (e.g., OEC23 and OEC17). The integration of the Cab (Lhcb) protein into the thylakoid membrane follows a third pathway and involves a chloroplast homologue of SRP54, a component of the signal recognition particle. It is not clear whether these pathways are truly separate or whether they share certain components.

Two nuclear mutants of maize, *hcf106* and *tha1* (*thylakoid assembly*), have pleiotropic reductions in the accumulation of the photosynthetic complexes resulting in pale-green, nonphotosynthetic seedlings (Barkan *et al.*, 1986; Martienssen *et al.*, 1989; Voelker and Barkan, 1995b). In these two mutants, two different subsets of lumenal proteins accumulate as intermediate-sized precursors probably retaining the lumenal targeting sequences (Voelker and Barkan, 1995b). These accumulated precursors are located outside of the thylakoids. The mutations thus identify two nuclear loci that are involved in targeting polypeptides to the lumen. The subsets of proteins that are affected in each mutant correspond to two of the classes defined *in vitro* for the different thylakoid-targeting pathways: OEC33 and plastocyanin in *tha1*, and OEC16 and OEC23 in *hcf106*. The integration of Cyt*f* is also retarded in *tha1*, indicating that this plastid-encoded protein also depends on at least one component of the machinery involved in targeting nuclear-encoded proteins. The *hcf106* and *tha1* mutations are both unstable due to the insertion of transposable elements, and the genes have been cloned (Martienssen *et al.*, 1989). The Hcf106 protein is found in thylakoid and in envelope membranes, suggesting that it may be part of a more general pathway (R. A. Martienssen, personal communication). The *Tha1* gene encodes a polypeptide similar to the bacterial *SecA*, which is involved in protein secretion, in good agreement with the defect in thylakoid targeting caused by the *tha1* mutation and with the *in vitro* studies (Voelker *et al.*, 1997). In the chloroplast mutant *pm7* of *Oenothera*, there is an accumulation of a precursor of Cytf, as well as of precursors of the nucleus-encoded OEC23 and OEC16 (Johnson and Sears, 1990b; Johnson *et al.*, 1991). The basis of this defect is unknown, but it again suggests that nucleus- and

chloroplast-encoded polypeptides share common thylakoid-targeting or processing components.

In *C. reinhardtii,* a genetic approach has identified nuclear loci involved in Cyt*f* targeting (Smith and Kohorn, 1994). Chloroplast transformation allows the introduction of site-directed mutations in the cleavable presequence of Cyt*f.* Some of these mutations cause a lack of Cyt*f* and the accumulation of Cyt*f* precursor and impair photosynthetic function. The mutations thus apparently interfere with the targeting of Cyt*f* to the thylakoid. Starting from these mutants, selection for restoration of photosynthetic growth yields many extragenic suppressors, including some at five distinct *TIP* nuclear loci (*thylakoid insertion protein;* B. Kohorn, personal communication). These new loci should help identify components of the thylakoid-targeting machinery.

V. Signaling between the Plastid and the Nucleocytosol

In the preceding sections, we have seen many examples of how nucleus-encoded proteins are imported into the plastid where they are involved in different aspects of plastid gene expression and thus mediate control of the plastid by the nucleocytoplasmic compartment. In other cases, mutations that affect plastid development or gene expression reveal control, but it is not clear whether the gene products are imported and directly implicated or whether they indirectly signal to the plastid. There are also examples of nuclear genes whose products are clearly not in the plastid, but influence its genetic activity, implying that some sort of signal must be transduced from one compartment to the other. Little is known on the nature of such signals or how they are transduced and perceived in the plastid. Obviously the signals could be other polypeptides that are imported into the plastid. Whether metabolites or secondary messengers are also involved is not known, nor is it known whether nucleic acids can be imported into the plastids.

The expression of some nuclear genes is influenced by the state of the plastid. The best example comes from studies with agents or mutations that lead to photo-oxidative destruction of the chloroplast (Mayfield and Taylor, 1984; Oelmüller, 1989; Taylor, 1989; Susek and Chory, 1992). When the synthesis of carotenoids is blocked by mutation or prevented by treatment with the herbicide Norflurazon, exposure of the plant to light causes damage to the plastids. Excited triplet chlorophyll is not quenched for lack of carotenoids, leading to the formation of reactive radicals. This damage to the plastid is associated with a reduction in the expression of certain nuclear

genes implicated in plastid function. The expression of the *Cab* (or *Lhcb*) genes encoding the light-harvesting chlorophyll a/b proteins (LHCII) is notably reduced, at least in part by a decrease in transcription (Batschauer *et al.*, 1986; Burgess and Taylor, 1988). The expression of certain other nuclear genes encoding plastid proteins, or proteins with plastid-related functions, can be similarly affected to various degrees (Oelmüller and Briggs, 1990; Tonkyn *et al.*, 1992; Conley and Shih, 1995; Bolle *et al.*, 1994; 1996; Kusnetsov *et al.*, 1996). The inhibition is not observed in the absence of photodamage, for instance, if carotenoid-deficient seedlings are kept in far-red light or low-intensity white light, or in mutants lacking chlorophyll (Oelmüller, 1989). The accumulation of *Cab* mRNA or *Elip* mRNA (early light-induced protein) can also be depressed by treatments that interfere with chloroplast development such as inhibitors of chloroplast transcription or translation applied at early stages (Rapp and Mullet, 1991; Susek *et al.*, 1993; Adamska, 1995). Mutations that block plastid development early have similar effects, for example, the plastome mutation SR1V35 in tobacco (Bolle *et al.*, 1994), the *chm* nuclear mutation in *Arabidopsis* (Susek *et al.*, 1993; see section II.C), or the *dag* and *dcl* mutations (described in section III.A). This regulation of nuclear genes is observed in developing seedlings from many species of plants (Susek and Chory, 1992), and also in mature spinach, more prominently in young leaves than in older ones (Tonkyn *et al.*, 1992). The general conclusion from these studies is that the expression of many nuclear genes is influenced by the functional state of the plastids and that the regulation is at least in part due to changes in transcriptional activity. The nature of the signal(s) emanating from the plastid is still elusive.

The *gun* mutants of *A. thaliana* (*genomes uncoupled*) are defective in the response of nuclear genes to plastid photodamage (Susek *et al.*, 1993). The endogenous *Cab* and *Rbcs* genes are expressed in *gun1-1* mutant plants photobleached by treatment with Norflurazon. Reporter genes driven by a *Cab* promoter are also expressed in bleached mutant plants, indicating that the effect is (at least in part) at the transcription level. The *gun1-1* mutation can also relieve the reduced expression of the reporter when plastid development has been affected by treatment with chloramphenicol or by the *chm* mutation. The *gun* mutations identify at least three nuclear genes (*GUN1, GUN2,* and *GUN3*) that are required for the coordination of nuclear gene expression with chloroplast function. Because the *gun* mutations are recessive, it can be argued that they probably affect a repressive signaling pathway from the plastid to the nucleus.

In *C. reinhardtii,* inhibitors or mutations that interfere with the later steps of chlorophyll synthesis cause a decrease in the levels of specific nuclear gene transcripts like *Cab* or *RbcS1,* but inhibitors of the early steps of the pathway do not (Johanningmeier and Howell, 1984; Johanningmeier,

1988). It is therefore not the lack of chlorophyll synthesis but rather the particular intermediates that accumulate which are relevant. These observations indicate that the accumulation of porphyrin compounds produced in the plastid may influence the activity of certain nuclear genes. In tobacco, the accumulation of coproporphyrinogen and coproporhyrin can be caused by the reduced activity of coproporphyrinogen oxidase in transformed plants expressing antisense RNA (Kruse *et al.*, 1995). This leads to a reduction of *Cab* (*Lhcb*) mRNA levels and a deregulation of *Elip* mRNA accumulation. However, in these experiments, the plants were exposed to light and the accumulation of coproporphyrin(ogen) led to photo-oxidative damage. It is therefore probable that the effect on *CAB* and *ELIP* mRNAs is due to photodamage, although it remains possible that the porphyrin compounds have a more direct effect similar to that observed in *C. reinhardtii.*

In the green alga *Dunaliella tertiolecta,* acclimation to changes in light intensity are accompanied by converse changes in chlorophyll, in LHCII protein amounts, and in the Cab (*Lhcb*) mRNA levels which are controlled at the transcriptional level (Escoubas *et al.,* 1995). Treatments with inhibitors of photosynthetic electron transport that modify the redox state of the plastoquinone pool can similarly affect the accumulation of *Cab* mRNA. This leads to a model where a sensor of the redox state in the chloroplast transduces a signal that can regulate *Cab* gene expression in the nucleocytoplasmic compartment.

The chloroplast and photosynthesis are involved in nitrogen metabolism. There are observations that may indicate an effect of the chloroplast on nuclear gene expression in this respect, although alternative explanations are possible. Nitrate is reduced to nitrite by nitrate reductase in the cytoplasm, and nitrite is then reduced to ammonium in the chloroplast. Norflurazon-induced photo-oxidation of squash cotyledons in the light interferes with the accumulation of nitrate reductase mRNA and enzyme in the cytoplasm (Oelmüller and Briggs, 1990; Oelmüller, 1989). Whether this is due to a feedback mechanism in the cytoplasm because nitrite cannot be reduced in the damaged plastid or is due to a signal from the plastid is not known. Nitrogen limitation in *C. reinhardtii* causes a chlorophyll deficiency, which is accompanied by a moderate reduction of chloroplast transcripts from photosynthetic genes, but a marked reduction in the *Cab* and *RbcS* mRNAs (Plumley and Schmidt, 1989). Although this reduction could be influenced by the chloroplast, it may alternatively result from a more direct regulation of nuclear gene expression as a function of nitrogen availability.

Although protein import into the chloroplast is not discussed in this review and is only part of gene expression in a broad sense as briefly discussed above (section IV.F), there is an interesting coupling of protein import to plastid function. In etioplasts, a major component of the prolamel-

lar body is PorA (protochlorophyllide oxydoreductase; see section III.B). PorA accumulates as a complex with its substrate (and NADPH), but is degraded in its free form (Reinbothe *et al.*, 1996a). Import of PorA into isolated etioplasts and chloroplasts is quantitatively dependent on protochlorophyllide (Reinbothe *et al.*, 1995). Thus the accumulation of protochlorophyllide synthesized in the plastid envelope may regulate the amount of POR A protein that is imported. This specific effect is distinct from the general import capacity of plastids for various polypeptides, which changes during development and declines from high competence in young tissue to lower levels in mature leaves (Dahlin and Cline, 1991). The import competence of mature etioplasts for Cab (Lhcb) protein is transiently elevated during greening. Whether this import capacity is controlled by the nucleocytoplasmic compartment, by the plastid, or both is not known.

While there is considerable evidence for signaling from the plastids to the nuclear genome, many questions remain concerning the nature of the signal(s), the transduction pathways, and their targets. The signals could be metabolites, second messengers, or macromolecules although there is so far no evidence for protein or nucleic acid export from the plastids. While the lack of nuclear gene expression in the absence of plastid development could be taken to suggest the requirement for positive signaling from the differentiating plastid, the recessive *gun* mutations suggest repressive components from damaged plastids. Protochlorphyllide is required for PorA import into the etioplast and thus acts positively, while some porphyrin precursors of chlorophyll biosynthesis may inhibit nuclear gene expression in *C. reinhardtii*. There is clearly still much to be learned about these multiple regulatory interactions.

VI. Concluding Remarks

A striking feature of the nuclear mutants that affect chloroplast gene expression is that most of them act at posttranscriptional levels. This is in keeping with observations on chloroplast gene expression that show that regulation is also largely, but not exclusively, at posttranscriptional steps. This review is concerned with gene expression, focusing on transcription, mRNA accumulation, and translation. However, additional steps are involved to make or keep a gene product functional, like posttranslational modification and protein processing, import into organelles, suborganellar targeting, assembly with cofactors, assembly into multisubunit complexes, and protein degradation. Some of these have been briefly approached here because they are altered by mutations with phenotypes similar to those affecting gene expression. But there are many more interesting implications of these post-

translational steps for the coordination of chloroplast and nucleocytosolic function. Many of the factors mediating these steps in the chloroplast, like chaperones, components of the import apparatus or proteases must be encoded in the nucleus.

One of the more surprising aspects concerning the interactions of the nucleus with the plastid is the large number of nuclear loci which are specifically required for the expression of single chloroplast genes or transcription units. This is particularly true in *C. reinhardtii* for *psaA trans*-splicing, for the stability of different RNAs, and for translation. Some mutations in vascular plants also show high specificity, but often a small set of genes is affected. This does not imply that more general factors affecting many or all genes are not also involved, and obviously many nuclear genes encode "housekeeping" components of the chloroplast gene expression machinery. It is likely that mutations in such factors would have the effect of blocking chloroplast function completely, as illustrated, for example, by the plastid deficiency in the *iojap* mutant of maize, which is probably due to a defect in a ribosomal protein. Similar mutants in *C. reinhardtii* would be lethal and would not be recovered in genetic screens. Thus, for instance, screening for plastids which are functional enough to exhibit high chlorophyll fluorescence may actually favor mutations in the class of gene-specific nuclear factors.

The large number of loci that the mutations reveal raises the question of how many nuclear genes are involved in chloroplast function. For many chloroplast genes in *C. reinhardtii,* there are several loci involved, sometimes in different steps of gene expression. Many of these loci are represented by single alleles, implying that genetic saturation has not been reached and that some loci remain to be identified. This would suggest that hundreds of nuclear genes may be involved in chloroplast gene expression.

The nuclear localization of genes for a vast majority of plastid proteins places the plastid under the control of nuclear gene regulation. Hence the question of whether this regulation of imported proteins is sufficient to explain how plastid development is dependent on the differentiation of the cell type in which it resides or whether the expression of plastid genes is regulated by other types of signals from the nucleocytosolic compartment. This is, for example, illustrated by the control of photomorphogenesis. The regulatory processes that govern the differentiation of the plant are being elucidated, as well as their effect on plastid differentiation. But it is not known whether the signal transduction pathways have branches in the plastid. For instance, the pleiotropic *det/cop/fus* mutations affect chloroplast gene expression, but it is not yet clear whether this is mediated by regulating the expression of nuclear proteins imported into the organelle or by a signal transduction chain that alters plastid gene expression more directly. Conversely, plastid development regulates nuclear gene expression and,

perhaps in some cases, cell differentiation, as suggested by *dag* and *dcl* effects on palisade cells. Little is known about the molecular nature of signals that can travel in either direction between the plastids and the nucleocytoplasmic compartment.

A similar question arises about the circadian control of gene expression in the nucleocytosolic compartment (Anderson and Kay, 1996) and in the plastid (Salvador *et al.,* 1993b; Hwang *et al.,* 1996). Is it a clock in the nucleocytosolic compartment that also regulates plastid gene expression and, if so, how is the signal transduced to the plastids? The observation of circadian oscillations of free calcium in the cytosol and in the chloroplast is intriguing in this respect (Johnson *et al.,* 1995). Or do plastids have a separate clock (possibly encoded in the nucleus), perhaps derived from the circadian clock of a cyanobacterial ancestor of the endosymbiotic organelle (Roenneberg and Morse, 1993; Kondo *et al.,* 1994)?

The function of the plastids and of the cell are tightly integrated. Considerable progress has been made in the elucidation of the mechanisms involved. Many plastid genomes have been sequenced, and the cloning of relevant nuclear genes is advancing at an increasing pace. Novel genetic screens have identified mutants that are providing new insight. Much excitement is in store.

Acknowledgments

I thank numerous colleagues in Geneva and elsewhere who have communicated their unpublished data and discussed many aspects of the field. I am grateful to Drs. J.-D. Rochaix and W. Zerges for their critical comments on the manuscript. Some of the work described here is supported by a grant from the Swiss National Fund (31.34014.92) to J.-D. Rochaix and M.G.C.

References

Adamska, I. (1995). Regulation of early light-inducible protein gene expression by blue and red light in etiolated seedlings involves nuclear and plastid factors. *Plant Physiol.* **107,** 1167–1175.

Ahmad, M., and Cashmore, A. R. (1993). HY4 gene of A. thaliana encodes a protein with characteristics of a blue -light receptor. *Nature (London)* **366,** 162–166.

Allen, J. F. (1993). Control of gene expression by redox potential and the requirement for chloroplast and mitochondrial genomes. *J. Theor. Biol.* **165,** 609–631.

Allison, L. A., and Maliga, P. (1995). Light-responsive and transcription-enhancing elements regulate the plastid psbD core promoter. *EMBO J.* **14,** 3721–3730.

Allison, L. A., Simon, L. D., and Maliga, P. (1996). Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants. *EMBO J.* **15,** 2802–2809.

Anderson, S. L., and Kay, S. A. (1996). Illuminating the mechanism of the circadian clock in plants. *Trends Plant Sci.* **1,** 51–57.

Armbrust, E. V., Ferris, P. J., and Goodenough, U. W. (1993). A mating type-linked gene cluster expressed in Chlamydomonas zygotes participates in the uniparental inheritance of the chloroplast genome. *Cell* **74,** 801–811.

Baeza, L., Bertrand, A., Mache, R., and Lerbs Mache, S. (1991). Characterization of a protein binding sequence in the promoter region of the 16S rRNA gene of the spinach chloroplast genome. *Nucleic Acids Res.* **19,** 3577–3581.

Barkan, A. (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. *Plant Cell* **5,** 389–402.

Barkan, A., Miles, D., and Taylor, W. C. (1986). Chloroplast gene expression in nuclear, photosynthetic mutants of maize. *EMBO J.* **5,** 1421–1427.

Barkan, A., Walker, M., Nolasco, M., and Johnson, D. (1994). A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. *EMBO J.* **13,** 3170–3181.

Barkan, A., Voelker, R., Mendel-Hartvig, J., Johnson, D., and Walker, M. (1995). Genetic analysis of chloroplast biogenesis in higher plants. *Physiol. Plant.* **93,** 163–170.

Barnes, S. A., Nishizawa, N. K., Quaggio, R. B., Whitelam, G. C., and Chua, N. H. (1996). Far-red light blocks greening of Arabidopsis seedlings via a phytochrome A-mediated change in plastid development. *Plant Cell* **8,** 601–615.

Barnes, S. A., McGrawth, R. B., and Chua, N. H. (1997). Light signal transduction in plants. *Trends Cell Biol.* **7,** 21–25.

Bartley, G. E., Scolnik, P. A., and Giuliano, G. (1994). Molecular biology of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **45,** 287–301.

Batschauer, A., Mösinger, E., Kreuz, K., Dörr, I., and Apel, K. (1986). The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light-harvesting chlorophyll a/b protein. *Eur. J. Biochem.* **154,** 625–634.

Bendich, A. (1987). Why do chloroplasts and mitochondria contain so many copies of their genome. *BioEssays* **6,** 279–282.

Bennoun, P. (1994). Chlororespiration revisited: Mitochondrial–plastid interactions in Chlamydomonas. *Biochim. Biophys. Acta* **1186,** 59–66.

Bennoun, P., and Delepelaire, P. (1982). Isolation of photosynthesis mutants of Chlamydomonas. *In* Methods in Chloroplast Molecular Biology (M. Edelman, R. B. Hallick, and N. H. Chua, eds.), pp. 25–38. Elsevier, Amsterdam.

Berends-Sexton, T., Christopher, D. A., and Mullet, J. E. (1990). Light-induced switch in barley psbD–psbC promoter utilization: A novel mechanism regulating chloroplast gene expression. *EMBO J.* **9,** 4485–4494.

Blasko, K., Kaplan, S. A., Higgins, K. G., Wolfson, R., and Sears, B. B. (1988). Variation in copy number of a 24-base pair tandem repeat in the chloroplast DNA of Oenothera hookeri strain Johansen. *Curr. Genet.* **14,** 287–292.

Blowers, A. D., Klein, U., Ellmore, G. S., and Bogorad, L. (1993). Functional in vivo analyses of the 3′ flanking sequences of the Chlamydomonas chloroplast rbcL and psaB genes. *Mol. Gen. Genet.* **238,** 339–349.

Bock, R., and Maliga, P. (1995). In vivo testing of a tobacco plastid DNA segment for guide RNA function in psbL editing. *Mol. Gen. Genet.* **247,** 439–443.

Bock, R., Hermann, M., and Kössel, H. (1996). In vivo dissection of cis-acting determinants for plastid RNA editing. *EMBO J.* **15,** 5052–5059.

Bogorad, L. (1975). Evolution of organelles and eukaryotic genomes. Science **188,** 891–898.

Bogorad, L. (1991). Replication and transcription of plastid DNA. *In* "The Molecular Biology of Plastids," Vol. 7A, "Cell Culture and Somatic Cell Genetics" (L. Bogorad and I. K. Vasil, eds.), pp. 93–124. Academic Press, San Diego.

Boinski, J. J., Wang, J. L., Xu, P., Hotchkiss, T., and Berry, J. O. (1993). Post-transcriptional control of cell type-specific gene expression in bundle sheath and mesophyll chloroplasts of Amaranthus hypochondriacus. *Plant Mol. Biol.* **22,** 397–410.

Bolle, C., Sopory, S., Lübberstedt, R. B., Klösgen, R. B., Herrmann, R. G., and Oelmüller, R. (1994). The role of plastids in the expression of nuclear genes for thylakoid proteins studied with chimeric beta-glucuronidase gene fusions. *Plant Physiol.* **105,** 1355–1364.

Bolle, C., Kusnetsov, V. V., Herrmann, R., and Oelmüller, R. (1996). The spinach AtpC and AtpD genes contain elements for light-regulated, plastid-dependent and organ-specific expression in the vicinity of the transcription start sites. *Plant J.* **9,** 21–30.

Bonen, L. (1993). Trans-splicing of pre-mRNA in plants, animals and protists. *FASEB J.* **7,** 40–46.

Bonnema, A. B., Castillo, C., Reiter, N., Cunningham, M., Adams, H. P., and O'Connell, M. (1995). Molecular and ultrastructural analysis of a nonchromosomal variegated mutant. *Plant Physiol.* **109,** 385–392.

Bonnett, H. T., Djurberg, I., Fajardo, M., and Glimelius, K. (1993). A mutation causing variegation and abnormal development in tobacco is associated with an altered mitochondrial DNA. *Plant J.* **3,** 519–525.

Börner, T., and Sears, B. B. (1986). Plastome mutants. *Plant Mol. Biol. Rep.* **4,** 69–92.

Bowler, C., and Chua, N. H. (1994). Emerging themes of plant signal transduction. *Plant Cell* **6,** 1529–1541.

Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N. H. (1994). Cyclic GMP and calcium mediate phytochrome phototransduction. *Cell* **77,** 73–81.

Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., and Sanford, J. C. (1988). Choroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science* **240,** 1534–1538.

Boynton, J. E., Gillham, N. W., Newman, S. M., and Harris, E. H. (1992). Organelle genetics and transformation of Chlamydomonas. *In* "Cell Organelles. Plant Gene Research. Basic Knowledge and Application" (R. G. Herrmann, ed.), pp. 3–64. Springer-Verlag, Vienna.

Buchanan, B. B. (1991). Regulation of CO_2 assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. *Arch. Biochem. Biophys.* **288,** 1–9.

Buchanan, B. B., Schürmann, P., and Jacquot, J. P. (1994). Thioredoxin and metabolic regulation. *Semin. Cell Biol.* **5,** 285–293.

Bülow, S., and Link, G. (1988). Sigma-like activity from mustard (Sinapis alba L.) chloroplasts comferring DNA-binding and transcription specificity to E.coli core RNA polymerase. *Plant Mol. Biol.* **10,** 349–357.

Burgess, D. G., and Taylor, W. C. (1988). The chloroplast affects the transcription of a nuclear gene family. *Mol. Gen. Genet.* **214,** 89–96.

Cabrera y Poch, H. L., Peto, C. A., and Chory, J. (1993). A mutation in the Arabidopsis DET3 gene uncouples photoregulated leaf development from gene expression and chloroplast biogenesis. *Plant J.* **4,** 671–682.

Carpousis, A. J., van Houwe, G., Ehretsman, C., and Krisch, H. M. (1994). Copurification of E. coli RNase E and PNPase: Evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell* **76,** 889–900.

Castle, L. A., and Meinke, D. W. (1994). A FUSCA gene of Arabidopsis encodes a novel protein essential for plant development. *Plant Cell* **6,** 25–41.

Chatterjee, M., Sparvoli, S., Edmunds, C., Garosi, P., Findlay, K., and Martin, C. (1996). DAG, a gene required for chloroplast differentiation and palisade development in Antirrhinum majus. *EMBO J.* **15,** 4194–4207.

Chaudhuri, S., and Maliga, P. (1996). Sequences directing C to U editing of the plastid psbL mRNA are located within a 22 nucleotide segment spanning the editing site. *EMBO J.* **15,** 5958–5964.

Chaudhuri, S., Carrer, H., and Maliga, P. (1995). Site-specific factor involved in the editing of the psbL mRNA in tobacco plastids. *EMBO J.* **14,** 2951–2957.

Chen, Q., Adams, C. C., Usack, L., Yang, J., Monde, R. A., and Stern, D. B. (1995). An AU-rich element in the 3′ untranslated region of the spinach chloroplast petD gene participates in sequence-specific RNA–protein complex formation. *Mol. Cell. Biol.* **15,** 2010–2018.

Cheung, A. Y., Bogorad, L., Van Montagu, M., and Schell, J. (1988). Relocating a gene for herbicide tolerance: A chloroplast gene is converted into a nuclear gene. *Proc. Natl. Acad. Sci. USA* **85,** 391–395.

Chiu, W. L., Johnson, E.-M., Kaplan, S. A., Blasko, K., Sokalski, M. B., Wolfson, R., and Sears, B. B. (1990). Oenothera chloroplast DNA polymorphisms associated with plastome mutator activity. *Mol. Gen. Genet.* **221,** 59–64.

Choquet, Y., Goldschmidt-Clermont, M., Girard-Bascou, J., Kück, U., Bennoun, P., and Rochaix, J. D. (1988). Mutant phenotypes support a trans-splicing mechanism for the expression of the tripartite psaA gene in the C. reinhardtii chloroplast. *Cell* **52,** 903–913.

Chory, J. (1993). Out of darkness: Mutants reveal pathways controlling light-regulated development in plants. *Trends Genet.* **9,** 167–172.

Chory, J., Nagpal, P., and Peto, C. A. (1991). Phenotypic and genetic analysis of det2, a new mutant that affects light-regulated seedling development in Arabidopsis. *Plant Cell* **3,** 445–459.

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A role for cytokinins in de-etiolation in Arabidopsis. *Plant Physiol.* **104,** 339–347.

Christopher, D. A., Kim, M., and Mullet, J. E. (1992). A novel light-regulated promoter is conserved in cereal and dicot chloroplasts. *Plant Cell* **4,** 785–798.

Conley, T. R., and Shih, M. C. (1995). Effects of light and chloroplast functional state on expression of nuclear genes encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase in long hypocotyl (hy) mutants and wild-type Arabidopsis thaliana. *Plant Physiol.* **108,** 1013–1022.

Cook, W. B., and Walker, J. C. (1992). Identification of a maize nucleic acid-binding protein (NBP) belonging to a family of nuclear-encoded chloroplast proteins. *Nucleic Acids Res.* **20,** 359–364.

Dahlin, C., and Cline, K. (1991). Developmental regulation of the plastid protein import apparatus. *Plant Cell* **3,** 1131–1140.

Danon, A., and Mayfield, S. P. Y. (1991). Light regulated translational activators: Identification of chloroplast gene specific mRNA binding proteins. *EMBO J.* **10,** 3993–4001.

Danon, A., and Mayfield, S. P. (1994a). ADP-dependent phosphorylation regulates RNA-binding in vitro: Implications in light-modulated translation. *EMBO J.* **13,** 2227–2235.

Danon, A., and Mayfield, S. P. (1994b). Light-regulated translation of chloroplast messenger RNAs through redox potential. *Science* **266,** 1717–1719.

Deng, X. W. (1994). Fresh view of light signal transduction. *Cell* **76,** 423–426.

Deng, X. W., Matsui, M., Wei, N., Wagner, D., Chu, A. M., Feldmann, K. A., and Quail, P. H. (1992). COP1, an Arabidopsis regulatory gene, encodes a protein with both a zinc-binding motif and a Gβ homologous domain. *Cell* **71,** 791–801.

dePamphilis, C. W., and Palmer, J. D. (1990). Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant. *Nature (London)* **348,** 337–339.

Dinkins, R. D., Bandaranayake, H., Green, B. R., and Griffiths, A. J. F. (1994). A nuclear photosynthetic electron transport mutant of Arabidopsis thaliana with altered expression of the chloroplast petA gene. *Curr. Genet.* **25,** 282–288.

Drager, R. G., Zeidler, M., Simpson, C. L., and Stern, D. B. (1996). A chloroplast transcript lacking the 3′ inverted repeat is degraded by 3′–5′ exoribonuclease activity. *RNA* **2,** 652–663.

Drapier, D., Girard-Bascou, J., and Wollman, F. A. (1992). Evidence for nuclear control of the expression of the atpA and atpB chloroplast genes in Chlamydomonas. *Plant Cell* **4,** 283–295.

Dürrenberger, F., and Rochaix, J. D. (1991). Chloroplast ribosomal intron of Chlamydomonas reinhardtii: In vitro self-splicing, DNA endonuclease activity and in vivo mobility. *EMBO J.* **10,** 3495–3501.

Elich, T. D., and Chory, J. (1994). Initial events in phytochrome signaling: Still in the dark. *Plant Mol. Biol.* **26,** 1315–1327.

Erickson, J. M., Rahire, M., Malnöe, P., Girard-Bascou, J., Pierre, Y., Bennoun, P., and Rochaix, J. D. (1986). Lack of D2 protein in a Chlamydomonas reinhardtii psbD mutant affects photosystem II stability and D1 expression. *EMBO J.* **5,** 1745–1754.

Escoubas, J. M., Lomas, M., LaRoche, J., and Falkowski, P. G. (1995). Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. *Proc. Natl. Acad. Sci. USA* **92,** 10237–10241.

Falk, J., Schmidt, A., and Krupinska, K. (1993). Characterization of plastid DNA transcription in ribosome deficient plastids of heat-bleached barley leaves. *J. Plant. Physiol.* **141,** 176–181.

Fischer, N., Stampacchia, O., Redding, K., and Rochaix, J. D. (1996). Selectable marker recycling in the chloroplast. *Mol. Gen. Genet.* **251,** 373–380.

Frances, S., White, M. J., Edgerton, M. D., Jones, A. M., Elliott, R. C., and Thompson, W. F. (1992). Initial characterization of a pea mutant with light-independent photomorphogenesis. *Plant Cell* **4,** 1519–1530.

Fukuzawa, H., Kohchi, T., Shirai, H., Ohyama, K., Umesono, K., Inokuchi, H., and Ozeki, H. (1986). Coding sequences for chloroplast ribosomal protein S12 from the liverwort, Marchantia polymorpha, are separated far apart on the different DNA strands. *FEBS Lett.* **198,** 11–15.

Furbank, T. F., and Tayor, W. C. (1995). Regulation of photosynthesis in C3 and C4 plants: A molecular approach. *Plant Cell* **7,** 797–807.

Furuya, M. (1993). Phytochromes: Their molecular species, gene families, and functions. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **44,** 617–645.

Gallie, D. R. (1993). Posttranscriptional regulation of gene expression in plants. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **44,** 77–105.

Gamble, P. E., and Mullet, J. E. (1989). translation and stability of proteins encoded by the plastid psbA and psbB genes are regulated by a nuclear gene during light-induced chloroplast development in barley. *J. Biol. Chem.* **264,** 7236–7243.

Gillham, N. W. (1978). "Organelle Heredity." Raven Press, New York.

Gillham, N. W. (1994). "Organelle Genes and Genomes." Oxford Univ. Press, New York.

Gillham, N. W., Boynton, J. E., and Harris, E. H. (1991). Transmission of plastid genes. *In* "The Molecular Biology of Plastids," Vol. 7A, "Cell Culture and Somatic Cell Genetics" L. Bogorad and I. K. Vasil, eds., pp. 55–92. Academic Press, San Diego.

Gillham, N. W., Boynton, J. E., and Hauser, C. R. (1994). Translational regulation of gene expression in chloroplasts and mitochondria. *Annu. Rev. Genet.* **28,** 71–93.

Girard, J., Chua, N. H., Bennoun, P., Schmidt, G., and Delosme, M. (1980). Studies on mutants deficient in the photosystem I reaction centers in Chlamydomonas reinhardtii. *Curr. Genet.* **2,** 215–221.

Girard-Bascou, J., Pierre, Y., and Drapier, D. (1992). A nuclear mutation affects the synthesis of the chloroplast psbA gene production in Chlamydomonas reinhardtii. *Curr. Genet.* **22,** 47–52.

Glick, R. E., and Sears, B. B. (1993). Large unidentified open reading frame in plastid DNA (ORF2280) is expressed in chloroplasts. *Plant Mol. Biol.* **21,** 99–108.

Goldberg, A. L. (1991). The mechanism and function of ATP-dependent proteases in bacterial and animal cells. *Eur. J. Biochem.* **203,** 9–23.

Goldschmidt-Clermont, M., Girard Bascou, J., Choquet, Y., and Rochaix, J. D. (1990). Trans-splicing mutants of Chlamydomonas reinhardtii. *Mol. Gen. Genet.* **223,** 417–425.

Goldschmidt-Clermont, M., Choquet, Y., Girard Bascou, J., Michel, F., Schirmer Rahire, M., and Rochaix, J. D. (1991). A small chloroplast RNA may be required for trans-splicing in Chlamydomonas reinhardtii. *Cell* **65,** 135–143.

Goodenough, U. W., Armbrust, E. V., Campbell, A. M., and Ferris, P. J. (1995). Molecular genetics of sexuality in Chlamydomonas. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 21–44.

Gordon, K. H. J., Crouse, E. J., Bohnert, H. J., and Herrmann, R. G. (1982). Physical mapping of differences in chloroplast DNA of the five wild-type plastomes in Oenothera subsection Euoenothera. *Theor. Appl. Genet.* **61,** 373–384.

Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalrymple, B., Kuramitsu, H., Shiroza, T., Foster, T., Clark, W. P., Ross, B., Squires, C. L., and Maurizi, M. R. (1990). Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proc. Natl. Acad. Sci. USA* **87,** 3513–3517.

Gray, M. W. (1991). Origin and evolution of plastid genomes and genes. *In* "The Molecular Biology of Plastids, Vol. 7A, "Cell Culture and Somatic Cell Genetics" (L. Bogorad and I. K. Vasil, eds.), pp. 303–330. Academic Press, San Diego.

Gray, M. W. (1988). Organelle origins and ribosomal RNA. *Biochem. Cell Biol.* **66,** 325–348.

Grevelding, C., Suter-Crazzolara, C., von Menges, A., Kemper, E., Masterson, R., Schell, J., and Reiss, B. (1996). Characterization of a new allele of pale cress and its role in greening in Arabidopsis thaliana. *Mol. Gen. Genet.* **251,** 532–541.

Gruissem, W., and Schuster, G. (1993). Control of mRNA degradation in organelles. *In* "Control of Messenger RNA Stability" (J. G. Belasco and G. Brawerman, eds.), pp. 329–365. Academic Press, San Diego.

Gruissem, W., and Tonkyn, J. C. (1993). Control mechanisms of plastid gene expression. *Crit. Rev. Plant Sci.* **12,** 19–55.

Gu, J., Miles, D., and Newton, K. (1993). Analysis of leaf sectors in the NCS6 mitochondrial mutant of maize. *Plant Cell* **5,** 963–971.

Gumpel, N. J., and Purton, S. (1994). Playing tag with Chlamydomonas. *Trends Cell Biol.* **4,** 299–301.

Gumpel, N. J., Ralley, L., Girard-Bascou, J., Wollman, F. A., Nugent, J. H. A., and Purton, S. (1995). Nuclear mutants of Chlamydomonas reinhardtii defective in the biogenesis of the cytochrome b6f complex. *Plant Mol. Biol.* **29,** 921–932.

Hagemann, R. (1992). Plastid genetics in higher plants. *In* "Cell Organelles. Plant Gene Research. Basic Knowledge and Application" (R. G. Herrmann, ed.), pp. 65–96. Springer-Verlag, Vienna.

Han, C., Coe, E. H., and Martienssen, R. A. (1992). Molecular cloning and characterization of iojap (ij), a pattern striping gene of maize. *EMBO J.* **11,** 4037–4046.

Han, C., Patrie, W., Polacco, M., and Coe, E. H. (1993). Aberrations in plastid transcripts and deficiency of plastid DNA in striped and albino mutants in maize. *Planta* **191,** 552–563.

Hanley-Bowdoin, L., and Chua, N. H. (1987). Chloroplast promoters. *Trends Biochem. Sci.* **12,** 67–70.

Hanson, M. R., Sutton, C. A., and Lu, B. (1996). Plant organelle gene expression: Altered by editing. *Trends Plant Sci.* **1,** 57–64.

Harris, E. H., Boynton, J. E., and Gillham, N. W. (1994). Chloroplast ribosomes and protein synthesis. *Microbiol. Rev.* **58,** 700–754.

Hauser, C. R., Gillham, N. W., and Boynton, J. E. (1996). Translational regulation of chloroplast genes. Proteins binding to the 5′-untranslated regions of chloroplast mRNAs in Chlamydomonas reinhardtii. *J. Biol. Chem.* **271,** 1486–1497.

Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P., and Gruissem, W. (1996). Chloroplast mRNA 3′-end processing by a high molecular weight complex is regulated by nuclear encoded RNA binding proteins. *EMBO J.* **15,** 1132–1141.

Herrin, D. L., and Schmidt, G. W. (1988). Trans-splicing of transcripts for the chloroplast psaA1 gene. *J. Biol. Chem.* **263,** 14601–14604.

Herrin, D. L., Chen, Y. F., and Schmidt, G. W. (1990). RNA splicing in Chlamydomonas chloroplasts. Self-splicing of 23S preRNA. *J. Biol. Chem.* **265,** 21134–21140.

Herrin, D. L., Bao, Y., Thompson, A. J., and Chen, Y. F. (1991). Self-splicing of the Chlamydomonas chloroplast psbA introns. *Plant Cell* **3,** 1095–1107.

Herrmann, R. G., Westhoff, P., and Link, G. (1992). Biogenesis of plastids in higher plants. *In* "Cell Organelles. Plant Gene Research. Basic Knowledge and Application" (R. G. Herrmann, ed.), pp. 275–349. Springer-Verlag, Vienna.

Hess, W. R., Prombona, A., Fieder, B., Subramanian, A. R., and Börner, T. (1993). Chloroplast rps15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: Evidence for a functioning non-chloroplast-encoded RNA polymerase. *EMBO J.* **12,** 563–571.

Hess, W. R., Hoch, B., Zeltz, P., Hübschmann, T., Kössel, H., and Börner, T. (1994). Inefficient rpl2 splicing in barley mutants with ribosome-deficient plastids. *Plant Cell* **6,** 1455–1465.

Hirose, T., and Sugiura, M. (1996). Cis-acting elements and trans-acting factors for accurate translation of chloroplast psbA mRNAs: Development of an in vitro translation system from tobacco chloroplasts. *EMBO J.* **15,** 1687–1695.

Hong, S., and Spreitzer, R. J. (1994). Nuclear mutation inhibits expression of the chloroplast gene that encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol.* **106,** 673–678.

Hornung, S., Fulgosi, H., Dorfel, P., and Herrmann, R. G. (1996). Sequence variation in the putative replication origins of the five genetically distinct basic Euoenothera plastid chromosomes (plastomes). *Mol. Gen. Genet.* **251,** 609–612.

Howe, G., and Merchant, S. (1992). The biosynthesis of membrane and soluble plastidic c-type cytochromes of Chlamydomonas reinhardtii is dependent on multiple common gene products. *EMBO J.* **11,** 2789–2801.

Hsu-Ching, C., and Stern, D. B. (1991a). Specific binding of chloroplast proteins in vitro to the 3′ untranslated region of spinach chloroplast petD mRNA. *Mol. Cell. Biol.* **11,** 4380–4388.

Hsu-Ching, C., and Stern, D. B. (1991b). Specific ribonuclease activities in spinach chloroplasts promote mRNA maturation and degradation. *J. Biol. Chem.* **266,** 24205–24211.

Hu, J., and Bogorad, L. (1990). Maize chloroplast RNA polymerase: the 180-, 120- and 38-kilodalton polypeptides are encoded in chloroplast genes. *Proc. Natl. Acad. Sci. USA* **87,** 1531–1535.

Hu, J., Troxler, R. F., and Bogorad, L. (1991). Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid rpoC1 gene. *Nucleic Acids Res.* **19,** 3431–3434.

Huang, C., Wang, S., Chen, L., Lemieux, C., Otis, C., Turmel, M., and Liu, X. Q. (1994). The Chlamydomonas chloroplast clpP gene contains translated large insertion sequences and is essential for growth. *Mol. Gen. Genet.* **244,** 151–159.

Hurt, E. C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J. D., and Schatz, G. (1986). The cleavable pre-sequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. *EMBO J.* **5,** 1343–1350.

Hwang, S., Kawazoe, R., and Herrin, D. L. (1996). Transcription of tufA and other chloroplast-encoded genes is controlled by a circadian clock in Chlamydomonas. *Proc. Natl. Acad. Sci. USA* **93,** 996–1000.

Igloi, G. L., and Kössel, H. (1992). The transcriptional apparatus of chloroplasts. *Crit. Rev. Plant Sci.* **10,** 525–558.

Iratni, R., Baeza, L., Andreeva, A., Mache, R., and Lerbs-Mache, S. (1994). Regulation of rDNA transcription in chloroplasts: Promoter exclusion by constitutive repression. *Genes Dev.* **8,** 2928–38.

Jenkins, B. D., Kulhanek, D. J., and Barkan, A. B. (1997). Nuclear mutations that block group II RNA splicing in maize chloroplasts reveal several intron classes with distinct requirements for splicing factors. *Plant Cell* **9,** 283–296.

Johanningmeier, U. (1988). Possible control of transcript levels by chlorophyll precursors in Chlamydomonas. *Eur. J. Biochem.* **177,** 417–424.

Johanningmeier, U., and Howell, S. H. (1984). Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in Chlamydomonas reinhardtii. *J. Biol. Chem.* **259,** 13541–13549.

Johnson, C. H., and Schmidt, G. W. (1993). The psbB gene cluster of the Chlamydomonas reinhardtii chloroplast: Sequence and transcriptional analyses of psbN and psbH. *Plant Mol. Biol.* **22,** 645–658.

Johnson, E. M., and Sears, B. B. (1990a). Membrane composition and physiological activity of plastids from an Oenothera plastome mutator-induced chloroplast mutant. *Plant Physiol.* **92,** 254–261.

Johnson, E. M., and Sears, B. B. (1990b). Structure and expression of cytochrome fin an Oenothera plastome mutant. *Curr. Genet.* **17,** 529–534.

Johnson, E. M., Schnabelrauch, L. S., and Sears, B. B. (1991). A plastome mutation affects processing of both chloroplast and nuclear DNA-encoded plastid proteins. *Mol. Gen. Genet.* **225,** 106–112.

Johnson, H. C., Knight, M. R., Kondo, T., Masson, P., Sedbrook, J., Haley, A., and Trewavas, A. (1995). Circadian oscillations of cytosolic and chloroplastic free calcium in plants. *Science* **269,** 1863–1866.

Kanevski, I., and Maliga, P. (1994). Relocation of the plastid rbcL gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc. Natl. Acad. Sci. USA* **91,** 1969–1973.

Keddie, J. S., Carroll, B., Jones, J. D. G., and Gruissem, W. (1996). The DCL gene of tomato is required for chloroplast development and palisade morphogenesis in leaves. *EMBO J.* **15,** 4208–4217.

Kendrick, R. E., and Kronenberg, G. H. M. (1993). "Photomorphogenesis in Plants," 2nd Ed. Kluwer Academic, Dordrecht.

Khrebtukova, I., and Spreitzer, R. J. (1996). Elimination of the Chlamydomonas gene family that encodes the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Proc. Natl. Acad. Sci. USA* **93,** 13689–13693.

Kim, J., Gamble-Klein, P., and Mullet, J. E. (1991). Ribosomes pause at specific sites during synthesis of membrane-bound chloroplast reaction center protein D1. *J. Biol. Chem.* **266,** 14931–14938.

Kim, J., Klein, P. G., and Mullet, J. E. (1994a). Vir-115 gene product is required to stabilize D1 translation intermediates in chloroplasts. *Plant Mol. Biol.* **25,** 459–467.

Kim, J. K., Eichacker, L. A., Rudiger, W., and Mullet, J. E. (1994b). Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apo-protein stability. *Plant Physiol.* **104,** 907–916.

Kim, M., and Mullet, J. E. (1995). Identification of a sequence-specific DNA binding factor required for transcription of the barley chloroplast blue light-responsive psbD-psbC promoter. *Plant Cell* **7,** 1445–1457.

Kirk, J. T. O., and Tilney-Bassett, R. A. E. (1978). "The Plastids," 2nd Ed. Elsevier/North-Holland, Amsterdam.

Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Characterization of cDNA for a dehydration-inducible gene that encodes a ClpA,B-like protein in Arabidopsis thaliana L. *Biochem. Biophys. Res. Commun.* **196,** 1214–1220.

Klaff, P. (1995). mRNA degradation in spinach chloroplasts: psbA mRNA degradation is initiated by endonucleolytic cleavages within the coding region. *Nucleic Acids Res.* **23,** 4885–4892.

Klaff, P., and Gruissem, W. (1991). Changes in chloroplast mRNA stability during leaf development. *Plant Cell* **3,** 517–529.

Klein, U., De Camp, J. D., and Bogorad, L. (1992). Two types of chloroplast gene promoters in Chlamydomonas reinhardtii. *Proc. Natl. Acad. Sci. USA* **89,** 3453–3457.

Klein, U., Salvador, M. L., and Bogorad, L. (1994). Activity of the Chlamydomonas chloroplast rbcL gene promoter is enhanced by a remote sequence element. *Proc. Natl. Acad. Sci. USA* **91,** 10819–10823.

Koncz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G. P., and Schell, J. (1990). Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in Arabidopsis thaliana. *EMBO J.* **9,** 1337–1346.

Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S., and Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. *Science* **266,** 1233–1236.

Kraepiel, Y., Jullien, M., Cordonnier-Pratt, M. M., and Pratt, L. (1994). Identification of two loci involved in phytochrome expression in Nicotiana plumbaginifolia and lethality of the corresponding double mutant. *Mol. Gen. Genet.* **242,** 559–565.

Kruse, E., Mock, H. P., and Grimm, B. (1995). Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and affects the oxidative defense system. *EMBO J.* **14,** 3712–3720.

Kubicki, A., Steinmüller, K., and Westhoff, P. (1994). Differential transcription of plastome-encoded genes in the mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme-type C4 plants maize and sorghum. *Plant Mol. Biol.* **25,** 669–679.

Kuchka, M., Mayfield, S. P., and Rochaix, J. D. (1988). Nuclear mutations specifically affect the synthesis and/or degradation of the chloroplast-encoded D2 polypeptide of photosystem II in Chlamydomonas reinhardtii. *EMBO J.* **7,** 319–324.

Kuchka, M. R., Goldschmidt-Clermont, M., van Dillewijn, J., and Rochaix, J. D. (1989). Mutation at the Chlamydomonas NAC2 locus specifically affects the stability of the chloroplast psbD transcript encoding polypeptide D2 of PSII. *Cell* **58,** 869–876.

Kück, U., Choquet, Y., Schneider, M., Dron, M., and Bennoun, P. (1987). Structural and transcriptional analysis of two homologous genes for the P700 chlorophyll a apoproteins in Chlamydomonas reinhardtii: Evidence for trans-splicing. *EMBO J.* **6,** 2185–2195.

Kudla, J., Hayes, R., and Gruissem, W. (1996). Polyadenylation accelerates degradation of chloroplast mRNA. *EMBO J.* **15,** 7137–7146.

Kushnir, S., Babiychuk, E., Bannikova, M., Momot, V., Komarnitsky, I., Cherep, N., and Gleba, Y. (1991). Nucleo-cytoplasmic incompatibility in cybrid plants possessing an Atropa genome and a Nicotiana plastome. *Mol. Gen. Genet.* **225,** 225–330.

Kusnetsov, V., Bolle, C., Lübberstedt, T., Sopory, S., Herrmann, R. G., and Oelmüller, R. (1996). Evidence that the plastid signal and light operate via the same cis-acting elements in the promoters of nuclear genes for plastid proteins. *Mol. Gen. Genet.* **252,** 631–639.

Kwok, S. F., Piekos, B., Misera, S., and Deng, X. W. (1996). A complement of ten essential and pleiotropic Arabidopsis COP/DET/FUS genes is necessary for repression of photomorphogenesis in darkness. *Plant Physiol.* **110,** 731–742.

Langdale, J. A., and Kidner, C. A. (1994). bundle sheath defective, a mutation that disrupts cellular differentiation in maize leaves. *Development* **120,** 673–681.

Landgdale, J. A., and Nelson, T. (1991). Spatial regulation of photosynthetic development in C4 plants. *Trends Genet.* **7,** 191–196.

Lerbs, S., Bräutigam, E., and Mache, R. (1988). DNA-dependent RNA polymerase of spinach chloroplasts: Characterization of alpha-like and sigma-like polypeptides. *Mol. Gen. Genet.* **211,** 459–464.

Lerbs-Mache, S. (1993). The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complexes? *Proc. Natl. Acad. Sci. USA* **90,** 5509–5513.

Li, H. M., Culligan, K., Dixon, R. A., and Chory, J. (1995). CUE1: A mesophyll cell-specific positive regulator of light-controlled gene expression in Arabidopsis. *Plant Cell* **7,** 1599–1610.

Li, J., Nagpal, P., Vitart, V., McMorris, T. C., and Chory, J. (1996). A role for brassinosteroids in light-dependent development of Arabidopsis. *Science* **272,** 398–401.

Li, Y., and Sugiura, M. (1990). Three distinct ribonucleoproteins from tobacco chloroplasts: Each contains a unique amino terminal acidic domain and two ribonucleoprotein consensus motifs. *EMBO J.* **9,** 3059–3066.

Lisitsky, I., and Schuster, G. (1995). Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA. *Nucleic Acids Res.* **23,** 2506–2511.

Lisitsky, I., Liveanu, V., and Schuster, G. (1994). RNA-binding activities of the different domains of a spinach chloroplast ribonucleoprotein. *Nucleic Acids Res.* **22,** 4719–4724.

Lisitsky, I., Liveanu, V., and Schuster, G. (1995). RNA-binding characteristics of a ribonucleoprotein from spinach chloroplasts. *Plant Physiol.* **107,** 933–941.

Lisitsky, I., Klaff, P., and Schuster, G. (1996). Addition of destabilizing poly(A)-rich sequences to endonuclease cleavage sites during the degradation of chloroplast mRNA. *Proc. Natl. Acad. Sci. USA* **93,** 13398–13403.

Liu, B., and Troxler, R. F. (1996). Molecular characterization of a positively photoregulated nuclear gene for a chloroplast RNA polymerase sigma factor in Cyanidium caldarium. *Proc. Natl. Acad. Sci. USA* **93,** 3313–3318.

Lopez-Juez, E., Nagatani, A., Tomizawa, K. I., Deak, M., Kern, R., Kendrick, R. E., and Furuya, M. (1992). The cucumber long hypocotyl mutant lacks a light-stable PhyB-like phytochrome. *Plant Cell* **4,** 241–251.

Maier, R. M., Nechermann, K., Igloi, G. L., and Kössel, H. (1995). Complete sequence of the maize chloroplast genome: Gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *J. Mol. Biol.* **251,** 614–628.

Malnoe, P., Mayfield, S. P., and Rochaix, J. D. (1988). Comparative analysis of the biogenesis of photosystem II in the wild-type and y-1 mutant of Chlamydomonas reinhardtii. *J. Cell Biol.* **106,** 609–616.

Martienssen, R. A., Barkan, A., Freeling, M., and Taylor, W. C. (1989). Molecular cloning of a maize gene involved in photosynthetic membrane organization that is regulated by Robertson's Mutator. *EMBO J.* **8,** 1633–1639.

Martinez-Zapater, J. M., Gil, P., Capel, J., and Somerville, C. R. (1992). Mutations at the Arabidopsis CHM locus promote rearrangements of the mitochondrial genome. *Plant Cell* **4,** 889–899.

Mayer, R., Raventos, D., and Chua, N. H. (1996). det1, cop1 and cop9 mutations cause inappropriate expression of several gene sets. *Plant Cell* **8,** 1951–1959.

Mayfield, S. P. (1990). Chloroplast gene regulation: Interaction of the nuclear and chloroplast gneomes in the expression of photosynthetic proteins. *Curr. Opin. Cell Biol.* **2,** 509–513.

Mayfield, S. P., and Taylor, W. C. (1984). Carotenoid-deficient maize seedlings fail to accumulate light- harvesting chlorophyll a/b binding protein (LHCP) mRNA. *Eur. J. Biochem.* **144,** 79–84.

Mayfield, S. P., Cohen, A., Danon, A., and Yohn, C. B. (1994). Translation of the psbA mRNA of Chlamydomonas reinhardtii requires a structured RNA element contained within the 5′ untranslated region. *J. Cell Biol.* **127,** 1537–1545.

Mayfield, S. P., Yohn, C. B., Cohen, A., and Danon, A. (1995). Regulation of chloroplast gene expression. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 147–166.

McNellis, T. W., and Deng, X. W. (1995). Light control of seedling morphogenetic pattern. *Plant Cell* **7,** 1749–1761.

Meurer, J., Berger, A., and Westhoff, P. (1996). A nuclear mutant of Arabidopsis with impaired stability on distinct transcripts of the plastid psbB, psbD/C, ndhH and ndhC operons. *Plant Cell* **8,** 1193–1207.

Mieszczak, M., Klahre, U., Levy, J. H., Goodall, G. J., and Filipowicz, W. (1992). Multiple plant RNA binding proteins identified by PCR: Expression of cDNA encoding RNA binding proteins targeted to chloroplasts in Nicotiana plumbaginifolia. *Mol. Gen. Genet.* **234,** 390–400.

Miles, D. (1994). The role of high chlorophyll fluorescence photosynthesis mutants in the analysis of chloroplast thylakoid membrane assembly and function. *Maydica* **39,** 35–45.

Millar, A. J., McGrath, R. B., and Chua, N. H. (1994). Phytochrome phototransduction pathways. *Annu. Rev. Genet.* **28,** 325–349.

Miséra, S., Müller, A. J., Weiland-Heidecker, U., and Jürgens, G. (1994). The FUSCA genes of Arabidopsis: Negative regulators of light responses. *Mol. Gen. Genet.* **244,** 242–252.

Monod, C., Goldschmidt-Clermont, M., and Rochaix, J. D. (1992). Accumulation of chloroplast psbB RNA requires a nuclear factor in Chlamydomonas reinhardtii. *Mol. Gen. Genet.* **231,** 449–459.

Moore, T., and Keegstra, K. (1993). Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. *Plant Mol. Biol.* **21,** 525–537.

Morden, C. W., Wolfe, K. H., dePamphilis, C. W., and Palmer, J. D. (1991). Plastid translation and transcription in a non-photosynthetic plant: Intact, missing and pseudo genes. *EMBO J.* **10,** 3281–3288.

Morse, D., Salois, P., Markovic, P., and Hastings, J. W. (1995). A nuclear-encoded form II RuBisCO in dinoflagellates. *Science* **268,** 1622–1624.

Mourad, G. S., and White, J. A. (1992). The isolation of apparently homoplastidic mutants induced by a nuclear recessive gene in Arabidopsis thaliana. *Theor. Appl. Genet.* **84,** 906–914.

Mullet, J. E. (1993). Dynamic regulation of chloroplast transcription. *Plant Physiol.* **103,** 309–313.

Mullet, J. E., Gamble Klein, P., and Klein, R. R. (1990). Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proc. Natl. Acad. Sci. USA* **87,** 4038–4042.

Myers, A. M., Harris, E. H., Gillham, N. W., and Boynton, J. E. (1984). Mutations in a nuclear gene of Chlamydomonas cause the loss of two chloroplast ribosomal proteins, one synthesized in the chloroplast and the other in the cytoplasm. *Curr. Genet.* **8,** 369–378.

Nagley, P., and Devenish, R. J. (1989). Leading organellar proteins along new pathways: The relocation of mitochondrial and chloroplast genes to the nucleus. *Trends Biochem. Sci.* **14,** 31–35.

Nelson, T., and Langdale, J. A. (1992). Developmental genetics of C4 photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 25–47.

Neuhaus, G., Bowler, C., Kern, R., and Chua, N. H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* **73,** 937–952.

Newton, K. J., Knudsen, C., Gabay-Laughnan, S., and Laughnan, J. R. (1990). An abnormal growth mutant in maize has a defective mitochondrial cytochrome oxidase gene. *Plant Cell* **2,** 107–113.

Nickelsen, J., and Link, G. (1991). RNA–protein interactions at transcript 3′ ends and evidence for trnK–psbA cotranscription in mustard chloroplasts. *Mol. Gen. Genet.* **228,** 89–96.

Nickelsen, J., and Link, G. (1993). The 54 kDa RNA-binding protein from mustard chloroplasts mediates endonuleolytic transcript 3′ end formation in vitro. *Plant J.* **3,** 537–544.

Nickelsen, J., van Dillewijn, J., Rahire, M., and Rochaix, J. D. (1994). Determinants for stability of the chloroplast psbD RNA are located within its short leader region in Chlamydomonas reinhardtii. *EMBO J.* **13,** 3182–3191.

Oelmüller, R. (1989). Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. *Photochem. Photobiol.* **49,** 229–239.

Oelmüller, R., and Briggs, W. R. (1990). Intact plastids are required for nitrate- and light-induced accumulation of nitrate reductase activity and mRNA in squash cotyledons. *Plant Physiol.* **92,** 434–439.

Offermann-Steinhard, K., and Herrmann, R. G. (1990). Nucleotide sequences of psbB and psbH, the plastid encoded genes for CP47 and the 10 kDa phosphoprotein of photosystem II in Oenothera hookeri and argillicola. *Nucleic Acids Res.* **18,** 6452.

Ohta, M., Sugita, M., and Sugiura, M. (1995). Three types of nuclear genes encoding chloroplast RNA-binding proteins (cp29, cp31 and cp33) are present in Arabidopsis thaliana: Presence of cp31 in chloroplasts and its homologue in nuclei/cytoplasm. *Plant Mol. Biol.* **27,** 529–539.

Palmer, J. D. (1991). Plastid chromosomes: Structure and evolution. *In* "The Molecular Biology of Plastids," Vol. 7A, "Cell Culture and Somatic Cell Genetics" (L. Bogorad and I. K. Vasil, eds.), pp. 5–53. Academic Press, San Diego.

Palmer, J. D. (1992). Comparison of chloroplast and mitochondrial genome evolution in plants. *In* "Cell Organelles. Plant Gene Research. Basic Knowledge and Application" (R. G. Herrmann, ed.), pp. 99–133. Springer-Verlag, Vienna.

Pepper, A., Delaney, T., Washburn, T., Poole, D., and Chory, J. (1994). DET1, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear-localized protein. *Cell* **78,** 109–116.

Perl, A., Aviv, D., and Galun, E. (1991). Nuclear-organelle interaction in Solanum: Interspecific cybridizations and their correlation with a plastome dendrogram. *Mol. Gen. Genet.* **228,** 193–200.

Pfannschmidt, T., and Link, G. (1994). Separation of two classes of plastid DNA-dependent RNA polymerases that are differentially expressed in mustard (Sinapis alba L.) seedlings. *Plant Mol. Biol.* **25,** 69–81.

Plumley, F. G., and Schmidt, G. W. (1989). Nitrogen-dependent regulation of photosynthetic gene expression. *Proc. Natl. Acad. Sci. USA* **86,** 2678–2682.

Popot, J. L., de Vitry, C., and Atteia, A. (1994). Folding and assembly of integral membrane proteins: An introduction. *In* "Membrane Protein Structure. Experimental Approaches" (S. H. White, ed.), pp. 41–96. Oxford Univ. Press, Oxford.

Pyke, K. A., and Leech, R. M. (1992). Chloroplast division and expansion is radically altered by nuclear mutations in Arabidopsis thaliana. *Plant Physiol.* **99,** 1005–1008.

Pyke, K. A., and Leech, R. M. (1994). A genetic analysis of chloroplast division and expansion in Arabidopsis thaliana. *Plant Physiol.* **104,** 201–207.

Pyke, K. A., Rutherford, M. R., Robertson, E., and Leech, R. M. (1994). arc6, a fertile Arabidopsis mutant with only two mesophyll cell chloroplasts. *Plant Physiol.* **106,** 1169–1177.

Quail, P. H. (1994). Photosensory perception and signal transduction in plants. *Curr. Opin. Genet. Dev.* **4,** 652–661.

Quail, P. H., Boylan, M. T., Parks, B. M., Short, T. W., Xu, Y., and Wagner, D. (1995). Phytochromes: Photosensory perception and signal transduction. *Science* **268,** 675–680.

Rapp, J. C., and Mullet, J. E. (1991). Chloroplast transcription is required to express the nuclear genes rbcS and cab. Plastid DNA copy number is regulated independently. *Plant Mol. Biol.* **17,** 813–823.

Reardon, E. M., and Price, C. A. (1995). Plastid genomes of three non-green algae are sequenced. *Plant Mol. Biol. Rep.* **13,** 320–323.

Reinbothe, S., and Reinbothe, C. (1996). Regulation of chlorophyll biosynthesis in angiosperms. *Plant Physiol.* **111,** 1–7.

Reinbothe, S., Reinbothe, C., Heintzen, C., Seidenbecher, C., and Parthier, B. (1993). A methyl jasmonate-induced shift in the length of the 5′ untranslated region impairs the translation of the plastid rbcL transcript in barley. *EMBO J.* **12,** 1505–1512.

Reinbothe, S., Runge, S., Reinbothe, C., van Cleve, B., and Apel, K. (1995). Substrate-dependent transport of the NADPH:protochlorophyllide oxidoreductase into isolated plastids. *Plant Cell* **7,** 161–172.

Reinbothe, S., Reinbothe, C., Lebedev, N., and Apel, K. (1996a). PORA and PORB, two light-dependent protochlorophyllide-reducing enzymes of angiosperm chlorophyll biosynthesis. *Plant Cell* **8,** 763–769.

Reinbothe, S., Reinbothe, C., Apel, K., and Lebedev, N. (1996b). Evolution of chlorophyll biosynthesis—The challenge to survive photoxidation. *Cell* **86,** 703–705.

Reiter, R. S., Coomber, S. A., Bourett, T. M., Bartley, G. E., and Scolnik, P. A. (1994). Control of leaf and chloroplast development by the Arabidopsis gene pale cress. *Plant Cell* **6,** 1253–1264.

Reith, M. (1995). Molecular biology of Rhodophyte and Chromophyte plastids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 549–575.

Robertson, E. J., Pyke, K. A., and Leech, R. M. (1995). arc6, an extreme chloroplast division mutant of Arabidopsis also alters proplastid proliferation and morphology in shoot and root apices. *J. Cell Sci.* **108,** 2937–2944.

Robinson, C., and Klösgen, R. B. (1994). Targeting of proteins into and across the thylakoid membrane—A multitude of mechanisms. *Plant Mol. Biol.* **26,** 15–24.

Rochaix, J. D. (1992). Post-transcriptional steps in the expression of chloroplast genes. *Annu. Rev. Cell Biol.* **8,** 1–28.

Rochaix, J. D. (1995). Chlamydomonas reinhardtii as the photosynthetic yeast. *Annu. Rev. Genet.* **29,** 209–230.

Rochaix, J. D. (1996). Post-transcriptional regulation of chloroplast gene expression in Chlamydomonas reinhardtii. *Plant Mol. Biol.* **32,** 327–341.

Rochaix, J. D., Kuchka, M., Mayfield, S., Schirmer-Rahire, M., Girard-Bascou, J., and Bennoun, P. (1989). Nuclear and chloroplast mutations affect the synthesis or stability of the chloroplast psbC gene product in Chlamydomonas reinhardtii. *EMBO J.* **8,** 1013–1021.

Rodermel, S., Haley, J., Jiang, C. Z., Tsai, C. H., and Bogorad, L. (1996). A mechanism for intergenomic integration: Abundance of ribulose bisphosphate carboxylase small-subunit protein influences the translation of the large-subunit mRNA. *Proc. Natl. Acad. Sci. USA* **93,** 3881–3885.

Roenneberg, T., and Morse, D. (1993). Two circadian oscillators in one cell. *Nature (London)* **362,** 362–364.

Roitgrund, C., and Mets, L. J. (1990). Localization of two novel choroplast genome functions: Trans-splicing of RNA and protochlorophyllide reduction. *Curr. Genet.* **17,** 147–153.

Roth, R., Hall, L. N., Brutnell, T. P., and Langdale, J. A. (1996). bundle sheath defective2, a mutation that disrupts the coordinated development of bundle sheath and mesophyll cells in the maize leaf. *Plant Cell* **8,** 915–927.

Rott, R., Drager, R. G., Stern, D. B., and Schuster, G. (1996). The 3′ untranslated regions of chloroplast genes in Chlamydomonas reinhardtii do not serve as efficient transcriptional terminators. *Mol. Gen. Genet.* **252,** 676–683.

Roussell, D. L., Thompson, D. L., Pallardy, S. G., Miles, D., and Newton, K. J. (1991). Chloroplast structure and function is altered in the NCS2. *Plant Physiol.* **96,** 232–238.

Rowan, R., Whitney, S. M., Fowler, A., and Yellowlees, D. (1996). Rubisco in marine symbiotic dinoflagellates: Form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. *Plant Cell* **8,** 539–553.

Runge, S., Sperling, U., Frick, G., Apel, K., and Armstrong, G. A. (1996). Distinct roles for light-dependent NADPH:protochlorophyllide oxidoreductase (POR) A and B during greening in higher plants. *Plant J.* **9,** 513–523.

Sakamoto, W., Kindle, K., and Stern, D. B. (1993). In vivo analysis of Chlamydomonas chloroplast petD gene expression using stable transformation of beta-glucuronidase translational fusions. *Proc. Natl. Acad. Sci. USA* **90,** 497–501.

Sakamoto, W., Chen, X., Kindle, K. K., and Stern, D. B. (1994a). Function of the Chlamydomonas reinhardtii petD 5′ untranslated region in regulating the accumulation of subunit IV of the cytochrome b6/f complex. *Plant J.* **6,** 503–512.

Sakamoto, W., Sturm, N. R., Kindle, K., and Stern, D. B. (1994b). petD mRNA maturation in Chlamydomonas reinhardtii chloroplasts: Role of 5′ endonucleolytic processing. *Mol. Cell. Biol.* **14,** 6180–6186.

Sakamoto, W., Kondo, H., Murata, M., and Motoyoshi, F. (1996). Altered mitochondrial gene expression in a maternal distorted leaf mutant of Arabidopsis induced by chloroplast mutator. *Plant Cell* **8,** 1377–1390.

Salvador, M. L., Klein, U., and Bogorad, L. (1993a). 5′ Sequences are important positive and negative determinants of the longevity of Chlamydomonas chloroplast gene transcripts. *Proc. Natl. Acad. Sci. USA* **90,** 1556–1560.

Salvador, M. L., Klein, U., and Bogorad, L. (1993b). Light-regulated and endogenous fluctuations of chloroplast transcript levels in Chlamydomonas. Regulation by transcription and RNA degradation. *Plant J.* **3,** 213–219.

Schaller, A., and Ryan, C. A. (1995). Cloning of a tomato cDNA (GenBank L38581) encoding the proteolytic subunit of a Clp-like energy dependent protease (PGR95-001). *Plant Physiol.* **108,** 1341.

Schultes, N. P., Brutnell, T. P., Allen, A., Dellaporta, S. L., Nelson, T., and Chen, J. (1996). Leaf permeasel gene of maize is required for chloroplast development. *Plant Cell* **8,** 463–475.

Schuster, G., and Gruissem, W. (1991). Chloroplast mRNA 3′ end processing requires a nuclear-encoded RNA-binding protein. *EMBO J.* **10,** 1493–1502.

Sears, B. B. (1980). Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* **4,** 233–255.

Sears, B. B., and Sokalski, M. B. (1991). The Oenothera plastome mutator: Effect of UV irradiation and nitroso-methyl urea on mutation frequencies. *Mol. Gen. Genet.* **229,** 245–252.

Shanklin, J., DeWitt, N. D., and Flanagan, J. M. (1995). The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of Escherichia coli ClpP and ClpA: An archetypal two-component ATP-dependent protease. *Plant Cell* **7,** 1713–1722.

Sharma, R., Lopez-Juez, E., Nagatani, A., and Furuya, M. (1993). Identification of photo-inactive phytochrome A in etiolated seedlings and photo-active phytochrome B in green leaves of the aurea mutant of tomato. *Plant J.* **4,** 1035–1042.

Short, T. W., and Briggs, W. R. (1994). The transduction of blue light signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 143–171.

Sieburth, L. E., Berry-Lowe, S., and Schmidt, G. (1991). Chloroplast RNA stability in Chlamydomonas: Rapid degradation of psbB and psbC transcripts in two nuclear mutants. *Plant Cell* **3,** 175–189.

Smith, T. A., and Kohorn, B. D. (1994). Mutations in a signal sequence for the thylakoid membrane identify multiple transport pathways and nuclear suppressors. *J. Cell Biol.* **126,** 365–374.

Squires, C., and Squires, C. L. (1992). The Clp proteins: Proteolysis regulators or molecular chaperones. *J. Bacteriol.* **174,** 1081–1085.

Stampacchia, O., Girard-Bascou, J., Zanasco, J. L., Zerges, W., Bennoun, P., and Rochaix, J. D. (1997). A nuclear-encoded function essential for translation of the chloroplast *psaB* mRNA in Chlamydomonas. *Plant Cell* **9,** 773–782.

Staub, J. M., and Maliga, P. (1994). Translation of psbA mRNA is regulated by light via the 5′-untranslated region in tobacco plastids. *Plant J.* **6,** 547–553.

Stern, D. B., and Gruissem, W. (1987). Control of plastid gene expression: 3′ Inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. *Cell* **51,** 1145–1157.

Stern, D. B., and Kindle, K. L. (1993). 3′ End maturation of the Chlamydomonas reinhardtii chloroplast atpB mRNA is a two-step process. *Mol. Cell. Biol.* **13,** 2277–2285.

Stern, D. B., Jones, H., and Gruissem, W. (1989). Function of plastid mRNA 3′ inverted repeats. *J. Biol. Chem.* **264,** 18742–18750.

Stern, D. B., Radwanski, E. R., and Kindle, K. L. (1991). A 3′ stem/loop structure of the Chlamydomonas chloroplast atpB gene regulates mRNA accumulation in vivo. *Plant Cell* **3,** 285–297.

Stubbe, W. (1989). Oenothera—An ideal system for studying the interactions of genome and plastome. *Plant Mol. Biol. Rep.* **7,** 245–257.

Subbaiah, C. C., and Tewari, K. K. (1993). Purification and characterization of ribonucleoproteins from pea chloroplasts. *Eur. J. Biochem.* **211,** 171–179.

Subramanian, A. R. (1993). Molecular genetics of chloroplast ribosomal proteins. *Trends Biochem. Sci.* **18,** 177–181.

Subramanian, A. R., Stahl, D., and Prombona, A. (1991). Ribosomal proteins, ribosomes and translation in plastids. *In* "The Molecular Biology of Plastids," Vol. 7A, "Cell Culture and Somatic Cell Genetics" (L. Bogorad and I. K. Vasil, eds.), pp. 191–215. Academic Press, San Diego.

Sugiura, M. (1991). Transcript processing in plastids: Trimming, cutting, splicing. *In* "The Molecular Biology of Plastids," Vol. 7A, "Cell Culture and Somatic Cell Genetics" (L. Bogorad and I. K. Vasil, eds.), pp. 125–137. Academic Press, San Diego.

Surzycki, S. J., and Shellenbarger, D. L. (1976). Purification and characterization of a putative sigma factor from Chlamydomonas reinhardi. *Proc. Natl. Acad. Sci. USA* **73,** 3961–3965.

Susek, R. E., and Chory, J. (1992). A tale of two genomes: Role of a chloroplast signal in coordinating nuclear and plastid genome expression. *Aust. J. Plant Physiol.* **19,** 387–399.

Susek, R. E., Ausubel, F., and Chory, J. (1993). Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. *Cell* **74,** 787–799.

Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990). Stable transformation of plastids in higher plants. *Proc. Natl. Acad. Sci. USA* **87,** 8526–8530.

Szekeres, M., Németh, K., Koncz-Kalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Rédei, G., Nagy, F., Schell, J., and Koncz, C. (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. *Cell* **85,** 171–182.

Takahashi, T., Gasch, A., Nishizawa, N., and Chua, N. H. (1995). The DIMINUTO gene of Arabidopsis is involved in regulating cell elongation. *Genes Dev.* **9,** 97–105.

Taylor, W. C. (1989). Regulatory interactions between nuclear and plastid genomes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **406,** 211–233.

Terzaghi, W. B., and Cashmore, A. R. (1995). Light-regulated transcription. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 445–474.

Theg, S. M., and Scott, S. V. (1993). Protein import into chloroplasts. *Trends Cell Biol.* **3,** 186–190.

Thompson, A. J., and Herrin, D. L. (1991). In vitro self-splicing reactions of the chloroplast group I intron Cr.LSU from Chlamydomonas reinhardtii and in vivo manipulation via gene-replacement. *Nucleic Acids Res.* **19,** 6611–6618.

Thorsness, P. E., and Weber, E. R. (1996). Escape and migration of nucleic acids between chloroplasts, mitochondria and the nucleus. *Int. Rev. Cytol.* **165,** 207–234.

Tiller, K., and Link, G. (1993). Phosphorylation and dephosphorylation affect functional characteristics of chloroplast and etioplast transcription systems from mustard (Sinapis alba L.). *EMBO J.* **12,** 1745–1753.

Tiller, K., Eisermann, A., and Link, G. (1991). The chloroplast transcription apparatus from mustard (Sinapis alba L.). Evidence for three different transcription factors which resemble bacterial sigma factors. *Eur. J. Biochem.* **198,** 93–99.

Tonkyn, J. C., Deng, X. W., and Gruissem, W. (1992). Regulation of plastid gene expression during photooxidative stress. *Plant Physiol.* **99,** 1406–1415.

Torazawa, K., Hayashida, N., Obokata, J., Shinozaki, K., and Sugiura, M. (1986). The 5′ part of the gene for ribosomal protein S12 is located 30 kbp downstream of its 3′ part in tobacco chloroplast genome. *Nucleic Acids Res.* **14,** 3143.

Troxler, R. F., Zhang, F., Hu, J., and Bogorad, L. (1994). Evidence that sigma factors are components of chloroplast RNA polymerase. *Plant Physiol.* **104,** 753–759.

van Tuinen, A., Kerckhoffs, L. H., Nagatani, A., Kendrick, R. E., and Korneef, M. (1995). Far-red light-insensitive, phytochrome A-deficient mutants of tomato. *Mol. Gen. Genet.* **246,** 133–141.

Vera, A., and Sugiura, M. (1995). Chloroplast rRNA transcription form structurally different tandem promoters: An additional novel-type promoter. *Curr. Genet.* **27,** 280–284.

Vierstra, R. D. (1993). Protein degradation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 385–410.

Voelker, R., and Barkan, A. (1995a). Nuclear genes required for post-translational steps in the biogenesis of the chloroplast b6f complex in maize. *Mol. Gen. Genet.* **249,** 507–514.

Voelker, R., and Barkan, A. (1995b). Two nuclear mutations disrupt distinct pathways for tarteting proteins to the chloroplast thylakoid. *EMBO J.* **14,** 3905–3914.

Voelker, R., Mendel-Hartvig, J., and Barkan, A. (1997). Transposon-disruption of a maize nuclear gene, tha-1, encoding a chloroplast SecA homolog: In vivo role of cp-SecA in thylakoid targeting. Genetics **145,** 467–478.

von Arnim, A., and Deng, X. W. (1996). A role for transcriptional repression during light control of plant development. *BioEssays* **18,** 905–910.

von Wettstein, D., Gough, S., and Kannangara, C. G. (1995). Chlorophyll biosynthesis. *Plant Cell* **7,** 1039–1057.

Wada, T., Tunoyama, Y., Shiina, T., and Toyoshima, Y. (1994). In vitro analysis of light-induced transcription in the wheat psbD/C gene cluster using plastid extracts from dark-grown and short-term-illuminated seedlings. *Plant Physiol.* **104,** 1259–1267.

Walbot, V. (1992). Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 49–82.

Walbot, V., and Coe, E. H. (1979). Nuclear gene iojap conditions a programmed change to ribosome-less plastids in Zea mays. *Proc. Natl. Acad. Sci. USA* **76,** 2760–2764.

Wei, N., and Deng, X. W. (1996). The role of COP/DET/FUS genes in light control of Arabidopsis seedling development. *Plant Physiol.* **112,** 871–878.

Wei, N., Chamovitz, D. A., and Deng, X. W. (1994). Arabidopsis COP9 is a component of a novel signalling complex mediating light control of development. *Cell* **78,** 117–124.

Wolfe, K. H., Morden, C. W., and Palmer, J. D. (1992). Function and evolution of a minimal plastid genome from a nonphotosynthetic parasitic plant. *Proc. Natl. Acad. Sci. USA* **89,** 10648–10652.

Wolfson, R., Higgins, K. G., and Sears, B. B. (1991). Evidence for replication slippage in the evolution of Oenothera chloroplast DNA. *Mol. Biol. Evol.* **8,** 709–720.

Wu, H. Y., and Kuchka, M. R. (1995). A nuclear suppressor overcomes defects in the synthesis of the chloroplast psbD gene product caused by mutations in two distinct nuclear genes of Chlamydomonas. *Curr. Genet.* **27,** 263–269.

Xie, Z., and Merchant, S. (1996). The plastid-encoded ccsA gene is required for heme attachment to chloroplast c-type cytochromes. *J. Biol. Chem.* **271,** 4632–4639.

Xu, R., Bingham, S. E., and Webber, A. N. (1993). Increased mRNA accumulation in a psaB frame-shift mutant of Chlamydomonas reinhardtii suggests a role for translation in psaB mRNA stability. *Plant Mol. Biol.* **22,** 465–474.

Yang, J., Schuster, G., and Stern, D. B. (1996). CSP41, a sequence-specific chloroplast mRNA binding protein, is an endoribonuclease. *Plant Cell* **8,** 1409–1420.

Ye, L., Li, Y., Fukami-Kobayashi, K., Go, M., Konishi, T., Watanabe, A., and Sugiura, M. (1991). Diversity of a ribonucleoprotein family in tobacco chloroplasts: Two new chloroplast ribonucleoproteins and a phylogenetic tree of ten chloroplast RNA-binding domains. *Nucleic Acids Res.* **19,** 6485–6490.

Yohn, C. B., Cohen, A., Danon, A., and Mayfield, S. P. (1996). Altered mRNA binding activity and decreased translation initiation in a nuclear mutant lacking translation of the chloroplast psbA mRNA. *Mol. Cell. Biol.* **16,** 3560–3566.

Zeltz, P., Hess, W. R., Neckermann, K., Börner, T., and Kössel, H. (1993). Editing of the chloroplast rpoB transcript is independent of chloroplast translation and shows different patterns in barley and maize. *EMBO J.* **12,** 4291–4296.

Zerges, W., and Rochaix, J. D. (1994). The 5′ leader of a chloroplast mRNA mediates the translational requirements for two nucleus-encoded functions in Chlamydomonas reinhardtii. *Mol. Cell. Biol.* **14,** 5268–5277.

Zerges, W., Girard-Bascou, J., and Rochaix, J. D. (1997). Translation of the chloroplast *psbC* mRNA is controlled by interactions between its 5′ leader and the nuclear loci *TBC1* and *TBC3* in *Chlamydomonas reinhardtii. Mol. Cell. Biol.* **17,** 3440–3448.

Signaling in Unicellular Eukaryotes

Søren T. Christensen,* Vagn Leick,* Leif Rasmussen,† and Denys N. Wheatley‡
*Department of Medical Biochemistry and Genetics, Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark; †Institute of Medical Biology, Department of Anatomy and Cell Biology, Odense University, DK-5230 Odense M, Denmark; and ‡Cell Pathology Unit, Department of Pathology, University Medical School, Aberdeen AB25 2ZD, United Kingdom.

Aspects of intercellular and intracellular signaling systems in cell survival, proliferation, differentiation, chemosensory behavior, and programmed cell death in free-living unicellular eukaryotes have been reviewed. Comparisons have been made with both bacteria and metazoa. The central organisms were flagellates (*Trypanosoma, Leishmania,* and *Crithidia*), slime molds (*Dictyostelium*), yeast cells (*Saccharomyces cerevisiae*), and ciliates (*Paramecium, Euplotes,* and *Tetrahymena*). There are two novel aspects in this review. First, cellular responses are viewed in an evolutionary perspective, rather than from the more prevailing one, in which the unicellular eukaryotes are seen by the mammalian organisms. Second, results obtained with cell cultures in minimal, chemically defined nutrient media at low cell densities where intercellular signaling is strongly reduced are discussed. These results shed light on control mechanisms and their cooperation inside the living cell. Intracellular systems have many common features in unicellular and multicellular organisms.

KEY WORDS: Protozoans, Yeast, Cellular signaling, Apoptosis, Programmed cell death, Cell survival, Cell proliferation, Mating, Chemosensory behavior.

I. Introduction

Signaling systems in mammalian cells have been studied for years. Recently, investigators working with lower eukaryotes, such as flagellates, slime molds, ciliates, and yeast cells, have been surprised to find that many concepts and control mechanisms known from metazoa seem to apply also to the unicellular organisms. This is true for regulatory systems which

Copyright © 1998 by Academic Press.
All rights of reproduction in any form reserved.

have to do with fundamental features like cell survival, proliferation, differentiation, chemosensory behavior, and programmed cell death. So far, such results have most often been published without too much cross-referencing to other systems (an exception: Csaba and Müller, 1996). Here we attempt to remedy this situation. We are aware that we compare biological systems which were separated maybe more than a billion years ago and that we should be prepared to find huge differences between them. However, it is becoming increasingly obvious that nature has used many basic principles, building blocks, and control mechanisms throughout groups of eukaryote cells.

Studies on cellular control mechanisms in unicellular eukaryotes have proceeded along two lines, *in vitro* and *in vivo* experiments. The first line has dealt with gene sequences and isolation of control molecules from various cells. The second has dealt with the behavior of living cells in culture after exposure to activating or inhibitory compounds or to other adverse culture conditions. We want to contribute to this field by forming a bridge between these two lines. For some time, all of us have known of *checkpoints* in the cell division cycle in eukaryotes. Now we begin to discern another set of cellular control systems which so far has received little attention: control mechanisms operating in the transition of the cell from survival within the lag phase to *exponential multiplication,* to *differentiation,* or to *"programmed" cell death,* i.e., cell death which is under molecular control rather than being of an *"accidental"* nature.

Cells signal to each other when one influences another in a programmed and characteristic way. Often cell signaling requires both extracellular molecules and a complementary set of receptors in each cell. Signaling in unicellular eukaryotes was believed to be confined to mating factors in, e.g., ciliates and yeast cells. It is now evident that unicellular eukaryotes depend on extensive signaling systems for their existence (Janssens, 1988; Wheatley *et al.,* 1993, 1994; Vallesi *et al.,* 1995; Ameisen, 1996; Csaba and Müller, 1996; Rasmussen *et al.,* 1996). We can get insight into these systems under *in vivo* conditions by manipulation of nutrient media and by inhibition/activation of cellular functions.

Unicellular eukaryotes gave rise to metazoa during evolution. Hence we can ask, are unicellular inter- and intracellular communication systems homologous to those used by cells of metazoa? This may indeed be true for basic control mechanisms in cell survival, proliferation, differentiation, chemosensory behavior, and programmed cell death. After addressing this question, we will allude to some recent work on prokaryotes, primarily because it has now been appreciated that they also indulge in intercellular signaling, and the issue of how their systems compare will obviously be raised in the future (see IV and VI).

A. Cell Communication in Unicellular Eukaryotes

There are many examples of intercellular signaling in unicellular eukaryotes: (1) *Cell growth and proliferation* were long believed to depend solely on available nutrients. This idea was held in spite of findings over almost a century showing that there were clear stimulatory effects on cell multiplication related to the initial cell density in ciliate cultures (Schousboe *et al.*, 1997). Recent experiments have confirmed and extended these views. Thus proliferation is stimulated by autocrine compounds in *Paramecium* (Tanabe *et al.*, 1990) and *Dictyostelium* (Whitbread *et al.*, 1991). (2) Mating factors (pheromones) are important in cell recognition in *sexual reproduction* and are released into the environment by the yeast cells *Saccharomyces* and *Schizosaccharomyces* (Hirsch and Cross, 1992; Nielsen and Davey, 1995) and by the ciliates *Euplotes, Blepharisma,* and *Dileptus* (Parfenova *et al.*, 1989; Miyake, 1981; Luporini and Miceli, 1986). In addition, pheromones in *Euplotes raikovi* have mitogenic activity (Vallesi *et al.*, 1995). (3) Signaling plays a role in the *survival* of parasitic flagellates and of some ciliates; these organisms can die in the absence of appropriate signals (Christensen and Rasmussen, 1992; Ameisen *et al.*, 1995; Schousboe *et al.*, 1997). (4) There are examples of *differentiation* controlled by signals in slime molds. Thus starving *Dictyostelium* cells secrete many compounds, making free-living slime mold cells coalesce and differentiate into a multicellular organism (Clarke and Gomer, 1995).

B. Communication in Metazoa

1. The Roles of Signal Molecules

All cells respond to chemical changes in the environment. Many inter- and intracellular compounds affect cell survival, proliferation, differentiation, programmed cell death, etc. in metazoa (Fig. 1). Intercellular compounds are termed *primary messengers* and include *hormones, growth factors,* and *neurotransmitters* (Hardie, 1991). Water-soluble compounds can exert their effects via cell-surface receptors as secreted free, soluble molecules. The signals reach the cytoplasm or the nucleus of the target cells through the activation of intracellular enzyme systems (among them, *second messengers*), and this often results in the expression of new genes. Hydrophobic compounds, such as steroid hormones, exert their effects via intracellular receptors. Activated receptors increase the expression of specific genes by binding directly to specific DNA sequences. Signaling coordinates the behavior of individual cells and ensures integration of functions in the entire organism.

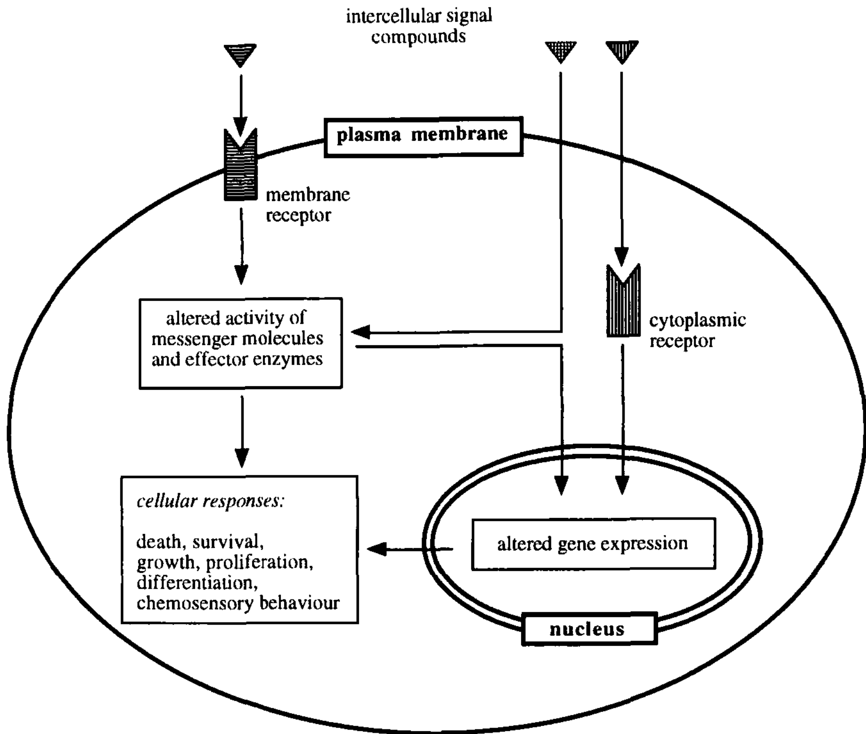


FIG. 1 Schematic presentation of signal processes in multicellular organisms. Intercellular signals operate in many ways. Two examples are endocrine and paracrine/autocrine signaling. Endocrine signals, e.g., hormones, act over long distances on target cells throughout the body via the bloodstream. Paracrine/autocrine signals, e.g., *growth factors*, are active over short distances, i.e., between individual cells in the immediate environment. Hydrophilic signal molecules often act on target cells through specific, *membrane associated* receptor systems. Hydrophobic compounds regularly bind to *cytoplasmic* receptors. In a few cases, the signals operate without a receptor. *Signaling via membrane-associated receptors.* An example: the peptide signal, platelet-derived growth factor, regulates cell survival, proliferation, differentiation, and chemosensory behavior in mammalian cells via a *protein tyrosine kinase receptor.* This leads to production and activation of several intracellular messenger molecules and effector enzymes, which may vary from one cellular response to another. In most cases (e.g., in proliferation) the intracellular signal pathway induces new gene expression. However, new gene activities are not required in chemosensory behavior (Bornfeldt *et al.*, 1995b). *Signaling via cytoplasmic receptors.* Glucocorticoids are steroid hormones and affect metabolism and suppress inflammatory responses. They are bound to carrier proteins in the intercellular environment. When the hormones are released from their carrier molecules they diffuse through the plasma membrane and bind to cytoplasmic receptors. The activated receptors bind to specific DNA sequences in the nucleus and induce the synthesis of regulatory proteins (Alberts *et al.*, 1994; Pepe and Albrecht, 1995; Knoebl *et al.*, 1996; Funder, 1996). *Signaling without receptors.* An example: nitric oxide (NO) is an intracellular messenger molecule which regulates many cellular activities, such as neurotransmission, vasomodulation, immune response, cell survival, and proliferation. Nitric oxide diffuses rapidly into the intercellular environment and enters neighboring cells. This leads to amplification of the original signal (Berdeaux, 1993; Marín and Govantes, 1995; Oh, 1995).

2. Signal Transduction via Protein Kinases

Many compounds take part in signal transduction. Binding of intercellular molecules to membrane-associated receptors leads to the activation of multiple downstream targets, which are intermediates between the receptor and the nucleus for immediate–early gene expression in programmed processes. Growth factors and chemoattractants often signal via two types of membrane-associated receptors: *tyrosine kinases (TK) receptors* and *G-protein linked receptors, e.g., seven-pass transmembrane helices (STM) receptors.* This is true for insulin; insulin-like growth factors (IGFs); epidermal, platelet, fibroblast, and nerve cell growth factors (EGF, PDGF, FGF, and NGF, respectively); and for the tripeptide fMet-Leu-Phe. Binding of signal molecules to both receptor types leads to the activation of many intracellular signal systems. Two families of protein kinases known as the *mitogen-activated protein kinase (MAPK) pathways* and the *protein kinase Cs (PKCs)* are central in this connection.

a. MAPK Pathways These consist of protein kinase cascades, including *GTP-binding proteins, MAP kinase kinase kinases, MAP kinase kinases,* and *MAP kinases. Ras GTPase* is one of the central GTP-binding enzymes. It regulates the activity of *Ral GTPase, PI-3 kinase,* and *Raf kinase* (Feig *et al.,* 1996), the latter being a MAP kinase kinase kinase. Signaling via TK receptors often results in the activation of *MEKs* (MAP kinase kinases) and *ERKs* (MAP kinases) through Ras GTPase and *Raf GTPase* and signaling via G-protein-linked receptors via *G proteins* and a *MEKK* (Lange-Carter *et al.,* 1993; Cano and Mahadevan, 1995; Marshall, 1996). MEKs are phosphorylated on serine residues, and MEK, a dual-specificity kinase, then phosphorylates ERKs on threonine and tyrosine residues. Other subfamilies of the MAPK cascade take part in signal transduction (Cano and Mahadevan, 1995; Canman and Kastan, 1996). One is *JNK/SAPK (c-jun amino-terminal kinase/stress-activated protein kinase).* JNK is activated by growth factors, cytokines, and stress factors, including heat shock, high osmolarity, and UV light. This activation occurs via MEKK by a MEK equivalent termed *SEK* and in pathways both dependent and independent of GTPase action (Minden *et al.,* 1994).

b. PKCs These are serine/threonine kinases and can be divided into *conventional PKCs (cPKCs), new PKCs,* and *atypical PKCs (aPKCs)* (reviewed by Nishizuka, 1992, 1995). Both cPKC and aPKC activate the Ras/Raf/MAPK cascades (Sözeri *et al.,* 1992; Kolch *et al.,* 1993; Hii *et al.,* 1995; Mitev *et al.,* 1995; Cano and Mahadevan, 1995; Nishizuka, 1995; Canman and Kastan, 1996). The cPKCs are Ca^{2+}/phospholipid-dependent enzymes. Their activities can be regulated by receptor-mediated hydrolysis of phos-

phatidylinositol by phospholipase C-α, forming the second messengers diacylglycerol (DAG) and inositol-1,5-triphosphate (IP_3). The aPKCs are regulated by ceramide and PIP_3, the latter being a product of the PI-3 kinase, rather than by Ca^{2+} and DAG (Liscovitch and Cantley, 1994; Nishizuka, 1995).

Evidently, the MAPK pathways can be activated simultaneously by distinct parallel cascades in response to the same stimuli in mammalian cells Cano and Mahadevan, 1995; Canman and Kastan, 1996). This shows a high degree of complexity in the regulatory mechanisms of signal transduction in these cells.

3. Cell Death—A Prerequisite for Multicellular Life

Complex mechanisms regulate development and maintenance of metazoa, some being dependent on the death of specific cells at appropriate times. These death processes require *de novo* gene expression and are called *programmed cell death* (PCD), in stark contrast to the "accidental" cell death associated with *necrosis* (Ellis *et al.*, 1991; Raff, 1992; Häcker and Vaux, 1995; Duke *et al.*, 1996). PCD eliminates cells in embryonic tissues during morphogenesis. As an example, 131 of a total of 1090 cells die by PCD during development of the nematode *Caenorhabditis elegans* (Ellis *et al.*, 1991). PCD removes unwanted or abnormal cells, maintains specific cell types, protects against infections, and regulates maturation of the immune and nerve systems in the adult organism (Baixeras *et al.*, 1994; Häcker and Vaux, 1995; Duke *et al.*, 1996). Evan (1994) and Vaux *et al.* (1994) suggested that PCD originated with the advent of metazoan systems. It will become obvious that we do not share their views.

Cytokines or inadequate amounts of survival and growth factors induce cell elimination (Raff, 1992). Raff suggested that all metazoan cells are genetically programmed to kill themselves; only constant support from neighboring cells keeps them alive, and even healthy cells take steps to commit suicide by PCD in the absence of this support. He proposed that limiting amounts of signaling compounds control the number of cells in a tissue and this selects for the most competitive cells, a form of "survival of the fittest" inside the organism. These signals can be both autocrine/paracrine and "endocrine" and in the latter case they are produced by cell types other than target cells (Bruckner *et al.*, 1989; Tschan *et al.*, 1990; Raff, 1993; Ishizaki *et al.*, 1993, 1994). This view—that only constant support from neighboring cells keeps the cells alive — may also be extended to some unicellular animals (see II.C, II.D, and III.E).

PCD is still only partly understood. It is regulated by the expression of early genes in the cell cycle, such as *c-fos, c-myc, c-jun,* and *cdc2* (Pandey and Wang, 1995), the tumor suppressor gene *p53,* ICE (interleukin-1-β-

converting enzyme) cysteine protease, and the proto-oncogene family of *bcl-2* in mammalian cells (Reed, 1994; Häcker and Vaux, 1995; Wertz and Hanley, 1996; Duke *et al.*, 1996). For example, the cMyc and cFos proteins induce PCD in cultured cells deprived of serum and growth factors (Evan *et al.*, 1992; Smeyne *et al.*, 1993; Harrington *et al.*, 1994) in a process mediated by p53 (Wagner *et al.*, 1994; Hermeking and Eick, 1994; Hermeking *et al.* 1995; Pandey and Wang, 1995). ICE protease destroys cells by attacking the scaffolding of DNA and by activating enzymes that break up nuclear chromatin (see below). The proteins Bax and Bcl-X_S induce PCD, whereas Bcl-2 and Bcl-X_L suppress it by dimerizing and inhibiting the activities of Bax and Bcl-X_S. Furthermore, Bcl-2 inhibits cMyc-mediated PCD (Fanidi *et al.*, 1992; Bisonnette *et al.*, 1992; Wagner *et al.*, 1993). Homologous gene products are involved in the development of *C. elegans* (Vaux, 1993; Steller, 1995).

PCD is associated with multiple cellular changes which depend on the nature and origin of the dying cell. Clarke (1990) distinguished between four morphological types of PCD: (1) apoptosis, (2) autophagic degeneration, (3) nonlysosomal vesiculate disintegration, and (4) a cytoplasmic type. Often cell death follows apoptosis or "shrinkage necrosis," as originally reported by Kerr (1971), Kerr *et al.* (1972), and Wyllie *et al.* (1980). Apoptosis leads to rapid cell dehydration, to an increase in the levels of free Ca^{2+}, and to activation of both endonucleases and transglutaminase (Arends *et al.*, 1990; Wyllie *et al.*, 1984; Ellis *et al.*, 1991; Wyllie, 1992; Wyllie *et al.*, 1992). Endonucleases fragment DNA into pieces of 50–300 kb (Filipski *et al.*, 1990), correlating with the size of chromatin loop domains of the nuclear scaffold, and into pieces of 180–200 bp and multiples thereof by so-called *internucleosomal cleavage* (Bortner *et al.*, 1995). Apoptosis involves (1) nuclear condensation, (2) pyknotic chromatin dispersed along the inner margin of the nuclear envelope, (3) convolution or "blebbing" of the cell membranes, (4) loss of ribosomes from rough endoplasmic reticulum and polysomes, and (5) reduction in the volume of the cytoplasm which becomes electron-dense (Wyllie *et al.*, 1980). The cells fragment into cytoplasmic bodies as the result of blebbing. These bodies are engulfed by macrophages and neighboring cells. In this way damage to the surrounding tissue is contained (Wyllie *et al.*, 1980). Many PCD processes are increasingly being shown not to exhibit all (or even most) of these "hallmarks."

PCD in metazoan cells does not always follow classical apoptosis. Dying mammalian neurons may degenerate by autophagy in which lysosome-derived vacuoles consume the cytoplasm. This process rarely leads to membrane blebbing, chromatin margination, or DNA fragmentation, but the nucleus often becomes pyknotic (Clarke, 1990). Examples of nonapoptotic, autophagic PCD come from studies on metamorphosis in insects. They concern the development of intersegmental muscles (Schwartz *et al.*, 1990,

1993) and the labial gland in the moth *Manduca sexta* (Zakeri *et al.*, 1996). Evidently, PCD is morphologically and biochemically diverse. Extensive subdivision of cell death is an attempt to grapple with the diversity of responses shown by injured and moribund cells. Apoptosis occurs in response to adverse agents, such as gamma irradiation, heat shock, and free radicals of oxygen (Columbano, 1995; Wertz and Hanley, 1996), and cell death may be accompanied by both apoptotic and necrotic features (Clarke, 1990; Columbano, 1995). However, the field of *pathological cell death* should become clearer, but many have expressed grave doubts as to what goes on. Columbano (1995) and Farber (1994) considered the present state of our concepts confusing and unintelligible and have underlined the naívity of the simplistic notion that "apoptotic" vs "necrotic" are the two main categories.

PCD was originally described as a phenomenon pertaining to metazoa. Since many groups have now found evidence for PCD or related processes in unicellular organisms (see II.C, II.D, III.E, and V), it is highly possible that it was present all along in these cells and exploited in connection with the *process of differentiation* in multicellular life.

C. Minimal Nutrient Substrates

Cells are often grown on rich nutrient substrates to ensure that they will "take." Kidder and Dewey (1951) grew unicellular organisms, ciliates, in minimal media in order to examine their specific nutrient requirements. Their media are protein- and lipid-free and consist of amino acids, vitamins, glucose, and salts. Minimal media can give information on relations between cells and their substrates with respect to cell survival, proliferation, differentiation, chemosensory behavior, etc. Use of minimal media is a *leitmotif* in this review.

Early studies on metazoan cells showed that minimal media are important for investigating signaling mechanisms. Thus Zwiller *et al.* (1982) grew transformed neuroblastoma and glioma cells in serum-free medium. They found that hemin and sodium nitroprusside activated cell proliferation, both compounds turning on a guanylate cyclase (cf. III.D.1). Furthermore, lshizaki *et al.* (1993) showed that autocrine signals inhibit PCD in lens epithelial cells in minimal media. These cells die at low initial densities, but cell-free medium from a culture with many cells rescues them. Addition of serum makes it impossible to see the effects of hemin and autocrine factors, because serum itself activates proliferation. Thus minimal media are useful for studying details of cell signaling.

The immediate effects of minimal nutrient medium are long lag phases, long generation times, or cell death. Compounds shortening lag phases and

rescuing cells from dying throw light on *in vivo* mechanisms involved. Three groups of compounds come to mind in this connection: phorbol esters, lipids, and tetrapyrroles, all of which have remarkable effects on proliferation of ciliates (see III.D) and yeast cells (V). The latter group of compounds — the tetrapyrroles, i.e., hemin, chlorophyllin, and protoporphyrin IX — also has spectacular effects on the proliferation of a flagellate, *Crithidia fasciculata* (see II.C).

We have used an experimental protocol for *Tetrahymena* in which we inoculated a known, *low number of cells* to give us predetermined low initial numbers of cells/ml. This density could be 1 or 5000 cells/ml or anything between these two values. This approach has been combined with *minimal nutrient media.* Furthermore, we reduced the amounts of cell-produced, proliferation-activating compounds from the mother culture by *three washings of the cells* in a centrifuge immediately before inoculation. In this way we reduced carryover of cell-produced, activating compounds at inoculation. The cells did not always grow as well as the controls in the complex media, but this approach yielded much biological information (see III).

D. Summary

Signaling systems exist in unicellular eukaryotes and have been studied in many laboratories. Gene sequences have been analyzed, enzymes have been isolated and characterized, and physiological/pharmacological experiments with cells in minimal media have been made, the latter showing connections between intracellular signaling pathways in living cells. All of these studies have contributed to showing similarities between genes, regulatory proteins, and pathways between cells of the two groups, the unicellular and the multicellular organisms.

II. Signaling Systems in Unicellular Eukaryotes

Here we are referring to "signaling systems" in the broadest sense of the term. They consist of primary messengers, receptors, effector enzymes, and messenger molecules. Many compounds affect signaling systems and alter cell behavior. To give examples, they can induce nonproliferating cells to proliferate, differentiate, survive, or die. They can also induce cells to change direction of locomotion or speed. The study of signaling is relatively new in unicellular organisms. The field is in its initial stage and our present

knowledge centers around a few activities in a few species. A more coherent picture should emerge with time.

A. Growth Factors in Paramecium

Autocrine growth factor-like compounds from unicellular eukaryotes were first purified from cultures of the ciliate *Paramecium.* The doubling times of the *jumyo* mutant of *P. tetraurelia* are long compared to those of wild-type cells (Takagi *et al.,* 1987). These doubling times are restored to normal values if the mutant cells are grown in mass cultures or if a concentrate of cell-free, conditioned medium is added to them (Takagi *et al.,* 1989). This indicates that signal compounds are being released to the medium. Using ultrafiltration, chromatography methods, and SDS–PAGE, Tanabe *et al.* (1990) isolated a 17-kDa protein (ParGF) from conditioned medium, and this protein stimulates proliferation of the mutant down to 10^{-9} *M*. The reason for the slow proliferation in the mutant is as yet unknown. Also, other cells secrete factors reducing the doubling times of the *jumyo* mutant. This is true for *P. tetraurelia, P. caudatum, P. multimicronucleatum* (Takagi *et al.,* 1993), and *Tetrahymena* (Tokusumi *et al.,* 1996). Even mammalian sera, epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α) stimulate proliferation in the *jumyo* mutant (Tokusumi *et al.,* 1996).

B. Pheromones in *E. raikovi*

Pheromones are intercellular signal compounds which control cell recognition in mating (Luporini and Miceli, 1986). In a marine ciliate, *E. raikovi,* the pheromones have diverse effects in starving and feeding cells. These compounds are constantly released, and they form a large family of homologous proteins (Luporini *et al.,* 1996) consisting of 37–40 amino acids (Raffioni *et al.,* 1988, 1989, 1992). So far, five pheromones have been characterized (Concetti *et al.,* 1986; Miceli *et al.,* 1983, 1991; Raffioni *et al.,* 1987, 1988, 1989, 1992). They share little overall sequence identity, but all of them have six cysteine residues at homologous positions and three alpha-helices with an up–down–up topology (Stewart *et al.,* 1992; Brown *et al.,* 1993; Luginbühl *et al.,* 1994). Binding between a pheromone and its receptor requires sequence and structure homology between the two molecules. A pheromone binds to, dimerizes, and activates two receptor molecules, which is possible because it carries a deep cleft and an asymmetrical charge distribution (Ortenzi *et al.,* 1990; Miceli *et al.,* 1992; Brown *et al.,* 1993; Luporini *et al.,* 1996). The receptor is produced from an isoform of a

prepro-pheromone, and this is immunologically equivalent to the mature pheromone. Thus it is possible that the pheromone and its receptor originate from the same precursor (Miceli *et al.*, 1992), as happens with EGF and TGF-α in mammalian cells (Massagué, 1990).

1. Starving Cells

Pheromones induce cells of different mating types to form pairs, and the paired cells exchange genetic material (Luporini *et al.*, 1996).

2. Feeding Cells

Ortenzi *et al.* (1990) and Luporini *et al.* (1992) studied pheromones in *E. raikovi* cells. They are structurally similar to the mammalian cytokin interleukin-2 (IL-2) (Ortenzi *et al.*, 1990; Luporini *et al.*, 1994). Furthermore, the pheromones cross-react with membrane components of the IL-2-dependent survival and proliferation system in T lymphocytes (Luporini *et al.*, 1996). Vallesi *et al.* (1995) showed that pheromones are mitogenic in *E. raikovi* in the presence of nutrients. A divalent pheromone antibody *activates* and its monovalent fragment *inhibits* mitogenesis. These authors suggested that the divalent antibody acts by binding to the immunologically equivalent region of the NH_2 end of the receptor which is then dimerized and activated. In contrast, the monovalent antibody prevents receptor dimerization. These results show that a *single* type of autocrine signal molecule activates sexual processes under starvation conditions, but proliferation under feeding conditions. Ortenzi *et al.* (1990) and Luporini *et al.* (1992) already consider the pheromones autocrine growth factors.

C. Apoptosis in Parasitic Flagellates

Apoptosis occurs in cultures of *Trypanosoma* and *Leishmania* as a result of host signals, low levels of autocrine survival factors, or heat shocks (Ameisen *et al.*, 1995; Welburn *et al.*, 1996; Moreira *et al.*, 1996). Death seems to be tied to changes in the life cycles of the parasites as they pass through their hosts (Schaub, 1994; Matthews and Gull, 1994). Thus epimastigotes of *T. cruzi* differentiate into metacyclic trypomastigotes passing from insect to mammalian hosts. Extensive internucleosomal DNA fragmentation accompanies *in vitro* cell death (Ameisen *et al.*, 1995), which may be inhibited by autocrine survival factors. Only cultures at high initial cell densities reach a threshold level of signal compounds sufficient to prevent death and support survival and proliferation. Also, apoptotic cell death (Ameisen *et al.*, 1995) occurs after a shift-up of the temperature,

mimicking the transition from the insect to the mammalian host, and after the epimastigotes are incubated with human complement (Nogueira *et al.*, 1975; Hall and Joiner, 1993). The temperature shift leads to apoptosis due to increased intracellular levels of Ca^{2+} in promastigotes of *L. amazonensis* (Moreira *et al.*, 1996).

Host signals influence survival and differentiation in procyclic (Vickerman, 1985) forms of trypanosomes. Glycosyl lectins secreted from the midgut of the host kill them. This is accompanied by nuclear condensation and surface blebbing (Maudlin and Welburn, 1987; Welburn *et al.*, 1989). Also, the plant lectin concanavalin kills *T. brucei rhodesiense* (Welburn *et al.*, 1996). This process is accompanied by *de novo* gene expression and includes DNA fragmentation (Welburn *et al.*, 1996), supporting the notion that PCD occurs in these flagellates.

These findings show that PCD and apoptosis are regulated by autocrine and host signals. Thus basic FGF-like activity has been found in African trypanosomes and in *Leishmania* (Kardami *et al.*, 1991), and the colony-stimulating factor from granulocyte macrophages acts as a growth factor and inhibits heat-induced apoptosis in *L. amazonensis* (Barcinski *et al.*, 1992; Welburn *et al.*, 1996). Moreover, the parasites respond to host cytokines supporting growth (Olsson *et al.*, 1992; Barcinski and Moreira, 1994; Lucas *et al.*, 1994) or inhibiting PCD. The downstream events in this signaling cascade are largely unknown. *T. brucei* cells have surface receptors for EGF (Hide *et al.*, 1989). The growth factor is mitogenic in serum-depleted medium and it stimulates protein kinase activity in procyclic trypanosomes (Hide *et al.*, 1989, 1994). In addition, EGF promotes proliferation of trypomastigotes (Sternberg and McGuigan, 1994). These findings indicate the presence of signal transduction pathways in these trypanosomes. *T. brucei* cells have many protein kinases that phosphorylate proteins at both serine/threonine and tyrosine residues, and they may play major roles in proliferation and differentiation (Parsons *et al.*, 1991, 1993; Gale *et al.*, 1994). Thus Wheeler-Alm and Shapiro (1992) showed that tyrosine kinases are required for proliferation and that both protein kinase activity and proliferation are reduced by genistein, a tyrosine kinase inhibitor. However, they found no evidence for EGF-induced phosphorylation of a tyrosine kinase specific substrate in an *in vitro* system. Also, PKC-related enzymes are implicated in proliferation in both *T. cruzi* and *T. brucei cells* (Gomez *et al.*, 1989; Keith *et al.*, 1990). In addition, epimastigotes of *T. cruzi* contain a functional inositol/DAG signaling pathway (DoCampo and Pignataro, 1991), but staurosporine at 1 μM, a serine/threonine kinase inhibitor, does not induce death in epimastigotes of *T. cruzi* (Ameisen *et al.*, 1995). This indicates that protein phosphorylations at serine/threonine residues do not reverse death by apoptosis in these cells, but effects of other serine/threonine kinase inhibitors and perhaps higher concentrations of staurosporine should be tested.

Crithidia fasciculata, also a parasitic flagellate, can be grown in a minimal chemically defined nutrient medium (Kidder and Dutta, 1958). Sørensen and Rasmussen (unpublished research) found that 6-anilino-5,8-quinolinedione (LY 83583) and [^{1}H]-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one, inhibitors of the soluble (NO-dependent) guanylate cyclase (Mulsch *et al.,* 1988; Randriamampita *et al.,* 1991; Garthwaite *et al.,* 1995), block cell proliferation. Addition of 8-bromo-cyclic GMP bypasses the effect of LY 83583 (Sørensen and Rasmussen, unpublished research). Similar effects are also seen in ciliates and yeast (see III.D and V.B). These results indicate that soluble guanylate cyclase, originally found in mammalian cells, plays a crucial role in the proliferation of lower groups of unicellular eukaryotes.

In this view, it would be interesting to see the effects of 8-bromo-cGMP and activators of the soluble guanylate cyclase system, namely, sodium nitroprusside and protoporphyrin IX (see III.D), on parasitic flagellates during programmed cell death.

D. Coalescence Factors in *Dictyostelium discoideum*

Many intercellular signal molecules take part in proliferation, differentiation, and chemosensory behavior in cells of the myxomycete *D. discoideum.* Starving, free-living cells assemble and differentiate into a multicellular aggregate of up to 10^5 individual cells. They form a migrating slug which differentiates into a foot plate, a stalk, and a fruiting body. Spore cells germinate into new free-living slime mold cells (Loomis, 1982). This life cycle is regulated by several autocrine/paracrine signal molecules, including cyclic AMP (cAMP), *differentiation-inducing factor* (DIF), *prestarvation factor* (PSF), and *conditioned medium factor* (CMF) (Gerisch, 1986, 1987; Clarke *et al.,* 1992; Clarke and Gomer, 1995). cAMP is the major chemotactic agent during cell aggregation. Upon starvation, cAMP is secreted in pulses, generating waves of the chemoattractant. These waves direct the cells to move toward the oscillating centers by sequential activation and adaptation of intracellular signaling pathways. In contrast, prespore and prestalk cells are exposed to a high, continuous level of cAMP (Van Haastert, 1991; Devreotes, 1994; Firtel, 1995). The cAMP binding sites are STM receptors (Devreotes and Zigmond, 1988). They activate multimeric G proteins, adenylate cyclase, guanylate cyclase, phospholipase C, influxes of Ca^{2+}, and effluxes of H^+ and K^+. All these effects induce gene expression (Reymond *et al.,* 1995) and trigger alterations in the cytoskeleton required for pseudopodial extensions implicated in chemotaxis (Fukui, 1985; Devreotes, 1994; Van Haastert, 1995; Noegel and Luna, 1995). These processes include the phosphorylations and dephosphorylations of myosin II heavy chain (Rahmsdorf *et al.,* 1978; Berlot *et al.,* 1987; Yomura and Kitanishi-

Yomura, 1992) and are regulated by cGMP (Liu and Newell, 1994; Newell *et al.*, 1995) and a membrane bound PKC isoform (Ravid and Spudich, 1989, 1992; Pasternak *et al.*, 1989; Egelhoff *et al.*, 1993; Abu-Elneel *et al.*, 1996). The MAP kinase ERK2 is also active in slug formation. Thus ERK2 is required for cell aggregation, development of the multicellular slug, and may be essential for morphogenesis and cell-type-specific gene expression (Segall *et al.*, 1995; Gaskins *et al.*, 1996). Maeda *et al.* (1996) proposed that cAMP through STM receptors stimulates the activity of adenylate cyclase by activating a calcium-dependent MAP kinase cascade.

Other autocrine signals are important in the developing slime mold. DIF, a chlorinated phenyl alkanone (Berks *et al.*, 1991), induces cell differentiation in the slug (Town *et al.*, 1976; Brookman *et al.*, 1982; Kay and Jermyn, 1983; Morris *et al.*, 1987; Williams *et al.*, 1987; Early *et al.*, 1995; Kawata *et al.*, 1996). PSF is a glycoprotein of about 70 kDa (Rathi *et al.*, 1991) that functions as a "cell-density-sensing factor." It induces gene functions leading to an increased synthesis of *discoidin* (regulating cell streaming), cAMP signaling, cell-to-cell adhesion, and lysosome functions (Clarke *et al.*, 1987; Rahti *et al.*, 1991; Rahti and Clarke, 1992; Schatzle *et al.*, 1991). PSF is continuously secreted by growing cells (Clarke *et al.*, 1988), but active only at high concentrations (i.e., at high cell densities) and when the food supply becomes scarce (Clarke *et al.*, 1987). Bacterial components can bind to the cells and block PSF signal transduction (Clarke *et al.*, 1988; Burdine and Clarke, 1995). CMF is an 80-kDa glycoprotein (Gomer *et al.*, 1991) active at high cell densities (Grabel and Loomis, 1978; Gomer and Firtel, 1987). The cells need high levels of CMF in order to transduce the cAMP-mediated signal during chemoattraction (Mehdy and Firtel, 1985; Gomer and Firtel, 1987; Gomer *et al.*, 1991; Yuen *et al.*, 1991, 1995; Van Haastert *et al.*, 1996). CMF is also implicated in cell aggregation in other ways (Jain *et al.*, 1992), but in contrast to PSF, CMF is slowly secreted upon starvation from both prespore and prestalk cells (Yuen *et al.*, 1991). The cells secrete small CMF-derived peptides with higher specific activities than the entire molecule after a long period of starvation (Yuen *et al.*, 1991).

The developing slime mold can be considered similar to a developing, multicellular embryo (Mutzel, 1995). The question therefore arises, does this development include *programmed cell death* (PCD)? Quite possibly. Formation of the stalk leads to nonviable, vacuolated, plant-like cells surrounded by a rigid wall (Mutzel, 1995), and this has been compared to PCD in metazoa (Cornillon *et al.*, 1994). Furthermore, DIF induces PCD in starved cells but not in growing cells of a mutant not producing this compound. DIF induces death only when cells have initiated differentiation (Cornillion *et al.*, 1994). These authors showed that the death process includes limited and focal condensation of chromatin, massive vacuolization, cytoplasmic condensation, and very late, membrane lesions. This mode of death has morphological and ultrastructural similarities to PCD in insect

cells during metamorphosis (Schwartz *et al.*, 1993; Zakeri *et al.*, 1993) and in some mammalian cell systems (Clarke, 1990). Cornillon *et al.* (1994) also showed that DIF induces an irreversible step leading to the inability to regrow in fresh medium even before any morphological alterations were seen. This supports the view that cell death associated with cell differentiation in *D. discoideum* follows a programmed course.

Signal molecules have been implicated in growth and proliferation in *D. discoideum* cells. First, a component of fetal calf serum stimulates growth (Loomis, 1987). Later, Whitbread *et al.* (1991) showed that conditioned medium from a dense culture stimulates proliferation and that it contains an active 12- to 14-kDa compound (*Dictyostelium* growth factor, DGF) resistant to SDS (sodium dodecyl sulfate) and heat treatments, but sensitive to mercaptoethanol or pronase treatments. These authors suggested that DGF supports proliferation only at high levels or at high cell densities. Details of the putative signal transduction pathway mediated by DGF are not yet known. The cells express a growth-specific ras gene (Ddras-G) (Robbins *et al.*, 1989). Furthermore, they express a MAP kinase termed ERK1 in both proliferating and differentiating cells, but this expression requires different promoters (Gaskins *et al.*, 1994). ERK1 is insensitive to cAMP and essential for proliferation in free-living cells (Maeda *et al.*, 1996; Gaskins *et al.*, 1994). Thus *ras*-like G proteins and ERK1 may take part in signal transduction in response to DGF.

In line with our general thesis that unicellular organisms presumably have a panoply of factors affecting many aspects of their life, behavior, and death, slime mold cells—as exemplified by *Dictyostelium*—clearly support this notion, having so many refined control systems available.

E. Tetrahymena and Yeast Cells

The fate of *Tetrahymena* cells in a culture in minimal, chemically defined medium depends on the initial cell density. The reason is that the cells release factors which prevent cell death (see III). In addition, compounds interfering with cell survival and proliferation also affect chemosensory behavior (see IV).

Overgaard *et al.* (unpublished research) did not find evidence for a *critical, initial* cell density in cultures of *Saccharomyces cerevisiae,* not even when grown in poor nutrient media. However, *S. cerevisiae* cells respond to and use both extra- and intracellular signaling mechanisms first discovered in multicellular cell systems (see V).

F. Summary

One basic feature common to all the unicellular systems investigated is the cascade leading to *cell survival and/or proliferation.* So far, we have mostly

mentioned enzyme systems in proliferation. Later, we will report on some of the effector molecules which are implicated here (see III). Some have been identified in the flagellate *C. fasciculata* (II.C), in *Tetrahymena* (III.C), and in yeast cells (V).

Increase of cell size should be called *cell growth;* increase in cell numbers should be called *cell proliferation.* We have often seen an increase in cell numbers referred to as "cell growth."

PCD was originally described as a phenomenon pertaining to metazoa. Since many groups have now found evidence for PCD in unicellular eukaryotes, it is highly possible that it was present all along in these cells and *exploited* in connection with the *process of differentiation* in multicellular life.

The interest in signaling mechanisms in unicellular organisms is now burgeoning. Here we selected results from phylogenetically distinct unicellular eukaryotes. Cellular mechanisms control whether the cells will stay alive, proliferate, differentiate, die, etc., and they have many common molecular features. This may not be surprising since we are dealing with fundamental mechanisms which have been in place for 1–2 billion years and are hardly likely to be altered without severe repercussions to the cells.

III. Signal Molecules in Cell Survival and Proliferation in *Tetrahymena*

Many microbes produce and release compounds first discovered in mammalian cells, such as hormones, growth factors, and other signal molecules. These microbes are both prokaryotes and unicellular eukaryotes (Csaba, 1994). The ciliate *Tetrahymena* has been singled out for studies of the effects of such compounds. At present, we do not know if autocrine signal molecules from *Tetrahymena* have any homology with mammalian survival and growth factors. However, activity of at least one factor from *Tetrahymena* can, in mammalian cells, mimic the effect of a mammalian hormone.

This review deals in part with cell survival and the lag phase and with the transition from this lag phase to exponential proliferation. The *T. thermophila* cells (wild-type inbred strain B-1868-III (Orias and Bruns, 1976)) in the lag phase have, unless anything else is stated, been transferred from 24-h-old cultures, i.e., from cultures in *early, stationary phase.* Cell proliferation has ceased for about 6 h in these cases. We know that we would get different results than those reported here if we had used 48-h-old cultures. Thus it is obvious that the stationary phase should be subdivided into many stages in the future.

A. General

1. Homologies in Signal Compounds in *Tetrahymena* and Mammalian Cells

Tetrahymena was long ago reported to release compounds with, for example, immunological similarity to insulin, somatostatin, adrenocorticotrophin (ACTH), β-endorphin, relaxin, vasotocin, calcitonin (LeRoith *et al.*, 1980, 1982, 1987; Shiloach *et al.*, 1985), dehydroepiandrosterone, estradiol (Csaba *et al.*, 1985), serotonin, and adrenaline (Janakidevi *et al.*, 1966; Blum, 1967; Brizzi and Blum, 1970; Csaba and Kovács, 1994). These results indicate that signaling processes are not confined to multicellular cell systems, but evolved with the origin of the unicellular eukaryotes. We agree with Csaba (1994, 1996) that signaling is an indispensable part of the biology of these cells.

Shiloach *et al.* (1985) reported that insulin-related material from *T. pyriformis* is similar in many respects to mammalian insulin. Thus it is highly comparable to insulin from swine with regard to effects on uptake and metabolism of glucose in porcine adipocytes. Excesses of anti-insulin antibody or antibodies directed against the insulin receptor greatly reduce the effect of *Tetrahymena* insulin (Shiloach *et al.*, 1985). In addition and reciprocally, Csaba and Lantos (1975, 1976) reported that insulin and adrenalin affect glucose uptake and metabolism in *T. pyriformis.* Insulin, ACTH, histamine, histidine, serotonin, etc. affect phagocytosis, possibly via multiple receptor mechanisms (for references, see Köhidai *et al.*, 1995). Furthermore, prototypic opioid agonists, such as morphine and β-endorphin, inhibited phagocytosis by a naloxone-reversible mechanism (De Jesús and Renaud, 1989; Salamán *et al.*, 1990; Chiesa *et al.*, 1993). Many mammalian signal molecules also had effects on the chemosensory behavior of *Tetrahymena.* These include insulin, PDGF, EGF, FGF, β-endorphin, and the leukocyte attractant fMet-Leu-Phe (Leick *et al.*, 1996b). Mechanisms in chemosensory behavior are described later (see IV).

There are also many similarities of the intracellular transduction systems in uni- and multicellular organisms. These similarities include G proteins, components of the inositol phospholipid pathway (Kovács *et al.*, 1996), phospholipase C and D (Florin-Christensen *et al.*, 1986; Kovács *et al.*, 1996), protein kinases (Hegyesi and Csaba, 1994a; Straarup *et al.*, 1997), adenylate cyclase/cAMP systems, guanylate cyclase/cGMP systems, calcium–calmodulin systems (Christensen *et al.*, 1996a; Csaba, 1994, 1996; Umeki and Nozawa, 1996), and nitric oxide systems (Köhidai *et al.*, 1992; Christensen *et al.*, 1996a).

2. Receptors

Tetrahymena cells bind insulin in the plasma and cilia membranes, in vesicles, and in the nuclear envelope (Csaba, 1985; Kovács and Csaba, 1987;

Christopher and Sundermann, 1992; Hegyesi and Csaba, 1992a,b, 1994b). The binding sites on the surface membranes are similar to those of mammalian receptors in terms of saturation kinetics and temperature and pH dependence (Kovács and Csaba, 1990). The latter results indicate that these binding sites have some similarities to those of mammalian receptors. Christopher and Sundermann (1995b) partially characterized a membrane protein of 62–67 kDa from cilia of *T. pyriformis* which is immunologically similar to mammalian insulin. They suggested that their protein may be a precursor of both an insulin-like growth factor and its receptor. More recently, Christopher and Sundermann (1996) found no immunogenic evidence for the presence of plasma and cilia membrane proteins identical to the mammalian receptor α-subunit. In contrast, such compounds are detected in mitochondria, cilia (microtubules), nuclei, and cytoplasm in *T. pyriformis.* Furthermore, immunoblotting with the α-subunit antibody reveals two active proteins of 38 and 97 kDa in whole cell lysates, and these were not found in cilia membranes. The authors suggested that insulin binds to the cell surface through interaction with the insulin-like protein in ciliary membranes.

O'Neill *et al.* (1988) and Zipser *et al.* (1988) found an opioid receptor in *Tetrahymena* with sequence homologies to receptors in leeches and rats. β-Endorphin was a chemoattractant in these cells, and this effect was blocked by (−)naloxone but not by (+)naloxone, suggesting the presence of stereo-specific opiate receptors (O'Neill *et al.*, 1988).

3. Signal Imprinting and Receptor Induction

Csaba (1980, 1996) proposed that *Tetrahymena* cells memorize their first encounter with growth factors or hormones and then alter their sensitivity to these compounds. This phenomenon was called *hormonal imprinting* (Csaba, 1980) or *receptor induction* (Christopher and Sundermann, 1992), and this could be subject to regulatory processes similar to those in multicellular organisms (Christopher and Sundermann, 1995a; Csaba, 1994, 1996). Many compounds, including single amino acids, dipeptides, and signal compounds, such as insulin (Csaba, 1996), elicit imprinting. This results either in an increase, "positive imprinting," or a decrease, "negative imprinting," in the binding of the signal molecules to the surface membrane of the cells in offspring generations (Csaba, 1994, 1996). Csaba and Kovács (1987) suggested that imprinting could be transferred to unexposed cells by the release of a "transfer factor" in their growth medium.

The molecular mechanism of imprinting is not understood, but Csaba (1996) has suggested three possibilities: (1) rearrangement of genes similar to the process of the formation of the antibody-coding genes in mammalian cells; (2) rearrangement of methylation patterns of the appropriate genes

influencing the expression of receptors, or (3) changes in the membrane subpatterns forming the receptors. Christopher and Sundermann (1996) found that insulin altered the protein composition in ciliary membrane extracts. SDS–PAGE analysis followed by silver staining revealed a stronger band at 30 kDa and several new protein bands in the 35- to 50-kDa range. These proteins may be candidates for cellular activities in insulin imprinting.

B. Autocrine Signals in Cell Survival and Proliferation

1. Minimal, Chemically Defined Nutrient Media

These media are useful in any critical study of regulation of cellular behavior in cultured cells. They offer ways of obtaining information not only on nutrient requirements, but also on signal molecules. Without recourse to fully defined, minimal media, many studies on signals languish because intercellular signaling occurs against a rich backdrop of empirical additives (i.e., supplements traditionally supplied, usually in excessive quantities, to "guarantee" that the cells will "take" and thrive). Signals become lost in the noise of these supplements. For growing cultures of unicellular pro- and eukaryotes, not only is proteose peptone supplied, but extracts of liver and yeast are often included. For growing cells from metazoa, media are usually enriched with serum and often growth factors or extracts are added, e.g., EGF and bovine pituitary extracts (Wheatley *et al.,* 1993, 1994; Christensen *et al.,* 1995).

Chemically defined media have been developed over the last 50 years for *Tetrahymena* (Kidder and Dewey, 1951; Holz *et al.,* 1962a; Rasmussen and Modeweg-Hansen, 1973; Szablewski *et al.,* 1991). The basic medium consists of amino acids, vitamins, glucose, nucleosides, and salts. With these media has come the realization that ciliates do not grow simply because they have adequate nutrition, but because certain signal molecules—as in multicellular organisms—activate cell survival and proliferation (Christensen and Rasmussen, 1992; Christensen *et al.,* 1993; Wheatley *et al.,* 1993; Schousboe *et al.,* 1997). The effects of such compounds were reported long ago by Cutler and Crump (1923), Robertson (1924), Hall and Loefer (1940), Kidder (1939, 1941), and Lilly and Stillwell (1965) and more recently by Tanabe *et al.* (1990), Takagi *et al.* (1993), and Vallesi *et al.* (1995), based on studies in free-living ciliates. Autocrine (paracrine) secretion of signaling compounds remains an obvious possibility.

2. Cell-to-Cell Interactions

The fate of *Tetrahymena* cells in a nutritionally complete but minimal chemically defined medium (CDM) (Szablewski *et al.,* 1991) depends on

the number of cells transferred to new medium. This is most apparent in cultures of *T. thermophila, T. pyriformis,* and *T. setosa* (Christensen and Rasmussen, 1992; Schousboe *et al.,* 1992; Christensen *et al.,* 1992, 1993; Christensen, 1993). *T. thermophila* cells die when transferred into conical culture flasks at low initial cell densities, i.e., <1000 cells/ml at the optimum temperature of 37°C (Fig. 2), despite the fact that the same nutrient medium can support proliferation up to a million cells/ml when inoculated at higher initial cell densities. In this way, *Tetrahymena* cells are similar to most cultured cells from metazoa. Other species (e.g., *T. pigmentosa* and *T. vorax;* see III.F) readily proliferate in CDM presumably at *any* initial cell density (Schousboe *et al.,* 1992). However, at low initial densities *T. vorax* cells have longer lag phases and longer generation times than cells at high

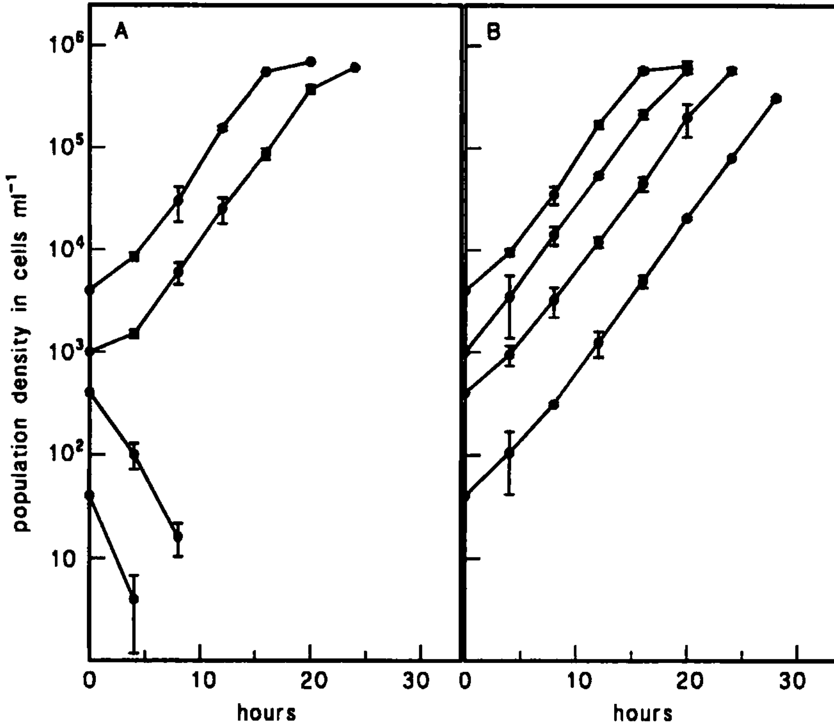


FIG. 2 Cell density of *Tetrahymena thermophila* in chemically defined nutrient medium in conical flasks as a function of time and the initial cell density in the absence, CDM (A), and in the presence of cell-free, conditioned medium, CCDM (B). Before inoculation, cells were washed three times either in defined nutrient medium or in a Tris/HCl buffer at pH 7.2. In both cases there was a $>10^5$-fold dilution of the original extracellular medium to reduce carryover of extracellular signals from the mother culture. Cell proliferation or cell death depends on the initial cell density. Redrawn from Christensen and Rasmussen (1992).

densities (Christensen, unpublished research). This suggests that cell-to-cell interactions promote cell survival and/or initiate cell multiplication. As will be shown later, this interaction can occur via autocrine signal molecules. In contrast, Kristiansen *et al.* (1996) reported that cells of *T. thermophila* at low densities do not die in CDM if their culture vessels are closed in such a way that there is no border between medium and air. They suggested that certain stress situations, e.g., at the culture surface, affect life and death of the cells. Furthermore, Hagemeister *et al.* (1996) reported (in an abstract) that cells at low densities survive in CDM in "open" cultures in medium with reduced levels of Ca^{2+}, Mg^{2+}, and certain amino acids.

3. Autocrine Signal Molecules

T. thermophila cells die rapidly when inoculated at low initial densities (e.g., at 250 cells/ml) into CDM in conical culture flasks (Christensen *et al.*, 1995). Cell-free, conditioned medium from high-density cultures prevents cell death and activates proliferation (Christensen and Rasmussen, 1992; Schousboe *et al.*, 1997) (Fig. 2). Similar experiments were made with single-cell cultures. Single cells were transferred into volumes of 1 μl (i.e., in droplets of CDM embedded by paraffin oil in a petri dish). These cultures have an initial cell density of 1000 cells/ml, and the single cells survive and multiply with a frequency of more than 90% (Table I). In contrast, single cells transferred to 10 μl under similar conditions usually died within 10–40 min (Christensen *et al.*, 1995, 1997). Ten cells transferred into 10 μl of medium all survived during a period of 60 min and subsequently proliferated

TABLE I

Single-Cell Experiments of *Tetrahymena thermophila* in 1, 10, and 1000 μl of Chemically Defined Nutrient Medium (CDM) and in Enriched Proteose Peptone Medium (PPYS)

Culture volume	CDM	PPYS
1 μl	29/32 (91%)	15/15 (100%)
10 μl	3/38 (8%)	24/25 (96%)
1000 μl	0/30 (0%)	29/30 (97%)

Note. 3/38 indicates that 3 of 38 single cells survived and formed a new culture. *One- and ten-microliter cultures:* single cells were transferred into microdrops embedded in sterile paraffin oil placed in a petri dish. *One thousand-microliter cultures:* single cells were transferred into short, 100 × 16-mm, loosely capped test tubes containing 1 ml of medium. From Christensen and Rasmussen (1992).

(Christensen *et al.*, 1997). Taken together, these results permit the conclusion that cell-to-cell communication in all these cases does not occur via debris from dead cells, but rather through autocrine (paracrine) signal compounds. We obtained similar results using various mutant cells: the *T. thermophila* mutant MS-1 [secreting lysosomal enzymes at low rates (Hünseler *et al.*, 1987)], mutant SB 281 [releasing no mucocysts (Maihle and Satir, 1985)], and mutant II8-G [defective in food vacuole formation (Tiedtke *et al.*, 1988; Christensen and Rasmussen, 1992)]. This indicates that the signal compounds are not released through mucocysts, by egestion of food vacuoles, or with lysosomal activity.

Schousboe *et al.* (1997) partially purified a 7- to 8-kDa protein from cultures of *T. thermophila* and from water in which the cells were suspended. This compound supported cell survival and proliferation and was isolated after dialysis, ultrafiltration, and anion exchange. It was characterized by SDS–PAGE analysis and dubbed *Tetrahymena* proliferation-activating factor (TPAF). This protein supports cell survival and proliferation even at low concentrations in cultures at low initial cell densities. About 10 μg TPAF/l is released from cells grown to 100,000 cells/ml in CDM. A 100-fold dilution of this fraction activates proliferation in cultures with 100 cells/ml, which indicates that TPAF is active at ca. 10^{-10} *M*. If TPAF is the single autocrine molecule for cell survival, it is active at 10^{-14} *M*. These results support previous findings (Christensen and Rasmussen, 1992) and demonstrate that specific signaling between individual cells affects life and death in *T. thermophila.*

Other autocrine factors affect cellular responses in *Tetrahymena*. Andersen and Houen (1989) found an autocrine protein of about 5 kDa which is released by multiplying cells. It accumulates in dense cultures and reduces the transcription rates by inhibiting the activity of RNA polymerase (Andersen and Islin, 1987). In addition, Schousboe *et al.* (1997) found evidence that a dialyzable factor produced by *T. thermophila* cells in stationary phase inhibits cell proliferation and is called *Tetrahymena* proliferation-inhibiting factor. They found that the extracellular medium from 48-h-old cultures (mixed 1 : 1 with fresh medium) does not activate cells to proliferate unless the medium is dialyzed. It is possible that the cells start to release an inhibitor of cell multiplication when their growth rates decrease. Rasmussen *et al.* (1996) proposed that cells survive starvation for longer durations if they do not reduce their size by cell divisions. A cell-produced block for division at the beginning of a starvation period could have been a great evolutionary advantage.

4. Cell Survival and Proliferation in Complex Media

Single *T. thermophila* cells survive and proliferate in 1-ml portions of a complex medium of proteose peptone enriched with yeast extracts and salts

(PPYS; Table I) (Orias and Rasmussen, 1976; Christensen and Rasmussen, 1992; Schousboe *et al.*, 1992). These single cells "took" with a frequency of about 90%. This fits the idea that the PPYS medium contains substances which either mimic or substitute for TPAF (Christensen and Rasmussen, 1992) or in other ways activate control mechanisms for proliferation. These substances can be lipids and/or proteins present in PPYS (Schousboe, unpublished research). This could explain the fact that PPYS has been a very successful nutrient medium for cultivation of many cells for almost a century.

CDM has opened a whole realm of possibilities for studying signaling mechanisms in terms of cell survival, proliferation, differentiation, chemosensory behavior, and programmed cell death in *Tetrahymena*. In the future, it will be very interesting to test other organisms using this kind of media.

C. Effects of Mammalian Growth Factors and Hormones

Signal compounds from mammalian sources have been tested in *Tetrahymena* cultures (see III.A). Andersen *et al.* (1984) showed that PDGF is mitogenic in a dose-dependent manner down to about 2×10^{-9} *M*. Furthermore, PDGF also stimulates DNA synthesis at similar low concentrations. Csaba *et al.* (1995) found that human cytokines IL-3 and IL-6 stimulate proliferation and increase insulin binding in *T. pyriformis.* Mammalian signal compounds promote cell survival in *T. thermophila*, including insulin (10^{-14} *M*) (Christensen *et al.*, 1996b), ACTH (3×10^{-7} *M*), EGF (10^{-10} *M*), PDGF (10^{-10} *M*), and NGF (10^{-11} *M*) (Kristiansen *et al.*, 1996). The following section discusses the effects of insulin in *T. thermophila* and relations between mammalian signal compounds and TPAF.

1. Biphasic Response to Insulin

The effect of human recombinant insulin on survival and proliferation has been studied in *T. thermophila* cells (B-III 1868; Orias and Bruns, 1976). Insulin stimulates cell survival and activates proliferation in two separate concentration ranges with effects down to about 3×10^{-8} *M* and again at 10^{-14}– 3×10^{-12} *M* (Christensen *et al.*, 1996b) (Fig. 3). The reason for this pattern is unknown, but it can be due either to the presence of two types of receptors which bind insulin with different affinities or to a constant inhibition/desensitization between separate receptor populations, as reported for receptors in mammalian cells (e.g., Housley *et al.*, 1987). A further observation needing to be explained is that insulin differs in its action in the two concentration intervals. Cells at the low level of insulin

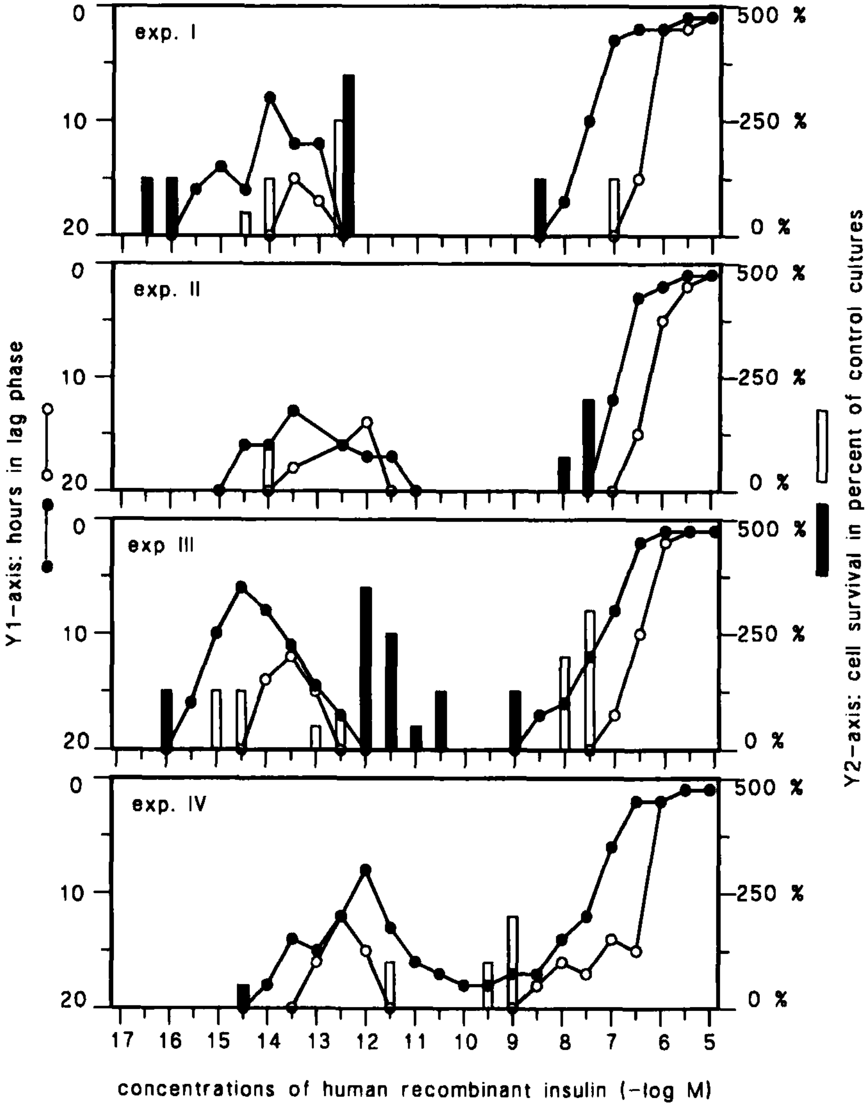


FIG. 3 Four individual experiments on the effects of human recombinant insulin on cell survival and proliferation in *Tetrahymena thermophila* in chemically defined nutrient medium in conical culture flasks at 400 cells/ml in the presence (solid symbols) and in the absence (open symbols) of 50 n*M* hemin. Solid lines show results from those cultures in which cells proliferated and the number of hours in the lag phase before the start of proliferation (Y1 axis). Bars show results from those cultures in which insulin promoted cell survival in a percentage of the control cultures (Y2 axis). From Christensen *et al.* (1996b).

from around 40 organisms/ml survive longer than the controls, but the cells do not proliferate, whereas they do from 400 cells/ml. Thus insulin has two effects (Christensen *et al.*, 1996b): at micro- and nanomolar levels it activates both *survival and proliferation,* whereas at the lower level it affects *survival* only. This may be caused either by promoting the secretion of TPAF or by lowering the threshold concentrations to such molecules (Wheatley *et al.*, 1993; Christensen *et al.*, 1996b).

2. Biological Activity of Insulin Fragments

Specific amino acid domains are important in the receptor binding and in the activity of peptide signals in mammalian cells. The C-terminal end of the insulin chain B has been singled out for particular attention (DeMeytz *et al.*, 1978; Saunders *et al.*, 1982; Gammeltoft, 1984). The amino acid residues in the aromatic triplet B24(Phe), B25(Phe), B26(Tyr) are especially important for directing insulin interaction with its receptor (Nagawa and Tager, 1987; Mirmira and Tager, 1989; Mirmira *et al.*, 1991). The effects of various peptide fragments of insulin on *T. thermophila* have also been explored. Cells incubated with a synthetic preparation of the insulin B22–B30 fragment survive about 3 times longer (10 h) than the control cultures (3 h) at 40 cells/ml. The same fragment—tested from 10^{-5} to 10^{-16} *M*—leads to cell survival *exclusively* in the low picomolar range, but is less potent than insulin (Christensen *et al.*, 1996b). These results indicate that domains of insulin may be linked with some activity of the entire molecule in *T. thermophila.*

Peptide fragments of the C-terminal end of the B chain of insulin are also active in mammalian cells. They mimic and/or enhance the activity of the entire insulin molecule. This activity can be both hormonal (with effects on carbohydrate metabolism) and mitogenic (with effects on cell growth and proliferation).

a. Hormonal Actions Weitzel *et al.* (1971, 1975) suggested that the peptide fragment B22–B25 forms a domain with the (B22, Arg) residue being a critical moiety for biological activites *in vivo* and *in vitro.* These activities can be both independent and dependent on the insulin receptor functions (Weitzel *et al.*, 1973, 1976; Kikuchi *et al.*, 1981a). More recently, Ng *et al.* (1989) showed that the B22–B25 fragment has both insulin-like and insulin-potentiating effects *in vivo* and proposed that it is internalized and acts through postbinding sites of the glucose metabolic pathway in target tissues. Also, other C-terminal fragments of the B chain are active. The B21–B26 fragment has insulin-like activity (Fujino *et al.*, 1977; Menuelle *et al.*, 1989) and this effect is enhanced by substitution of glycine for proline in the B23 position (Menuelle *et al.*, 1989). In addition, these and other peptide

fragments amplify and up-regulate the activity of the intact insulin molecule. These include B21–B26, B22–B25, B22–B26, and B23–B29 (Fujino *et al.*, 1977; Kikuchi *et al.*, 1980, 1981a,b; Menuelle *et al.*, 1989; Ng *et al.*, 1989). However, all are less potent and are needed at higher levels than the entire insulin molecule.

b. Mitogenic Actions Little is known about the effects of insulin fragments on cell survival and proliferation in mammalian cells. The N-terminal hexapeptide fragment of IGF II—identical to the C-terminal sequence of the Casiragua insulin B chain—promotes [^{3}H]thymidine incorporation at levels down to 10^{-8} *M* and up-regulates the IGF receptors in fibroblasts (Haselbacher *et al.*, 1992).

Results from *T. thermophila* cells show that biological effects of the C-terminal end of insulin are not restricted to cells from metazoa. Furthermore, the activity of these fragments is lower than that of the entire insulin molecule, as in metazoan cells. Thus the intact insulin molecule promotes cell survival in *T. thermophila* at concentrations up to 100 times lower than B22–B30 in the low-concentration range (Christensen *et al.*, 1996b). It is noteworthy that the pentapeptide B22–B26 at submaximal concentrations of insulin stimulates glucose oxidation in two separate concentration ranges in adipocytes (Kikuchi *et al.*, 1981a). In this light, it is possible that the B20–B30 fragment in *T. thermophila* cells cooperates with the cells' own signal molecules.

3. *Tetrahymena*-Produced Factors and Mammalian Growth Factors

Many mammalian growth factors can rescue moribund *Tetrahymena* cells. This raises the question, is there any homology between these factors and the cells' own compounds? The fact that insulin-related material is produced by *Tetrahymena* (Shiloach *et al.*, 1985) and the claim that these cells have receptors for insulin (Christopher and Sundermann, 1996; Csaba, 1996) point to the conclusion that insulin-related material may be autocrine signals for survival in these cells. Immunoblotting of fractionated CDM with anti-insulin (Schousboe *et al.*, 1997) shows a protein above 60 kDa, which may be similar to the one found by Christopher and Sundermann (1995b) in the cilia membranes of *T. pyriformis*. The filtered fraction of conditioned medium above 30 kDa does not rescue moribund cells in low-density cultures (Schousboe *et al.*, 1997) and therefore TPAF cannot be *identical* to insulin. They may, however, share some sequence homologies. Alternatively, the cells' own signals may have no obvious similarity to mammalian insulin-like material. To give an example, mammalian insulin and IGFs bind to the basic 7S globulin located in the middle lamella of the cell walls

and in the plasma membranes of soybean seeds (Komatsu and Hirano, 1991; Hirano *et al.*, 1992; Nishizawa *et al.*, 1994). This globulin has a protein kinase activity corresponding to about two-thirds of the activity of the tyrosine kinase of the rat insulin receptor (Komatsu *et al.*, 1994). It has therefore been proposed that the globulin has insulin receptor-like functions. Watanabe *et al.* (1994b) found that soybean seeds produce a 4-kDa signal peptide, *leginsulin.* The amino acid sequence of this molecule has no obvious similarity to insulin or IGFs, but it competes with insulin for binding of globulin. Thus, as for leginsulin, the compounds from *T. thermophila* cells may not necessarily show a close resemblance to mammalian insulin. Therefore the *Tetrahymena* insulin found by Leroith *et al.* (1980) and Shiloach *et al.* (1985) and TPAF need to be analyzed for amino acid sequence homology to insulin and IGFs. This also needs to be done with many other mammalian growth factors rescuing moribund cells of *Tetrahymena* before any conclusion as to their effects can be made.

D. Roles of Intracellular Messenger Systems

Many compounds rescue moribund cells of *T. thermophila*, including lipids, proteins, porphyrin molecules, and detergents (Christensen and Rasmussen, 1992; Schousboe *et al.*, 1992; Schousboe and Rasmussen, 1994; Kristiansen *et al.*, 1996). Some of these can act as second messenger molecules or activate intracellular primary and secondary effector enzymes activated by TPAF (Wheatley *et al.*, 1993; Rasmussen *et al.*, 1996). Others have suggested that these compounds prevent cell death due to inadequate culture conditions, leading to cell demise in the border between medium and air of the culture (Kristiansen *et al.*, 1996). Here we describe how these compounds can interfere with cellular signal transduction systems in *T. thermophila*, leading to cell survival and proliferation.

1. Cyclic GMP and Nitric Oxide

a. Metazoan Cells cGMP is a secondary messenger molecule in many cellular activities. It is produced by the guanylate cyclase which exists either as a membrane-bound isoform (*particulate* guanylate cyclase, pGC) or as a cytosolic isoform (*soluble* guanylate cyclase, sGC). pGC is activated by intercellular signal molecules binding to receptors associated with the enzyme. These primary messengers include natriuretic peptides and heat-stable enterotoxins (Garbers, 1992). sGC is activated by NO, which binds to iron in the heme moiety (hemin) attached to the enzyme (Ignarro, 1992). sGC is also activated by arachidonic acid, 15-lipooxygenase products, fatty acids (Gerzer *et al.*, 1983, 1986), and protoporphyrin IX (Ignarro *et al.*,

1984). The latter compound resembles the structure of the NO–hemin complex in the activated enzyme (Ignarro *et al.,* 1984). cGMP and NO regulate cellular activities, such as vasomodulation and neurotransmission (Moncada *et al.,* 1991; Nathan, 1992; Berdeaux, 1993; Oh, 1995). As an example, NO plays a major role in learning and memory functions in the mammalian brain. Excitatory amino acid neurotransmitters activate receptors for *N*-methyl-D-aspartate, leading to increased intracellular levels of NO via the action of nitric oxide synthase. NO, in turn, stimulates the formation of cGMP which then induces the release of other neurotransmitters, such as acetylcholine and dopamine. NO is also released to neighboring cells, resulting in an amplification of the original signal. In addition, the NO/cGMP system has various effects on cell survival and proliferation. Cytokines and growth factors increase intracellular levels of NO and cGMP and inhibit PCD and apoptosis in serum-deprived cultures of eosinophils, cells in ovarian follicles, PC12 cells, and sympathetic neurons (Beauvais *et al.,* 1995b; Chun *et al.,* 1995; Farinelli *et al.,* 1996; Park *et al.,* 1996). In contrast, NO and cGMP *inhibit* cell growth and proliferation in vascular smooth muscle cells (Cornwell *et al.,* 1994; Etienne *et al.,* 1996) and in osteoblast-like cells (Ralston *et al.,* 1994), whereas they *stimulate* cell proliferation in cultures of glioma and neuroblastoma cell lines (Zwiller *et al.,* 1982) and in T cells (Maghazachi, 1992).

b. Unicellular Eukaryotes cGMP is involved in cellular activities in unicellular organisms. It takes part in slug formation (Newell *et al.,* 1995; see IID), cell cycle events, metabolism, and ciliary beating in ciliates (Graves *et al.,* 1976; Schultz and Klumpp, 1984, 1994; Bonini and Nelson, 1988; Umeki and Nozawa, 1996). NO inhibits ADP–ribosyltransferase and slug formation in *D. discoideum.* This latter reaction is independent of cGMP formation (Tao *et al.,* 1992a,b). Paveto *et al.* (1995) found that *T. cruzi* cells have an NO/sGC system activated via NMDA receptors. These findings support the idea that the NO/cGMP transduction pathway has an old history in evolution.

Insulin and the NO donor sodium nitroprusside (SNP) (Ignarro *et al.,* 1981) induce the formation of cGMP in *T. pyriformis* (Kovács *et al.,* 1989; Köhidai *et al.,* 1992). Insulin supports cell survival in CDM (Christensen, 1993). Similarly, SNP, insulin, and 8-bromo cGMP (a permeable analogue of cGMP)—added separately—prevent cell death and induce *T. thermophila* cells to proliferate from low initial densities (Christensen *et al.,* 1996a,b). All these results support the view that an NO-dependent guanylate cyclase (sGC) is present in these cells and that this system plays a role in survival and proliferation. *T. thermophila* cells have cGMP-dependent protein kinases *(PKGs)* (Murofushi, 1973, 1974) and these enzymes are probably involved in signal transduction (see below). SNP produces a bipha-

sic response on cell survival and proliferation in *T. thermophila* over a wide concentration interval, like insulin does (Fig. 4). Furthermore, both insulin and SNP act in synergism with hemin (Christensen *et al.*, 1996a,b). SNP is toxic in the micromolar range, since it kills cells in high-density cultures.

Is NO an intercellular signal molecule in *T. thermophila?* NO may participate in some cell-to-cell interactions since NO is probably one of the first of a generation of "signaling gases" not acting through specific receptors. SNP is active at very low levels (10^{-14} *M*), showing that *T. thermophila*

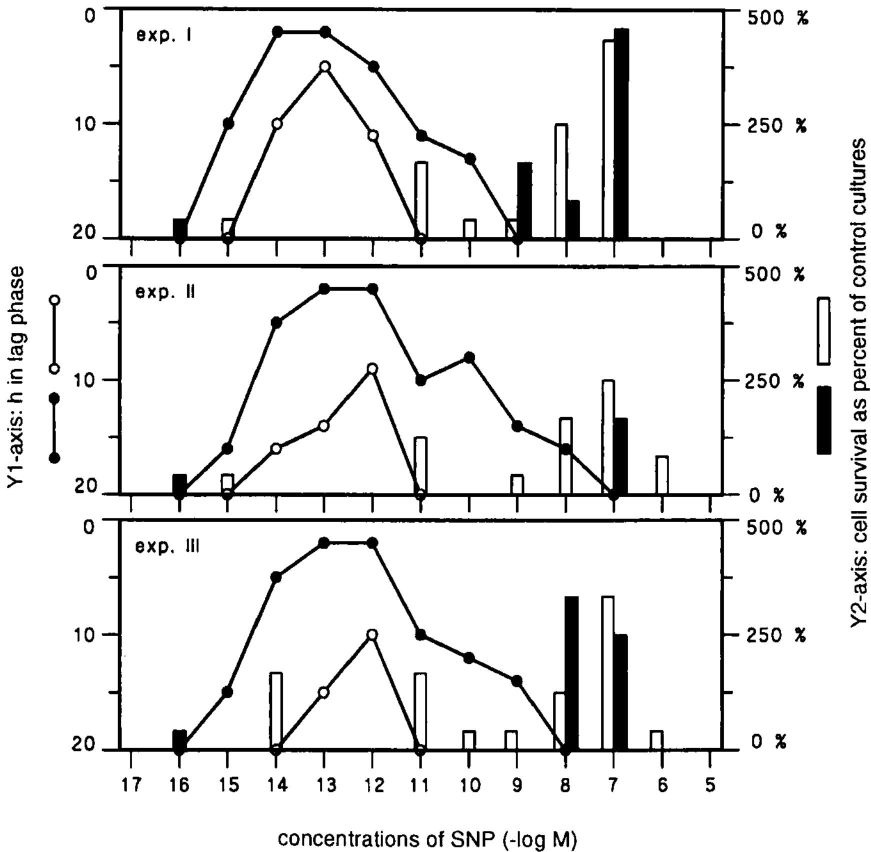


FIG. 4 Effects of various concentrations of sodium nitroprusside on survival and proliferation in *Tetrahymena thermophila* in chemically defined nutrient medium in conical culture flasks at 500 cells/ml in the presence (solid symbols) and in the absence (open symbols) of 50 n*M* hemin. Solid lines show results from those cultures in which the cells proliferated and the number of hours in the lag phase before the start of proliferation (Y1 axis). Bars show results from those cultures in which sodium nitroprusside promoted cell survival as a percentage of the control cultures (Y2 axis). From Christensen *et al.* (1996a).

cells can react on very low amounts of NO in the environment. NO at high levels disappears quickly by reacting rapidly with O_2^- or O_2, yielding $OONO^-$ and NO_2, respectively (Stamler *et al.*, 1992). However, at low concentrations it is rather stable, because two NO molecules have to react with one oxygen molecule to produce NO_2^- (Fukuto, 1995).

There are compounds known to inhibit NO production and the activity of the soluble guanylate cyclase in mammalian cells. These compounds also inhibit proliferation and induce death in *T. thermophila* (Christensen *et al.*, 1996a). They include N^G-methyl-L-arginine (NMA), methylene blue (MB), and LY 83583. NMA is a competitive inhibitor of L-arginine for NO-synthase (Gold *et al.*, 1990). MB blocks the activity of NO-dependent (soluble) guanylate cyclase by oxidizing the active hemo center (Martin *et al.*, 1985) or by inactivation of its hemo-deficient apoenzyme (Tsai *et al.*, 1983). Furthermore, MB binds to and inactivates NO (Wolin *et al.*, 1990), suppresses the release of NO from organic nitrates (Feelisch and Noack, 1987), and may also inhibit NO-synthase (Mayer *et al.*, 1993). LY 83583 lowers intracellular cGMP levels (Schmidt *et al.*, 1985) by inhibiting soluble guanylate cyclase (Mulsch *et al.*, 1988; Randriamampita *et al.*, 1991) and NO-synthase (Luo *et al.*, 1995). All of these compounds block the effects of insulin in *T. thermophila.* Effects of NMA on cell proliferation can be circumvented, bypassed, by an excess of L-arginine, or by SNP, protoporphyrin IX (PPIX), or 8-bromo cGMP. Among these compounds only 8-bromo cGMP circumvents cell death caused by MB and LY 83583 and makes the cells proliferate—again, a bypassing action (Christensen *et al.*, 1996a). We believe that cGMP activates a protein kinase G which in turn is involved in activation of immediate–early genes in cell proliferation. If this idea is correct then cGMP is the last known low-molecular-weight compound required for activation of these genes.

2. Protein Kinases

Enzyme activities are often controlled by protein phosphorylations in eukaryotes. Several systems are implicated in signal transduction pathways in mammalian cells in response to external stimuli, such as survival and growth factors. They include protein kinases which phosphorylate substrate enzymes at tyrosine and/or serine/threonine residues (see I.B), and similar systems seem to be at work in *Tetrahymena* with respect to survival and proliferation.

a. Protein Tyrosine Kinases Proliferating *T. thermophila* cells induce *in vivo* phosphorylation of at least one protein at 50–55 kDa. This phosphorylation is resistant to alkaline treatment (Christensen *et al.*, manuscript in preparation). Furthermore, genistein, a tyrosine kinase inhibitor, inhibited this phosphorylation. This inhibition is not seen in the presence of the

"negative control" daidzein. It was proposed that *T. thermophila* has protein tyrosine kinase activities. In line with these results, cell survival and proliferation induced by insulin are blocked by genistein, but not by daidzein. Leick and Christensen (unpublished research) observed that two proteins at 50–56 kDa are phosphorylated *in vitro* in extracts of cilia and, as in the *in vivo* experiments, these phosphorylations are resistant to alkaline treatment. Furthermore, the protein kinase in the cilia is active at 0°C in the absence of added Mg^{2+}, and its activity is inhibited by genistein and tyrphostins.

b. Protein Kinase C-like Activities Straarup *et al.* (1997) replaced TPAF with DAG, phorbol ester (PMA), and the synthetic diacylglycerol OAG, the latter two compounds substituting for DAG (Nishizuki, 1995). All of these compounds rescue moribund cells from dying. On the other hand, staurosporine, an inhibitor of serine/threonine kinase and PKC, induces cell death in cultures, even at high initial cell densities and when supplemented with PMA, OAG, and insulin (Christensen *et al.,* 1997). Furthermore, PMA (at 500 cells/ml) induces *in vivo* phosphorylations of several proteins with molecular masses of around 12–16, 42–46, 50–56, 60–64, and 72–78 kDa (Straarup *et al.,* 1997). Cells (at 250,000 cells/ml) induce *in vitro* phosphorylations of myelin basic protein peptide fragment 4-14. Both *in vivo* and *in vitro* phosphorylations are annulled by staurosporine.

c. Protein Kinase G PKG activities have been found in *Tetrahymena* by Murofushi (1973, 1974). They may be turned on by cGMP and may be involved in signal transduction in cell survival and proliferation.

These results suggest that serine/threonine and/or tyrosine kinases take part in survival and proliferation in *T. thermophila.* They also indicate that several protein kinases take part in a TPAF-induced phosphorylation cascade and that PKC-like activity is involved in this signal transduction pathway in *T. thermophila* (cf. Csaba, 1996). It is not known whether some of this activity is related to the 55-kDa calcium-dependent protein kinase found in these cells (Hegyesi and Csaba, 1994a). The kinase is activated directly by calcium, and H-7, a serine/threonine kinase inhibitor, has weak effects on its activity.

The cooperative interactions between protein phosphorylations and cGMP systems in survival and proliferation in *T. thermophila* are not well understood. Preliminary results indicate that some protein kinase activity may be located upstream in relation to a guanylate cyclase system (Rasmussen *et al.,* 1996).

E. Mechanisms in Cell Death

How do *T. thermophila* cells die in the absence of TPAF and in the presence of inhibitors of signal transduction? One might speculate that since signaling

between individual cells supports survival and allows proliferation in a sequence similar to that seen in multicellular organisms, then cell death may also share features similar to those seen in multicellular eukaryotes undergoing PCD. *T. thermophila* cells may have a genetic basis for PCD and apoptosis: these cells have a transcriptionally active polyploid macronucleus and an inactive diploid micronucleus which provides genetic continuity from one sexual generation to the next. The old macronucleus is replaced by a new one during conjugation. Chromatin condenses during this elimination (Wenkert and Allis, 1984; Orias, 1986; Weiske-Benner and Eckert, 1987) and the DNA fragments into oligonucleosome-sized pieces (Davis *et al.*, 1992). Actinomycin D inhibits the resorption of the old macronucleus (Ward and Herrick, 1996), and aurin, an endonuclease inhibitor (Batistatou and Greene, 1993), actinomycin D, or cycloheximide inhibits macronuclear condensation and DNA fragmentation (Mpoke and Wolfe, 1996). Thus the elimination of the old macronuclei can be regarded as a *developmental,* programmed process with nuclear alterations similar to apoptosis.

1. Cell Death in Low-Density Cultures

T. thermophila cells at low initial density die rapidly with first order kinetics (half-lives of <2 h), when transferred to CDM in conical culture flasks (Christensen *et al.*, 1995). Kristiansen *et al.* (1996) suggested that the cells die in a *"death zone"* in the border layer between the fluid medium and air of the culture. Cell death frequencies are reduced if their culture vessels are filled, the height of the medium is increased, or the temperature is decreased. The cells swim upward in the culture due to negative geotaxis and positive aerotaxis. Kristiansen *et al.* (1996) proposed that these increased survival frequencies result from a reduced probability of cells to enter the death zone and this gives more time for conditioning of the medium. Furthermore, low levels of the biosurfactant *surfactin* and detergents prevent cell death in CDM, and Kristiansen *et al.* (1996) suggested that these compounds make cells more resistant toward the "stress" at the border layer between medium and air.

Cells do not necessarily die at this border layer. Christensen *et al.* (1997) kept the cells at the bottom of culture tubes by slight centrifugation (about 12*g*) which does not alter cell viability or proliferation rates of ciliates inoculated at 1000 cells/ml. Single cells in 1 or 2 ml of CDM in glass test tubes die with similar frequencies whether the cultures are centrifuged or not. This result makes it unlikely that cells die in the layer between medium and air (see also note on page 232). Furthermore, filming of single cells in small volumes of CDM (10 μl) embedded in paraffin oil (corresponding to 100 cells/ml) shows that the cells are swimming at normal speeds and often die *at any position* in the culture by a quick "global" disintegration

observed photographically between two frames 0.2 s apart (Fig. 5). This is in contrast to the results of Kristiansen *et al.* (1996), who reported that their cells swelled and lysed before disintegration for about 10 s when observed on an object slide.

The question arises as to the role of the autocrine/paracrine signaling in preventing cell death. Kristiansen *et al.* (1996) suggested that growth factors from mammalian sources make the cells release detergents or surfactants which in turn prevent cell death in the border layer between medium and air. They doubt that cell demise follows a programmed mechanism. They found no internucleosomal degradation of the DNA. In contrast, using several extraction techniques, Schousboe (unpublished research) found no

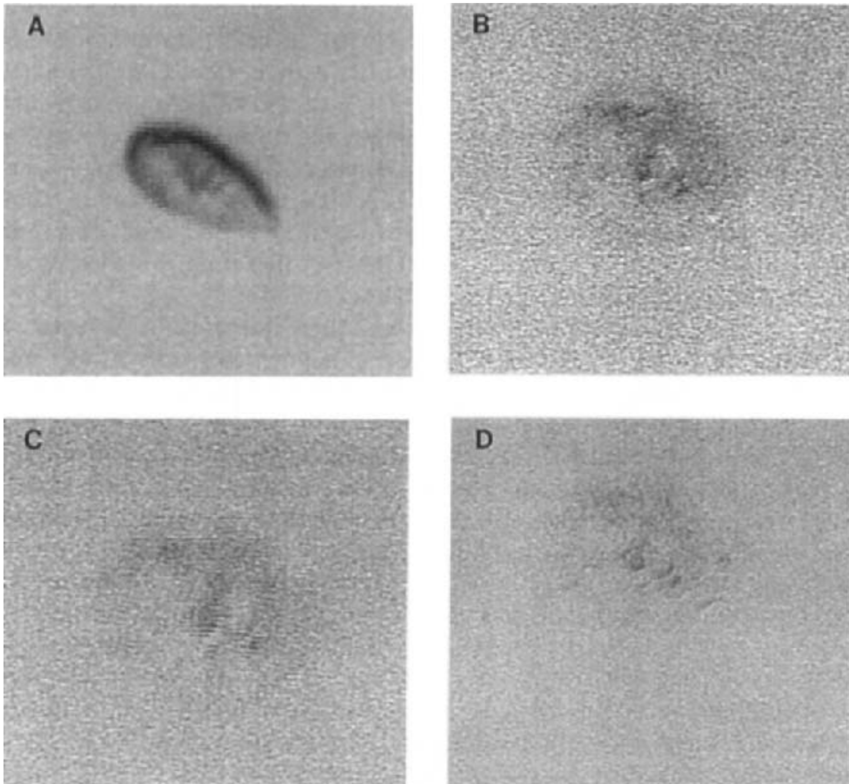


FIG. 5 The fate of single cells of *Tetrahymena thermophila* in 10-μl cultures of chemically defined medium embedded in paraffin oil. Frames 1–4 were recorded by a computer and the interval between each frame is 0.2 s, so the period required for cell disintegration is extremely short. Frames 1 to 4 are just one series of hundreds of similar recordings. From Christensen *et al.* (1997).

evidence for a release to the medium of hydrophobic compounds that could support survival and proliferation. Indeed, the components of the hydrophobic phases of conditioned medium have inhibitory effects on cell proliferation in *T. thermophila*. These results support the view that cell survival occurs via hydrophilic compounds, as shown with the identification of the cell-released protein TPAF.

Inhibition of protein synthesis dramatically postponed cell death (Christensen *et al.*, 1995). In the presence of either cycloheximide or puromycin, or in medium lacking a single essential amino acid, the cells survive 7–10 times longer than those in the control cultures. Furthermore, the critical initial density required to support survival is reduced by inhibition of *de novo* RNA synthesis by actinomycin D (Act D). Indeed, single cells inoculated into 10 μl of CDM die within 4–30 min, but in the presence of Act D, they survive and proliferate with long doubling times (Christensen *et al.*, 1995, 1997). Moreover, Act D does not affect the ability of the cells to swim upward in the culture at Act D levels that prevent cell death, nor does Act D reduce the swimming speed of the cells (Christensen *et al.*, 1997). All these results support the view that cell death is not simply the result of a direct accident in the layer between medium and air in the culture. Mammalian signal molecules can be mitogenic in *T. thermophila* (Andersen *et al.*, 1984) and certain intracellular signal systems seem to activate survival and proliferation in these cells. Cell death in the absence of TPAFs is, therefore, due to a default mechanism which may be related to PCD.

What mechanisms underlie the effects of the detergents on cell survival and proliferation in *Tetrahymena*? At low initial densities cells die by rapid disintegration, indicating that cell death is due to instability of the membrane construction. It is therefore possible that signal-induced cell survival involves activation of intracellular effector and messenger systems leading to *de novo* gene expression, which in turn stabilizes the plasma membrane system. If this is true, then the detergents have two functions: they alter the membrane composition either directly or indirectly by activating signal transduction pathways normally regulated via TPAFs. Low levels of detergents affect the activity of membrane-bound primary effector enzymes in signal transduction cascades by modulation of the fluidity of cell membranes as in mammalian cells. Active compounds in the case of mammalian cells are Tween, Brij, and Triton detergent classes and endogenous detergents, such as lysophosphatidylcholine, which also affect membrane receptors (Komori *et al.*, 1993; Bing *et al.*, 1993). The involved membrane-associated enzymes include PKC (Oishi *et al.*, 1988), adenylate cyclase (Salesse *et al.*, 1982), guanylate cyclase (Fujimoto and Okabayashi, 1975; Shier *et al.*, 1976; Fink and Cross, 1984), phospholipase A_2 (Van den Bosch, 1980), and nitric oxide-synthase (Komori *et al.*, 1993; Dudek *et al.*, 1994; Conforto *et al.*, 1994, Yoshida *et al.*, 1995). The latter leads to activation of an NO-dependent guanylate cyclase (Conforto *et*

al., 1994). These results show that detergents play a role in signal transduction processes in mammalian cells. Such compounds may also affect cell survival in a similar way in *T. thermophila*.

2. Cell Death Induced by Staurosporine

Inhibition of signal transduction in mammalian cell systems results in cell death. Death follows PCD either by apoptosis or by atypical death modes (Cohen *et al.*, 1992; Collins *et al.*, 1992). Darzynkiewicz (1995) suggested that inhibitors of signal transduction also block metabolic pathways of apoptosis, leading to an incomplete pattern of cell death. These inhibitors can also reduce or abolish effector molecules needed to execute the death process. Staurosporine induces PCD and apoptosis in mammalian cells (Sanchez *et al.*, 1992; Ishizaki *et al.*, 1993, 1994; Couldwell *et al.*, 1994; Bertrand *et al.*, 1994; Sabit *et al.*, 1994; Wiesner and Dawson, 1996). These effects have been ascribed to both PKC-dependent (Sanchez *et al.*, 1992) and PKC-independent processes (Raff *et al.*, 1993; Wiesner and Dawson, 1996).

Staurosporine at 20–40 n*M* induces death within about 30 h in *T. thermophila* cells in CDM. This process involves both autophagic degeneration similar to type 2 morphological PCD in metazoan cells and traits of apoptosis (Christensen *et al.*, 1997). The cells swim slowly, shrink in size, bulge out, and collect at the bottom of the culture (Fig. 6). They form star-like structures with cytoplasmic bodies or blebs at the end of the stalks after 25 h and they form many lysosomally derived autophagic vacuoles. Their nucleoli assemble, macronuclei become pycnotic with focal condensations of the chromatin, and often the mitochondria become electron dense. These changes are similar to those seen in starving *Tetrahymena* (Levy and Elliot, 1968; Nilsson and Leick, 1970; Nilsson, 1970a,b, 1976). Christensen *et al.* (1997) also found that cell death is postponed by Act D and this supports the view that cell demise in the presence of staurosporine requires *de novo* gene expressions.

Evidently, there are both similarities and differences between cell death induced in low-density cultures and by staurosporine. In both cases moribund cells are rescued by 8-bromo cGMP and cell death is inhibited by Act D. However, the morphological changes are quite different. The reason for this is as yet unknown. It is possible that staurosporine has effects other than those related to an inhibition of PKC-like activity and that the two types of death are dependent on different cellular activities. In both cases we need further evidence in order to conclude whether cell death is similar to PCD in metazoa. This evidence should come from studies on the role of transcriptional and translational changes in dying cells and whether such changes lead to the expressions of proteins with homology to those found in multicellular organisms.

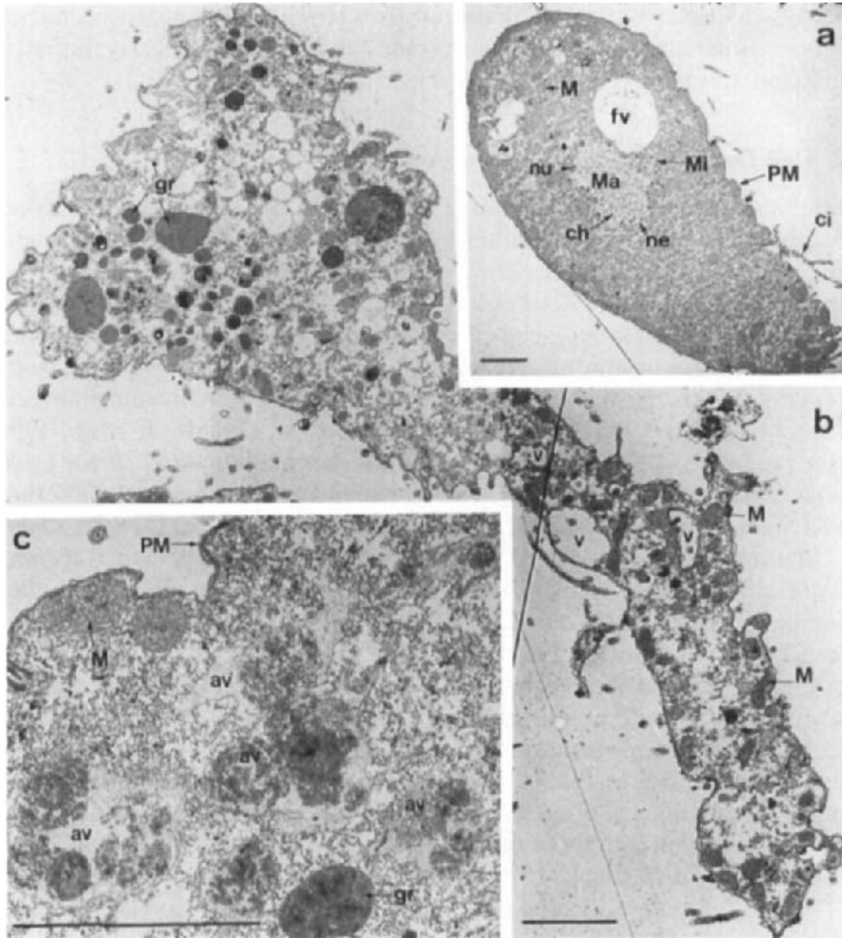


FIG. 6 Transmission electron micrographs of the effects of staurosporine (30 n*M*) on the ultrastructure of *Tetrahymena thermophila* in chemically defined nutrient medium in conical culture flasks after 18–24 h. (a) Control cells; (b and c) staurosporine-treated cells. *Abbreviations*: PM, plasma membrane; Ma, macronucleus; MI, micronucleus; ne, nuclear envelope; ch, chromatin; nu, nucleoli; ci, cilia; M, mitochondria; fv, food vacuole; v, vesicle/vacuole; av, autophagic vacuole; gr, granula. Bars, 5 μm. From Christensen *et al.* (1997).

F. Results from Other *Tetrahymena* Species

There are many species of *Tetrahymena* grown in the laboratory. *T. pyriformis* and *T. thermophila* were the first ones to grow in chemically defined

medium without lipids (Kidder and Dewey, 1951; Holz *et al.*, 1962a). It was reported that addition of lipids makes the cells grow faster (Rasmussen and Dive, 1980). Later, it was reported that other species, e.g., *T. setosa*, require lipids for cell proliferation (Holz *et al.*, 1962b). This requirement turned out to be cell density dependent (see below). It is not known today how many true lipid-requiring *Tetrahymena* species there are.

1. *T. pyriformis*

Cells of this species (Nanney and McCoy, 1976) behave in a way similar to *T. thermophila* in CDM. Christensen (1993) showed that *T. pyriformis* cells die in low-initial-density cultures, but insulin at 1 μM rescues these moribund cells. However, the insulin fragment B22–B30 at 1 μM activates cell proliferation, whereas it has no effect at this level in *T. thermophila* (Christensen *et al.*, 1996b) (see III.C). Thus peptide pieces from mammalian signal molecules have different effects in various *Tetrahymena* species. This may be due to different receptor affinities for such compounds.

2. *T. setosa*

Cells of this species have been grown (under the name of *T. setifera*) by Holz *et al.* (1962b), by Christensen *et al.* (1993), and by Boysen and Rasmussen (unpublished research). All experiments are made with the same cell line, HZ-1. The primary object was to shed light on nutritional requirements, which proved elusive, because they seemed to vary with the initial density of the cultures. This problem was solved when it was realized that both nutrients *and* cellular compounds promote proliferation.

a. Initial Densities, >4000 Cells/ml Cells have been grown for years in the lipid-free medium CDM and in complex proteose peptone/yeast extract medium with weekly transfers, provided the cell concentration did not fall below 4000 cells/ml (Boysen and Rasmussen, unpublished research).

b. Initial Densities, 700–2000 Cells/ml At this density, cells will not continue to grow in CDM unless it is supplemented with lipids, such as phospholipids, cholesterol, free fatty acids, or ethanol (Holz *et al.*, 1962b; Christensen *et al.*, 1993).

c. Initial Density, 1–2 Cells/ml All cultures die in CDM. If either cholesterol or cephalin (L-α-phosphatidylethanolamine) is added, then the cells in a small fraction of the cultures survive and proliferate. If both cephalin and cholesterol are added, cells in all cultures proliferate.

These results indicate that *T. setosa* cells require the nutrients present in CDM *and* cell-produced compounds for proliferation for many (>70) subcultivations. At densities lower than 4000 cells/ml, ethanol and cholesterol activate proliferation, possibly by making the cells more sensitive to the cell-produced compounds or by activating mechanisms which the cell-produced compounds would have activated. The idea that released compounds are essential is supported by the findings that proliferation of single cells inoculated into a volume of 0.5 μl of CDM (corresponding to an initial cell density of 2000 cells/ml) is activated by either ethanol or cell-free, conditioned medium from a high-density culture (Christensen *et al.*, 1993). At the single-cell level, both cephalin and cholesterol are necessary for activating putative control mechanisms, similar to those present in *T. thermophila.* Indeed, cell-free conditioned medium from cultures of these cells activates proliferation in *T. setosa* and vice versa (Christensen *et al.*, 1993).

It is surprising that it has taken so long to distinguish between a nutrient requirement and a requirement for activation of cell proliferation.

3. *T. vorax* and *T. pigmentosa*

Schousboe *et al.* (1992) showed that these two species (Nanney and McCoy, 1976)—in contrast to *T. thermophila*—multiply at very low initial densities in CDM. However, Christensen *et al.* (unpublished research) found that (1) the lag period before proliferation of *T. vorax* cells increases in a cell-density-dependent manner in cultures from high to low densities, (2) human insulin at picomolar levels dramatically reduces the duration of the lag period at low densities, and (3) cell-free conditioned medium from *T. vorax* activates proliferation in *T. thermophila.* These results indicate that *T. vorax* cells also release autocrine signal molecules with effects on survival and/or proliferation and that insulin mimics this effect.

T. vorax cells can change from a form with a small mouth to a form with a large mouth structure (Smith-Somerville and Buhse, 1984). This shift represents a case of differentiation and is influenced by extracellular compounds. So far, these compounds have proven elusive, but rapid inositol phospholipid turnover is implicated (Bae and Ryals, 1997).

G. Summary

1. Intercellular Systems

Cell survival and proliferation in *Tetrahymena* are activated both by compounds released by the cells themselves (TPAF) and by mitogenic signal

compounds known from mammalian cells. The latter compounds include insulin, PDGF, NGF, and EGF. The mammalian compounds also affect metabolism, phagocytosis, mitogenesis, DNA synthesis, and chemosensory behavior in ciliates. In one case, *Tetrahymena*-produced compounds had hormonal activities in mammalian cells.

2. Intracellular Systems

Pharmacological studies have shown that phorbol esters activate cell proliferation in *Tetrahymena* as well as in mammalian cells. Compounds which are known to activate a (soluble) guanylate cyclase, such as protoporphyrin IX and sodium nitroprusside (which releases nitric oxide), activate cell survival and proliferation both in *Tetrahymena* and in mammalian cells. Compounds which inhibit that enzyme system, e.g., N^{G}-methyl-L-arginine, methylene blue, and LY 83583, inhibit survival and proliferation in both systems. Finally, 8-bromo-cGMP rescues moribund *Tetrahymena* and mammalian cells in chemically defined nutrient media in the absence of appropriate amounts of survival signals. Thus cGMP may be the last known low-molecular-weight compound before the activation of immediate–early genes in cell proliferation. These results point to a striking similarity between control systems for survival and proliferation in ciliates and in cells from metazoa.

IV. Signal Molecules in Chemosensory Behavior in *Tetrahymena*

It is fairly simple to monitor the response of *Tetrahymena* cells to attractant and repellent compounds. These free-living cells swim fast and have therefore been used extensively for these purposes for many years. The best responses are obtained with starving cells.

A. Chemosensory Behavior in Various Biological Systems

All mobile cells probably show some kind of chemosensory response. Their reactions to chemical compounds and gradients fall into three categories: cells can alter their *direction* and *speed* of locomotion, and they may show *adaptation* (Wilkinson, 1990; Leick *et al.*, 1996b). A cell adapts when it adjusts its sensitivity to a certain compound and for the time being eliminates its ability to react to this compound. Thus an adapted cell responds when there is a change in stimulus intensity and it ceases to respond in the

absence of any further change. Adaptation permits a cell to operate with constant efficiency over a large range of stimulus intensities, e.g., while it migrates along a chemical gradient. Chemoattractants can affect all three parameters, viz., the direction of chemotaxis, the speed of the cell and its adaptation. To give examples, neutrophil leukocytes migrate in fairly straight paths toward the source of a chemical gradient (Wilkinson, 1987) and smooth muscle cells move toward an injury in response to the release of PDGF from blood platelets (Lynch *et al.*, 1987; Boyan *et al.*, 1994). In addition, stem cells migrate into embryonic thymus in the developing embryo (Bach and Papiernik, 1981). Individual *Dictyostelium* cells move toward higher levels of cAMP, unite with each other, and form a multicellular slime mold (Devreotes and Zigmond, 1988) (II.D). Finally, bacteria swim toward nutrients, such as amino acids and sugars, using adaptation mechanisms and altered swimming speeds to find them (Morimoto and Koshland, 1991).

B. Molecular Signal Transduction Mechanisms

Mechanisms in chemoattraction are varied and depend on the type of membrane receptor involved. Thus the tripeptide fMet-Leu-Phe activates a G-protein-dependent seven-transmembrane receptor system in leukocytes (Boulay *et al.*, 1990; Sengeløv *et al.*, 1994). This signal is sent on via MAP kinases (Thompson *et al.*, 1993). cAMP activates STM receptors (Devreotes and Zigmond, 1988) and possibly a MAPK cascade in *Dictyostelium* cells (Maeda *et al.*, 1996) (see II.D). In contrast, PDGF activates a receptor tyrosine kinase, leading to chemotaxis in smooth muscle cells (Bornfeldt *et al.*, 1995b). The signal is sent on via sphingosine-1-phosphate, PIP_2, and Ca^{2+} via the action of PLC-γ and PKA activity (Bornfeldt *et al.*, 1995a).

Receptors are covalently modified during the chemosensory response. Thus sensory receptors are methylated, which is required for the adaptation process in bacteria. The cAMP receptor plays a role in adaptation during chemotaxis and is phosphorylated in *D. discoideum* (Dunten and Koshland, 1991; Van Haastert, 1987). Also, systems other than those involving membrane receptors could lead to altered cell behavior. For example, exogenous NO elicits chemotaxis of neutrophils *in vitro* (Beauvais *et al.*, 1995a).

C. Chemosensory Behavior in *Tetrahymena*

Tetrahymena cells adapt to concentrations of a chemoattractant over 3–4 orders of magnitude. They can search relatively large volumes of water and assemble around an attractive source over long distances (Leick *et al.*,

1995). They are attracted to amino acids, peptides, and proteins but repelled by some organic chemicals and inorganic ions (Leick and Hellung-Larsen, 1985). These observations agree with the idea that these cells have evolved specific receptors for a number of chemicals.

1. Effects of Chemoattractants

Tetrahymena cells are attracted to proteins, peptides, and amino acids (Almagor *et al.,* 1981). They swim persistently forward when exposed to strong chemoattractants in semisolids, such as methylcellulose. Signaling peptides include mammalian growth factors, such as PDGF, insulin, FGF, and EGF. In general, potent peptide chemoattractants contain hydrophobic amino acids. PDGF, a strong chemoattractant, is a basic hydrophobic protein active at a few nanograms per milliliter. A weaker chemoattractant is the leukocyte attractant fMet-Leu-Phe, with three nonpolar amino acids and a formylated N-terminal group. This peptide is active at a few micrograms per milliliter. Insulin is a medium to strong attractant in the 50 n*M*–1 μM range. A literature survey lists more peptides with chemosensory effects (Leick *et al.,* 1996b).

Amino acids are weak chemoattractants that exert their effect in the millimolar range. Out of 23 amino acids tested, only 4 elicited a significant response in *T. thermophila.* L-phenylalanine, L-cysteine, L-histidine, and L-leucine were active at 1 m*M,* but sometimes at lower levels (Levandowsky *et al.,* 1984).

Of several short-chained aliphatic organic acids tested, *n*-butyrate has an effect in the micromolar range. Lipopolysaccharides at few nanograms per milliliter from *Escherichia coli* were strong chemoattractants (Kemp, unpublished research).

2. Receptors

T. pyriformis cells have β-endorphine-like receptors in their plasma membranes (Renaud *et al.,* 1996), and at 1–10 n*M* β-endorphin acts as a chemoattractant in these cells (O'Neill *et al.,* 1988) and in *T. thermophila* (Koppelhus *et al.,* 1994). This effect is blocked by (−)naloxone but not by (+)naloxone in *T. pyriformis,* suggesting the presence of a stereospecific opiate receptor in these cells (O'Neill *et al.,* 1988). Leick (1992) characterized the cellular binding sites for fMet-Leu-Phe with K_d values of 4–10 n*M* in *T. thermophila.* Christopher and Sundermann (1995b) presented suggestive evidence for an insulin-like receptor in the cilia of *T. pyriformis.* fMet-Leu-Phe (Leick, 1992) and insulin (Christopher and Sundermann, 1992) are internalized in vesicles.

Leick *et al.* (1996a) studied persistent swimming toward proteose peptone and FGF in semisolid methylcellulose. Concanavalin A (ConA) at 50 μg/ml blocked this behavior. Methyl-α-D-mannoside, but not methyl-α-D-galactoside, annulled the effect of ConA. This suggests that mannoside-containing ConA receptors mediate persistent swimming in these cells. ConA receptors are unequally distributed on the surface of starved cells. Fluorescent ConA (100 μg/ml) collected in the front of the cells. Methyl-α-D-mannoside, but not methyl-α-D-galactoside, reduces this binding. This indicates that mannoside-containing receptors are located in high concentrations at the anterior part of the plasma membrane and cilia (Leick *et al.*, 1996a). ConA neither inhibited the adaptation processes nor decreased the swimming speed of the cells. In contrast, Leick *et al.* (1996a) and Köhidai and Csaba (1996) found that ConA is a weak but significant chemoattractant in *T. thermophila* and *T. pyriformis,* respectively. These results suggest that ConA receptors are necessary for the persistent element of swimming and that ConA acts as a chemoattractant via nonspecific binding sites and/or via mannoside-containing receptors.

3. Intracellular Messenger Systems

Which compounds can block the adaptive response? Neither puromycin nor actinomycin D (inhibitors of gene expression) tested in a capillary assay was effective (Leick and Hellung-Larsen, 1985). Thus *de novo* syntheses of protein or RNA are not required for the cells to respond in this way. [Cycloheximide, also a protein synthesis inhibitor, blocks chemoattraction (Leick and Hellung-Larsen, 1985) but may represent a special case, since this compound also inhibits swimming speeds (Leick, unpublished research).] However, staurosporine, an inhibitor of protein serine/threonine phosphorylation, and genistein and tyrphostin A47, inhibitors of protein tyrosine kinase, block the adaptive response without reducing the swimming speeds of cells (Christensen and Leick, unpublished research). Chemosensory behavior was measured in a two-phase assay for chemoattraction, and dose/response curves were constructed for the inhibitors of tyrosine kinase. Thus genistein and tyrphostin A47 (both at 80 μM) added singly annul the attraction to both proteose peptone and insulin. In contrast, daidzein and tyrphostin A63, used as "negative control substances," had no effects. Swimming speeds of individual cells were unchanged or increased during the time of the experiments. Adaptation to a chemotactic stimulus was studied in *Paramecium.* Here microinjection of peptides inhibiting protein kinase C activity results in membrane depolarization, ciliary reversal, and backward swimming (Hinrichsen and Blackshear, 1993). Staurosporine annuls chemoattraction induced by proteose peptone and reducing the swimming speed of the cells (Christensen and Leick, unpublished research).

All these results taken together suggest that phosphorylations at serine/threonine and/or tyrosine residues are required in adaptive swimming, leading to chemoattraction in *T. thermophila.*

4. Signal Overlaps in Cell Survival, Proliferation, and Chemosensory Behavior?

Evidently, some mammalian signal compounds affect mitogenesis, cell survival and proliferation, and chemosensory behavior in *Tetrahymena* (Andersen *et al.,* 1984; Christensen *et al.,* 1996b; Kristiansen *et al.,* 1996; Leick *et al.,* 1996b). These results indicate that there is an overlap in signaling directing these distinct cellular responses. We draw attention to the fact that chemosensory behavior in ciliates is most often studied under starvation conditions, whereas proliferation is studied in a complete nutrient medium. These two different conditions could lead to different intracellular pathways.

Many mitogenic signal compounds stimulate both cell survival and proliferation and act as chemoattractants in mammalian cells. Some examples come from studies on FGFs, PDGFs, EGF, and IGFs (Matsuda *et al.,* 1992; Boyan *et al.,* 1994; Partanen *et al.,* 1992). Signaling begins at identical receptors, but the intracellular signaling pathways in chemosensory behavior can be quite distinct from those related to proliferation. This is true for the PDGF receptors (Rönnstrand *et al.,* 1992; Kashishian and Cooper, 1993; Bornfeldt *et al.,* 1994). Directed migration toward PDGF in smooth muscle cells requires a temporal balance between actin disassembly and assembly. This process is regulated by sphingosine-1-phosphate, PIP_2, and Ca^{2+} via the action of PLC-γ and PKA activity. In contrast, cell proliferation is regulated through Ras GTPase, PI-3 kinase, Raf kinase, and the MAPK cascade following *de novo* gene expressions (see IB) (Bornfeldt *et al.,* 1994, 1995a,b).

Putative overlapping components in signal transduction are as yet unknown in *Tetrahymena.* However, mannoside receptors for ConA seem to mediate cell survival on one hand (Christensen and Leick, unpublished research) and chemosensory behavior on the other hand (Leick *et al.,* 1996a). Furthermore, protein phosphorylations at serine/threonine and/or tyrosine residues seem to take part in both systems, and some of these components may be located close to or in the cilia: ConA receptors are present in the cilia (for references see Leick *et al.,* 1996a), the cilia membrane has immunogenically determined insulin-like activity (Christopher and Sundermann, 1995b), and the microtubules of the cilia contain a protein which is immunologically identical to the α-subunit of the mammalian insulin receptor (Christopher and Sundermann, 1996). In addition, the cilia have two proteins at 50–56 kDa which are *in vitro* phosphorylated but not in the

presence of tyrosine kinase inhibitors (Leick and Christensen, unpublished research). Growth factors, such as insulin, FGF, and EGF, activate protein kinases associated with microtubules, stimulate microtubule assembly, and phosphorylate native tubulin and microtubule-associated proteins in mammalian cells (Almas *et al.*, 1992; Sano and Kitajima, 1992; Wilden and Kahn, 1994). The identity of the phosphoproteins in the cilia is as yet unknown in *Tetrahymena.* However, they have molecular masses similar to those of their α- and β-tubulin. The latter is phosphorylated by a Ca^{2+}/calmodulin-dependent mechanism and was implicated in ciliary reversal (Hirano-Ohnishi and Watanabe, 1989).

Also other cellular responses may be regulated by overlapping signal systems. It was proposed that cell-produced chemoattractants lead to the accumulation of individual cells into shallow groups in old cultures of *Tetrahymena* (Levandowsky and Hauser, 1978). Kemp (unpublished research) found that a 1- to 14-kDa fraction of cell-free, conditioned medium is a chemoattractant with an activity similar to that of insulin. In contrast to, e.g., the ciliates *Euplotes* (see II.B) and *Blepharisma* (Honda and Miyake, 1975; Luporini *et al.*, 1996), there is no evidence for the presence of mating-type-specific pheromones serving as chemoattractants in *Tetrahymena.* In line with the work of Levandowsky and Hauser (1978) and Kemp (unpublished research), it is possible that autocrine and *nonspecific* mating-type signals (e.g., TPAF) lead to the accumulation of cells into clusters and thereby increased the probability of individual cells to meet and conjugate.

D. Summary

Tetrahymena cells respond to gradients of signal compounds by altered swimming patterns. These cells are attracted both to mammalian signal molecules and to *Tetrahymena*-released compounds. Protein kinases probably mediate the adaptive element of the chemosensory response and the regulatory system may be located in the cilia. A persistent swimming pattern is controlled by many mannoside-containing ConA receptors at the anterior part of the plasma membrane and in the cilia. Mammalian growth factors, such as insulin and PDGF, act as chemoattractants and survival signals in *T. thermophila.* This indicates a signal overlap for these cellular responses.

V. Control of Cell Proliferation in *S. cerevisiae*

S. cerevisiae, budding yeast, and *Schizosaccharomyces pombe,* fission yeast, are unicellular fungi. They have many transmembrane signal transduction

pathways homologous to mammalian cell systems. Good examples are MAPK kinase cascades and their upstream regulatory messenger systems (see I.B). Up to six functionally distinct but structurally homologous MAPK cascade systems take part in cellular behavior in *S. cerevisiae* and *S. pombe.* These include pheromone and invasive responses, growth, osmoregulation, cell wall integrity, and sporulation (Mordret, 1993; Herskowitz, 1995; Elion, 1995; Levin and Errede, 1995; Nielsen and Davey, 1995; Kothe, 1996). Many molecules regulate cell growth and cell cycle progression in yeast, but little is known about the transition from the lag phase to exponential proliferation. One reason is that traditional nutrient media are so rich that the cells proliferate fast and with such short lag phases that no effects of activators can be seen. To remedy this situation, minimal media without extracellular activators are needed in order to see how signaling pathways known from *in vitro* studies operate and cooperate in the living cell. Using such media, Overgaard *et al.* (1995) found that glucose acts as a signal for the induction of proliferation in *S. cerevisiae.*

A. Nutrient Media for Studies on Control of Proliferation

Friis *et al.* (1994) designed new minimal media for growing *S. cerevisiae.* They always started cultures with 300 cells/ml and grew them at 30°C in synthetic media. First they used Wickerham's medium (1946) containing salts, vitamins, and glucose, called $W_{glu.}$ The cells multiplied with doubling times around 2 h, stopping at ca. 0.3×10^9 organisms/ml. There was no stimulatory effect of compounds which activate cell survival and proliferation in mammalian cells and *Tetrahymena,* making this medium unsuitable for that type of *in vivo* studies on control mechanisms for cell proliferation. These authors then diluted all the nutrients—except glucose—1200-fold ($W_{1200,\ glu}$) and now the cultures reached about 3×10^6 cells/ml. They determined the percentage of cultures in the lag phase every day for a week after inoculation. This made it possible to establish the kinetics with which the cultures left the lag phase and entered proliferation. The "half-life" of the cultures spent in the lag phase—before proliferation—increased dramatically, from 4 to 28 h. The Ca^{2+} ionophore A23187 and extra Ca^{2+} reduced the long half-life from 28 to 6 h. Therefore, Ca^{2+}-dependent processes are important for the initiation of proliferation in *S. cerevisiae.* Such results are difficult to obtain in Wickerham's medium, because Ca^{2+} levels are high and lag phases short.

Overgaard *et al.* (1995) altered the composition of the medium even further. They replaced glucose with glycerol, $W_{gly,}$ and reduced the levels of all other components by either 120-fold ($W_{120,\ gly}$) or 1200-fold ($W_{1200,gly}$). They did this in order to get insight into the mechanisms involved

in shortening the lag phase of *S. cerevisiae.* The lag phases of the cultures in the W_{gly} medium lasted about 1 week and were followed by about 10 doublings, i.e., a 1000-fold increase in cell density. The lag phases lasted 1 and 2 weeks in $W_{120, gly}$ and $W_{1200, gly}$, respectively. Both of these media support proliferation up to 0.3×10^6 cells/ml.

B. Signal Molecules in Induction of Proliferation

Many of the compounds affecting survival and proliferation in the ciliates also have an effect on the duration of the lag phase of *S. cerevisiae* in the modified versions of Wickerham's medium. Thus there seem to be similarities in control mechanisms for proliferation in these two phylogenetically distinct unicellular eukaryotes.

1. Insulin and Compounds Affecting cGMP Systems

Many compounds shorten the lag phase dramatically from a week to a few hours. This is true for human insulin, sodium nitroprusside (Overgaard, unpublished research), PPIX, hemin, and 8-bromo-cGMP (Overgaard *et al.,* 1995). These compounds also activate proliferation in *T. thermophila* (see III.D). The inhibitor of the NO-dependent guanylate cyclase system, LY 83583, blocks activation of proliferation of insulin, PPIX, and hemin, but not the effect of 8-bromo-cGMP (Overgaard *et al.,* 1997). Thus cGMP bypasses the LY 83583-sensitive system and is the last-known low-molecular-weight compound before new gene expression, as it was argued for *Tetrahymena* (see III.D.1). It should be noted that insulin activates at the 100 f*M*–100 p*M* level and that 8-bromo-cGMP is active at nanomolar levels.

2. Bradykinin and Protein Kinase C Activators

PKC-activating compounds often induce cell proliferation in the absence of appropriate amounts of growth factors in cultures of mammalian cells. There are many PKC isotypes in *S. cerevisiae* and some are implicated in growth and proliferation in these cells (Levin *et al.,* 1990; Ogita *et al.,* 1990; Simon *et al.,* 1991; Iwai *et al.,* 1992; Watanabe *et al.,* 1994a; Kuo *et al.,* 1995a,b). Overgaard *et al.* (1997) found no stimulatory effects of the PKC-activating compounds, PMA and OAG, on proliferation when added singly to cultures of *S. cerevisiae* in $W_{120, gly}$. Bradykinin is an intercellular nonapeptide which induces an increase of intracellular Ca^{2+} via receptors (Burch and Kyle, 1992). This compound activates MAP kinases and isoforms of conventional PKC in mammalian cells (Dikic *et al.,* 1996; Tippmer *et al.,* 1994). Bradykinin at 1 n*M* plus PMA together reduce the lag phase from

a week to a few hours. Furthermore, Overgaard (unpublished research) found that staurosporine, the serine/threonine kinase and PKC inhibitor, annuls glucose-induced proliferation in $W_{1200,\ gly.}$ This supports the conclusion that a PKC is involved in the regulation of the exit from the lag phase to proliferation in *S. cerevisiae.* It also agrees with the findings that Ca ions play a role in the induction of proliferation in $W_{1200,\ glu}$ (Friis *et al.,* 1994).

3. Intercellular Signal Molecules and Interactions between Intracellular Messenger and Effector Enzymes

Many compounds have cooperative effects on the induction of proliferation in *S. cerevisiae.* Studies of synergisms indicate that a PKC activity may depend on cGMP-mediated processes. Thus PMA produces synergistic effects with insulin, hemin, or PPIX given singly. These effects were blocked by LY 83583, but not when 8-bromo-cGMP was present (Overgaard *et al.,* 1997). Furthermore, LY 83583 blocks proliferation induced by PMA and bradykinin. In addition, cAMP-mediated processes take part in the control of proliferation in *S. cerevisiae,* since the addition of 8-bromo cAMP reduces the lag phase of the cells (Overgaard *et al.,* 1997). However, 8-bromo-cAMP is much weaker than 8-bromo-cGMP, but in combination with PMA, the former produces an effect similar to that of the latter. Finally, LY 83583 does not inhibit proliferation induced by 8-bromo-cAMP.

These results show that intracellular signal systems, leading to the same "downstream" events, act either by operating together in one pathway or in a combination of multiple pathways. One can argue that a PKC system is located "upstream" in relation to guanylate cyclase activity in a signal transduction pathway as suggested for cells of *T. thermophila* (Rasmussen *et al.,* 1996). The effects of 8-bromo-cAMP are not blocked by LY 83583 and the nucleotide acts in synergism with PMA. This suggests that an adenylate cyclase may be activated independent of the PKC–guanylate cyclase system, although a cAMP-regulated pathway may overlap with a PKC-mediated system.

Overgaard *et al.* (unpublished research) found no evidence for autocrine/paracrine activation of cell proliferation in *S. cerevisiae.* Insulin and bradykinin at low levels both induce proliferation, which suggests that appropriate receptor systems are present on the cell surface. Glucose may act as a signal compound. Glucose or 6-deoxyglucose reduces the lag phase even at 55 μM in $W_{1200,\ gly}$ (Overgaard *et al.,* 1995), but this induction can be blocked by LY 83583 (Overgaard *et al.,* 1997).

Glucose-stimulated growth of *S. cerevisiae* may depend on both insulin and a glucose tolerance factor, both of which increased 2-deoxyglucose uptake (Berdicevski and Mirski, 1994; Mirsky and Berdicevski, 1994). Glucose has also been implicated as a signal molecule, increasing intracellular

levels of cAMP in starved cells of *S. cerevisiae* via a cdc25/Ras-GTP/adenylate cyclase pathway (Van Aelst *et al.*, 1990; Goldberg *et al.*, 1994). In this system the Ras-GTP/adenylase complex may receive the glucose signal directly, whereas the cdc25 protein is hyperphosphorylated by PKA and may constitute a prerequisite for the formation of this complex. Whether these signal transduction systems have overlapping functions with processes involved in the induction of proliferation remains to be investigated. There is a similarity between yeast cells and multicellular eukaryotes in the fact that the transition from the lag phase to proliferation can be brought about by putative membrane receptors or by direct activation of cytosolic systems. Their responses do not align with those of prokaryotes.

4. Senescence Factors

Chen *et al.* (1990) found that *S. cerevisiae* produce a senescence factor killing old cells. It is a 48-kDa protein which is continuously released by all cells, and they also release a protective protein which annuls the effect of the first factor. Old cells lose their ability to produce the protective protein and die.

5. Cell Death

Christiansen (unpublished research) found that inhibition of proliferation by LY 83583 leads to cell death after some days. This indicates that LY 83583 inhibits a basic level of cGMP production in the dilute versions of Wickerham's medium or that LY 83583 inhibits other cellular activities which support cell survival. LY 83583 has no general toxic effect on the cells, since the 8-bromo compounds of cGMP and cAMP rescue the cells from dying even in the presence of LY 83583. How the yeast cells die in the presence of this inhibitor alone is unknown. Expression of the *human* PCD-inducing *Bax-a* gene promotes growth arrest and cell death in *S. cerevisiae,* and moribund cells are rescued by coexpression of the human PCD-protecting Bcl-2 or Bcl-X_L (Sato *et al.*, 1994; Hanada *et al.*, 1995). This effect is dependent on mitochondrial function (Greenhalf *et al.*, 1996). Motizuki *et al.* (1995) showed that temperature-sensitive cell division cycle mutants of *S. cerevisiae* die by an active process related to autophagic degeneration when grown at restrictive temperatures. This cellular degradation is inhibited by cycloheximide, indicating that protein synthesis is involved in the activation of autophagic death. Thus cell death in these cells had some homology to PCD by autophagic degeneration in other cell systems (see I.B). PCD has been connected with the activities of the rRNA of the group 1 intron (omega) in *S. cerevisiae* (Griffiths, 1992). It is possible

that PCD is also induced by LY 83583. This can be tested by investigating the effect of Bcl-2 expression on LY 83583-induced death.

C. Summary

When cells are transferred into new medium they do not necessarily start proliferating, but go through a lag phase. Not much is known about this lag phase, but the results presented here indicate that there are many similarities between inductions of proliferation in *S. cerevisiae* and inductions of cell survival in *T. thermophila.* Compounds which stimulate PKC activities and soluble guanylate cyclase activities are involved. Also, cell proliferation is activated by 8-bromo-cGMP in both cases, probably because this latter compound activates a PKG, which again activates early genes in the cell division cycle.

VI. Signaling Systems in Prokaryotes Compared with Those in Eukaryotes

Prokaryotes have long been considered capable of growing on very basic requirements from low inocula, one bacterium being presumed adequate to produce a culture. But it has become clearer with time, especially in *in vivo* infections, that bacteria may need to be present in considerable numbers before their growth becomes rapid and exponential. The lag phase from low density before a culture reaches exponential phase can sometimes be very considerable, and certain substances can drastically reduce this period, as in unicellular eukaryotes. The tendency to use rich media for general culturing, however, has obscured the problem of signaling in bacteria, just as it did on eukaryotes (Wheatley *et al.,* 1993; Rasmussen *et al.,* 1996). Since bacteria do grow in very simple solutions of carbon sources (e.g., glucose) and nitrogen (ammonium salts) along other ions in culture, they are highly amenable to the sort of analysis which has been much more difficult in eukaryotes with their far more sophisticated "basic" nutritional requirements (except for yeast cells; see V). Surprisingly, it was not until clear evidence for specific signaling molecules was recently found that the problem began to be taken seriously in prokaryotes (Janssens, 1988; Koshland, 1988; Stock *et al.,* 1990; Parkinson, 1993). A good review of the origins of chemosignaling in prokaryotes is that of Pertseva (1991), and the topic has recently been surveyed by Gomer (1994) and by Kaprelyants and Kell (1996).

One of the first identified (families of) prokaryotic signaling molecules was the *N*-acyl-homoserine lactones (Fuqua *et al.,* 1994; Swift *et al.,* 1994, 1996), found in density (or *"quorum") signaling* among the marine organism *Vibrio fischeri.* Starvation sensing in *E. coli* has been linked with a homoserine lactone-dependent pathway (Huisman and Roberto, 1994). Furthermore, *E. coli* cells release aspartate, a chemoattractant which makes the cells assemble into "multicellular" aggregates (Budrene and Berg, 1995). One could argue that some of the primary messengers now being identified are going to be different from those in eukaryotes. Although the primary messenger molecules which have been found in unicellular eukaryotes to stimulate responses (see III and V) can be quite dissimilar from those which act in prokaryotes and vice versa (Wang, 1996), such compounds (e.g., insulin-like and somatostatin-like materials) have been reported to occur in *E. coli* and *Bacillus subtilis* (Lenard, 1992). Indeed, Zagon and McLaughlin (1992) showed that a modulator of metazoan development, the opioid growth factor [Met^5]-enkephalin, regulates cell growth and proliferation in *Staphylococcus aureus, S. marcesans,* and *P. aeruginosa,* possibly via a zeta opioid receptor.

It is also recognized that intracellular second messenger (signal transduction) pathways in bacteria have barely been considered and are a long way behind those already described in eukaryotes. There are, however, obvious points of similarity following the reception of signaling involving adenylate and guanylate cyclase, and also in the typical phosphorylation–dephosphorylation cascades that regulate so many functions in all organisms (Russo and Silhavy, 1993; Hellingwerf *et al.,* 1995). The GTP-dependent signaling of adenylate cyclase has features which clearly suggest mechanisms of the greatest antiquity (Stein *et al.,* 1985; Beuve *et al.,* 1990; Pertseva, 1991). The first report on nitric oxide synthase (see III.D) in a unicellular organism came from studies in the bacterial species *Nocardia* (Chen and Rosazza, 1994, 1995).

Within the past few years has come the realization that even bacteria have a genetic machinery to commit suicide. This is known as "postsegregational killing" or "plasmid addiction," which consists of a plasmid-encoded toxin–antidote pair (Jensen and Gerdes, 1995; Yarmolinsky, 1995). Although the regulatory components of this system can be very different from those seen in PCD in eukaryotes (see I.B), prokarytic and eukaryotic suicide may give the cells similar types of advantages. Bacterial death serves to eliminate individual cells which have lost their adaptability to environmental changes, such as fluctations in nutrient supply, viral infections, incidental radiation, and ability to defeat host immune response. Cell suicide thus maintains the survival of the fittest bacteria for the good of the species itself, and this may well apply to all unicellular organisms (see II.C, II.D, and III.E).

Since the present study is not about prokaryotes and there is a strong indication that prokaryotic microbiologists are being rapidly drawn into the whole signaling field as its importance begins to be appreciated (Losick and Kaiser, 1997), we trust that the few leads from the literature given in this section will entice the reader to follow this emerging field. Exactly when new mechanisms were developed or older functions adapted for signaling purposes to the point of divergence during the evolution of modern-day prokaryotes and eukaryotes is likely to be the focus of much investigation—and much controversy—in the future.

VII. Concluding Remarks

It has been customary to view unicellular eukaryotes with the condescending eyes of the metazoon biologist. We have here attempted to avoid that. Instead, we view the living cells in the perspective of evolution, from free-living to metazoan forms.

The free-living eukaryotes developed **intercellular signaling** for survival, proliferation, differentiation, mating, and chemosensory behavior. We propose here that the unicellular organisms have receptors with either very broad or very narrow specificity. To the first group may belong receptors for environmental (not autocrine/paracrine) compounds. To the second group may belong very specific receptors for cell-produced (autocrine) factors in all regulatory processes. The free-living cells have extensive mechanisms for *receptor induction*—signal imprinting—which is very well suited to their way of living and enables the cells to succeed under a varied set of conditions. The **intracellular pathways** were established early in the development of eukaryotes, probably as survival mechanisms: the cell which at the beginning of a starvation period could stop dividing and turn down metabolism in the most efficient way would have a decided advantage. This view goes a long way in explaining how primary messengers came to be quite varied and their receptors kept a broad specificity, whereas the elements of the intracellular signaling systems have changed little.

Metazoan cells originated from free-living cells and have inherited the latter's intracellular systems for proliferation controls, but have changed their own way of living. Their cells are always surrounded by food and stimulating compounds. And yet, in the fully grown body, the majority of these cells must divide at extremely low rates. This has been accomplished by building one "back-up" system after another into the inherited proliferation systems, in addition to refinements of the primary messenger and receptor systems, preventing proliferation-activating compounds from turning proliferation genes on at inappropriate times and under inappropriate

conditions, as well as regulating cellular activities. On top of this greater control of cell division also came the need for greater regulation of differentiation, already present in the free-living cells, but now exploited enormously.

Cell biology has progressed along three main routes: (1) natural history of living cells in culture, (2) work with isolated enzyme systems from cell homogenates, and (3) gene sequencing. Information has come from many channels, and we have stressed in this review that an important new route has been the combination of *low-initial-cell-density* experiments with *chemically defined* nutrient media. It seems quite obvious that it is our broader and more general knowledge of the behavior of the individual living cell which is limiting further advances today, whether it is free-living or part of a metazoon.

Acknowledgments

We thank Dr. Jørgen Friis, Dr. Karsten Kristiansen, and Helle Christiansen, Department of Molecular Biology; Kathrine Overgaard and Dr. Peter Schousboe, Institute of Medical Biology; and Jesper Dybvad, Institute of Biochemistry, Odense University; Professor Antonio Lima-de-Faria, University of Lund, Sweden; Professor Gyorgy Csaba, Semmelweis University, Hungary; Dr. Henrik Nielsen and Dr. Jytte Nilsson, Copenhagen University, for comments and critical reading of parts of the manuscript. This work was supported by grants from the Carlsberg Foundation (to S.T.C. and L.R.), the Novo Foundation (to L.R.), and the Danish Natural Science Research Council (to L.R. and V.L.).

Note added in proof. Similar to Kristiansen *et al.* (1996), we found that cell survival frequencies are increased at low initial population densities if the culture vessels are filled, i.e., have no medium/air interface. However, this increase may be due to an inhibition of macro molecular synthesis rather than to a reduced probability of the cells to enter a death zone at the medium surface. Thus, the final densities in the filled vessels are low (less than 30 cells/ml) as compared to the densities in the nonfilled vessels (*ca.* 100,000 cells/ml). The reason for this low cell density is unknown but could be due to a low oxygen tension in the cultures (Christensen *et al.,* 1997).

References

Abu-Elneel, K., Karchi, M., and Ravid, S. (1996). *Dictyostelium* myosin II is regulated during chemotaxis by a novel protein kinase C. *J. Biol. Chem.* **271,** 977–984.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). "Molecular Biology of the Cell," 3rd Ed. Garland, New York.

Almagor, M., Ron, A., and Bar-Tana, J. (1981). Chemotaxis in *Tetrahymena thermophila. Cell Motil.* **1,** 261–268.

Almas, B., Pryme, I. F., Vedeler, A., and Hesketh, J. E. (1992). Insulin: Signal transmission and short-term effects on the cytoskeleton and protein synthesis. *Int. J. Biochem.* **24,** 183–191.

Ameisen, J. C. (1996). The origin of programmed cell death. *Science* **272,** 1278–1279.

Ameisen, J. C., Idziorek, T., Billaut-Mulot, O., Loyens, M., Tissier, J.-P., Pontier, A., and Ouaissi, A. (1995). Apoptosis in a unicellular eukaryote (*Trypanosoma cruzi*): Implications for the evolutionary origin and the role of programmed cell death in the control of cell proliferation, differentiation and survival. *Cell Death Differ.* **2,** 285–300.

Andersen, H. A., and Houen, G. (1989). Purification and partial characterization of a transcription-inhibitory peptide from *Tetrahymena. Biol. Chem. Hoppe-Seyler* **370,** 41–46.

Andersen, H. A., and Islin, H. (1987). Peptides regulate the activity of RNA polymerases in *Tetrahymena. Biochem. Biophys. Acta* **908,** 1–11.

Andersen, H. A., Flodgaard, H., Klenow, H., and Leick, V. (1984). Platelet-derived growth factor stimulates chemotaxis and nucleic acid synthesis in the protozoan *Tetrahymena. Biochim. Biophys. Acta* **782,** 437–440.

Arends, M. J., Morris, R. G., and Wyllie, A. H. (1990). Apoptosis: The role of endonuclease. *Am. J. Pathol.* **136,** 593–608.

Bach, J. F., and Papiernik, M. (1981). Cellular and molecular signals in T cell differentiation. *Ciba Found. Symp.* **84,** 215–235.

Bae, S., and Ryals, P. E. (1997). Evidence that stomatin-induced differentiation in *Tetrahymena vorax* is mediated by signalling through rapid inositol phospholipid turnover. *J. Euk. Microbiol.* **44,** A116.

Baixeras, E., Bosca, L., Stauber, C., Gonzales, A., Carrera, A. C., Gonzalo, J. A., and Martinez-A. C. (1994). From apoptosis to autoimmunity: Insight from the signalling pathways leading to proliferation or to programmed cell death. *Immunol. Rev.* **142,** 53–91.

Barcinski, M. A., and Moreira, M. E. C. (1994). Cellular responses of protozoan parasites to host-derived cytokines. *Parasitol. Today* **10,** 352–355.

Barcinski, M. A., Schehtman, D., Quintao, L. G., Costa, D. A., Soares, L. R. B., Moreira, M. E. C., and Charlab, R. (1992). Granulocyte–macrophage colony stimulating factor increases the infectivity of *Leishmania amazonensis* by protecting promastigotes from heat-induced death. *Infect. Immun.* **60,** 3523–3527.

Batitastou, A., and Greene, L. (1993). Internucleosomal DNA cleavage and neuronal cell survival/death. *J. Cell Biol.* **122,** 523–532.

Beauvais, F., Michel, L., and Dubertret, L. (1995a). Exogeneous nitric oxide elicits chemotaxis of neutrophils in vitro. *J. Cell. Physiol.* **165,** 610–614.

Beauvais, F., Michel, L., and Dubertret, L. (1995b). The nitric oxide doners, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. *FEBS Lett.* **361,** 229–232.

Berdeaux, A. (1993). Nitric oxide: An ubiquitous messenger. *Fundam. Clin. Pharmacol.* **7,** 401–411.

Berdicevski, I., and Mirsky, N. (1994). Effects of insulin and glucose tolerance factor (GTF) on growth of *Saccharomyces cerevisiae. Mycoses* **37,** 405–410.

Berks, M., Traynor, D., Carrin, I., Insall., R. H., and Kay, R. R. (1991). Diffusible signal molecules controlling cell differentiation and patterning in *Dictyostelium. Development Suppl.* **1,** 131–139.

Berlot, C. H., Devreotes, P., and Spudich, J. A. (1987). Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localization and increases in kinase activity. *J. Biol. Chem.* **262,** 3918–3926.

Bertrand, R., Solary, E., O'Conner, P., Kohn, K. W., and Pommier, Y. (1994). Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.* **211,** 314–321.

Beuve, A., Boesten, B., Cranier, M., Danchin, A., and O'Gara, F. (1990). *Rhizobium meliloti* adenylate cyclase is related to eukaryotic adenylate and guanylate cyclases. *J. Bacteriol.* **172,** 2614–2621.

Bing, R. J., Termin, A., Conforto, A., Dudek, R., and Hoffmann, M. J. (1993). Membrane function and vascular reactivity. *Biosci. Rep.* **13,** 61–67.

Bissonette, R. P., Echeverri, F., Mahabubi, A., and Green, D. R. (1992). Apoptotic cell death induced by *c-myc* is inhibited by *bcl-2. Nature* **359,** 552–554.

Blum, J. J. (1967). An adrenergic control system in *Tetrahymena. Proc. Natl. Acad. Sci. USA* **51,** 81–88.

Bonini, N. M., and Nelson, D. L. (1988). Differential regulation of Paramecium ciliary motility by cAMP and cGMP. *J. Cell Biol.* **106,** 1615–1623.

Bornfeldt, K. E., Raines, E. W., Nakano, T., Graves, L. M., Krebs, E. G., and Ross, R. (1994). Insulin-like growth factor I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. *J. Clin. Invest.* **93,** 1266–1274.

Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., Hakomori, S.-I., and Ross, R. (1995a). Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: Spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell Biol.* **130,** 193–206.

Bornfeldt, K. E., Raines, E. W., Graves, L. M., Skinner, M. P., Krebs, E. G., and Ross, R. (1995b). Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. *Ann. NY Acad. Sci.* **766,** 416–430.

Bortner, C. D., Oldenburg, N. B. E., and Cidlowski, J. A. (1995). The role of DNA fragmentation in apoptosis. *Trends Biochem. Sci.* **5,** 21–26.

Boulay, F., Tardif, M., Brouchon, L., and Vignais, P. (1990). The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* **29,** 11123–11133.

Boyan, L. A., Bhargava, G., Nishimura, F., Orman, R., Price, R., and Terranova, V. P. (1994). Mitogenic and chemotactic responses of human peridontal ligament cells to the different isoforms of platelet-derived growth factor. *J. Dent. Res.* **73,** 1593–1600.

Brizzi, G., and Blum, J. J. (1970). Effect of growth and conditions on serotonin content of *Tetrahymena pyriformis. J. Protozool.* **17,** 553–555.

Brookman, J. J., Town, C. D., Jermyn, K. A., and Kay, R. R. (1982). Developmental regulation of stalk cell differentiation-inducing factor in *Dictyostelium dicoideum. Dev. Biol.* **77,** 687–699.

Brown, L. R., Mronga, S., Bradshaw, R. A., Ortenzi, C., Luporini, P., and Wüthrich, K. (1993). Nuclear magnetic resonance solution structure of the pheromone Er-10 from the ciliated protozoan *Euplotes raikovi. J. Mol. Biol.* **231,** 800–816.

Bruckner, P., Horler, I., Mendler, M., Houze, Y., Winterhalter, K. H., Eich-Bender, S. G., and Spycher, M. A. (1989). Induction and prevention of chondrocyte hypertrophy in culture. *J. Cell Biol.* **109,** 2537–2545.

Budrene, E. O., and Berg, H. C. (1995). Dynamics of formation of symmetrical patterns by chemotactic bacteria. *Nature* **376,** 49–53.

Burch, R. M., and Kyle, D. J. (1992) Minireview: Recent developments in the understanding of bradykinin receptors. *Life Sci.* **50,** 829–838.

Burdine, V., and Clarke, M. (1995). Genetic and physiological modulation of the prestarvation response in Dictyostelium discoideum. *Mol. Dev. Cell* **6,** 311–325.

Canman, C. E., and Kastan, M. B. (1996). Three paths to stress relief. *Nature* **384,** 213–214.

Cano, E., and Mahadevan, L. C. (1995). Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20,** 117–122.

Chen, J. B., Sun, J., and Jazwinski, S. M. (1990). Prolongation of the yeast life span by the v-HA-*RAS* oncogene. *Mol. Microbiol.* **4,** 281–286.

Chen, Y., and Rosazza, J. P. (1994). A bacterial nitric oxide synthase from a *Nocardia* species. *Biochem. Biophys. Res. Commun.* **203,** 1251–1258.

Chen, Y., and Rosazza, J. P. (1995). Purification and characterization of nitric oxide synthase (NOSNoc) from a *Nocardia* species. *J. Bacteriol.* **177,** 5122–5128.

Chiesa, R., Silva, W.I., and Renaud, F. L. (1993). Pharmacological characterization of an opioid receptor in the ciliate *Tetrahymena. J. Euk. Microbiol.* **40,** 800–804.

Christensen, S. T. (1993). Insulin rescues the unicellular eukaryote *Tetrahymena* from dying in a complete, synthetic nutrient medium. *Cell Biol. Int.* **17,** 833–838.

Christensen, S. T., and Rasmussen, L. (1992). Evidence for growth factors which control cell multiplication in *Tetrahymena thermophila. Acta Protozool.* **31,** 215–219.

Christensen, S. T., Schousboe, P., Ghiladi, M., and Rasmussen, L. (1992). Nutritional stress relieved by addition of hemin and phospholipids. *J. Comp. Physiol. B* **162,** 107–110.

Christensen, S. T., Schousboe, P., Nielsen, D. S., Søndergaard, G., and Rasmussen, L. (1993). Cell multiplication in *Tetrahymena setosa* and *Tetrahymena thermophila* in synthetic nutrient medium. Effects of ethanol, cholesterol and extracellular medium. *Acta Protozool.* **32,** 151–156.

Christensen, S. T., Wheatley, D. N., Rasmussen, M. I., and Rasmussen, L. (1995). Mechanisms controlling death, survival and proliferation in a model unicellular eukaryote *Tetrahymena thermophila. Cell Death Differ.* **2,** 301–308.

Christensen, S. T., Kemp, K., Quie, H., and Rasmussen, L. (1996a). Cell death, survival and proliferation in *Tetrahymena thermophila.* Effects of insulin, sodium nitroprusside, 8-bromo cyclic GMP, N^G-methyl-L-arginine and methylene blue. *Cell Biol. Int.* **20,** 653–666.

Christensen, S. T., Quie, H., Kemp, K., and Rasmussen, L. (1996b). Insulin produces a biphasic response in *Tetrahymena thermophila* by stimulating cell survival and activating cell proliferation in two separate concentration intervals. *Cell Biol. Int.* **20,** 437–444.

Christensen, S.T., Rasmussen, M.I., Chemnitz, J., Beyer, H., Olesen, J.D., Sørensen, H., Kristiansen, K., and Rasmussen, L. (1997). Cell death in *Tetrahymena thermophila* induced in low cell density cultures and by staurosporine. Submitted for publication.

Christopher, G. K., and Sundermann, C. A. (1992). Conventional and confocal microscopic studies of insulin receptor induction in *Tetrahymena pyriformis. Exp. Cell Res.* **201,** 301–308.

Christopher, G. K., and Sundermann, C. A. (1995a). Effect of long-term insulin exposure on insulin binding in *Tetrahymena pyriformis. Tissue Cell* **27,** 585–589.

Christopher, G. K., and Sundermann, C. A. (1995b). Isolation and partial characterization of the insulin binding site of *Tetrahymena pyriformis. Biochem. Biophys. Res. Commun.* **212,** 515–523.

Christopher, G. K., and Sundermann, C. A. (1996). Intracellular insulin binding in *Tetrahymena pyriformis. Tissue Cell* **28,** 427–437.

Chun, S. Y., Eisenhauer, K. M., Kubo, M., and Hsueh, A. J. (1995). Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology* **136,** 3120–3127.

Clarke, M., and Gomer, R. H. (1995). PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium. Experientia* **51,** 1124–1134.

Clarke, M., Kayman, S. C., and Riley, K. (1987). Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum. Differentiation* **34,** 79–87.

Clarke, M., Yang, J., and Kayman, S. (1988). Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum. Dev. Genet.* **9,** 315–326.

Clarke, M., Diminguez, N., Yuen, I. S., and Gomer, R. H. (1992). Growing and starving *Dictyostelium* cells produce distinct density-sensing factors. *Dev. Biol.* **152,** 403–406.

Clarke, P. G. H. (1990). Developmental cell death: Morphological diversity and multiple mechanisms. *Anat. Embryol.* **181,** 195–213.

Cohen, G. M., Sun, X., Snowden, R. T., Dinsdale, D., and Skilleter, D. N. (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal fragmentation. *Biochem. J.* **286,** 331–334.

Collins, R. J., Harmon, B. V., Gobe, G. C., and Kerr, J. F. R. (1992). Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Radiat.* Biol. **61,** 451–453.

Columbano, A. (1995). Cell death: Current difficulties in discriminating apoptosis from necrosis in the context of pathological processes *in vivo. J. Cell. Biochem.* **58,** 181–190.

Concetti, A., Raffioni, S., Miceli, C., Barra, D., and Luporini, P. (1986). Purification to apparent homogeneity of the mating pheromone of *mat-1* homozygous *Euplotes raikovi. J. Biol. Chem.* **261,** 10582–10587.

Conforto, A., Dudek, R., Hoffmann, M. R., and Bing, R. J. (1994). The production of nitric oxide in endothelial cells by amphiphiles. *Life Sci.* **54,** 1143–1153.

Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D., and Goldstein, P. (1994). Programmed cell death in *Dictyostelium. J. Cell Sci.* **107,** 2691–2704.

Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.* **267,** C1405–C1413.

Couldwell, W., Hinton, D., He, S., Chen, T., Sebat, I., Weiss, M., and Law, R. (1994). Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS Lett.* **345,** 43–46.

Csaba, G. (1980). Phylogeny and ontogeny of hormone receptors: The selection theory of receptor formation and hormonal imprinting. *Biol. Rev. Cambridge Philos. Soc.* **55,** 47–63.

Csaba, G. (1985). The unicellular *Tetrahymena* as a model cell for receptor research. *Int. Rev. Cytol.* **95,** 327–377.

Csaba, G. (1994). Phylogeny and ontogeny of chemical signalling: Origin and development. *Int. Rev. Cytol.* **155,** 1–48.

Csaba, G. (1996). Evolutionary significance of the hormone recognition capacity in unicellular organisms. Development of hormone receptors. *In* "Progress in Molecular and Subcellular Biology. Signalling Mechanisms in Protozoa and Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 1–28. Springer Verlag, Berein/New York.

Csaba, G., and Kovács, P. (1987). Transmission of hormonal imprinting in *Tetrahymena* cultures by intercellular communication. *Z. Naturforsch.* **42c,** 932–934.

Csaba, G., and Kovács, P. (1994). Effect of hormones and hormone-induced imprinting on the serotonin level of *Tetrahymena:* Immunocytochemical studies. *Microbios* **80,** 155–163.

Csaba, G., and Lantos, T. (1975). Effect of insulin on glucose uptake of protozoa. *Experientia* **31,** 1097–1098.

Csaba, G., and Lantos, T. (1976). Effect of epinephrine on glucose metabolism in *Tetrahymena. Endokrinologie* **68,** 239–240.

Csaba, G., and Müller, W. E. G. (1996). Signaling mechanisms in protozoa and invertebrates. In "Progress in Molecular and Subcellular Biology," Vol. 17. Springer-Verlag, Berein/New York.

Csaba, G., Inczefi-Gonda, Å., and Fehér, T. (1985). Induction of steroid binding sites (receptors) and presence of steroid hormones in the unicellular *Tetrahymena pyriformis. Comp. Biochem. Physiol. A* **82,** 567–570.

Csaba, G., Kovács, P., and Falus, A. (1995). Human cytokines interleukin (IL)-3 and IL-6 affect the growth and insulin binding of the unicellular organism *Tetrahymena. Cytokine* **7,** 771–774.

Cutler, D. W., and Crump, L. M. (1923). CIX. The rate of reproduction in artificial culture of *Colpidium colpoda. Biochem.* J. **17,** 174–186.

Darzynkiewicz, Z. (1995). Apoptosis in anti tumour strategies: Modulation of cell cycle or differentiation. *J. Cell. Biochem.* **58,** 151–159.

Davis, M. C., Ward, J. G., Herrick, G., and Allis, C. D. (1992). Programmed nuclear death: Apoptotic-like degredation of specific nuclei in conjugating *Tetrahymena. Dev. Biol.* **154,** 419–432.

De Jesús, S., and Renaud, F. L. (1989). Phagocytosis in *Tetrahymena thermophila: Naloxone-reversible inhibition by opiates. Comp. Biochem. Physiol.* C **92,** 139–142.

DeMeytz, P., Van Obberghen, E., Roth, J., Wolmer, A., and Brandenburg, D. (1978). Mapping of the residues responsible for the negative coorperative of the receptor-binding region of insulin. *Nature* **273,** 504–509.

Devreotes, P., and Zigmond, S. (1988). Chemotaxis in eukaryotic cells: A focus on leukocytes and Dictyostelium. *Annu. Rev. Cell Biol.* **4,** 649–686.

Devreotes, P. N. (1994). G protein-linked signalling pathways control the developmental program of *Dictyostelium. Neuron* **12,** 235–241.

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996). A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383,** 547–550.

DoCampo, R., and Pignataro, O. (1991). The inositol phosphate/diacylglycerol signalling pathway in *Trypanosoma cruzi. Biochem. J.* **275,** 407–411.

Dudek, R., Conforto, A., Pinto, V., and Bing, R. J. (1994). Effect of amphiphiles on nitric oxide synthase in endothelial cells. *Pharmacology* **48,** 374–379.

Duke, R. C., Ojcius, D. M., and Young, D.-E. (1996). Cell suicide in health and disease. *Sci. Am.* **275** (Dec.), 48–55.

Dunten, P., and Koshland, D. E., Jr. (1991). Tuning of the responsiveness of a sensory receptor via covalent modification. *J. Biol. Chem.* **266,** 1491–1496.

Early, A. E., Abe, T., and Williams, J. G. (1995). Evidence for positional differentiation of prestalk cells and for a morphogenetic gradient in *Dictyostelium. Cell* **83,** 91–99.

Egelhoff, T. T., Lee, R. J., and Spudich, J. A. (1993). *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization *in vivo. Cell* **75,** 363–371.

Elion, E. A. (1995). Ste5: A meeting place for MAP kinases and their associates. *Trends Cell Biol.* **5,** 322–327.

Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7,** 663–698.

Etienne, P., Pares-Herbute, N., and Monnier, L. (1996). Enhanced antiproliferative effect of nitric oxide in cultured smooth muscle cells from diabetic rats. *J. Cardiovasc. Pharmacol.* **27,** 140–146.

Evan, G. I. (1994). Old cells never die, they just apoptose. *Trends Cell Biol.* **4,** 191–192.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. S. M., Penn, L. Z., and Hancock, K. D. (1992). Induction of apoptosis in fibroblasts by *c-myc* protein. *Cell* **69,** 119–128.

Fanidi, A., Harrington, E. A., and Evan, G. I. (1992). Cooperative interaction between *c-myc* and *bcl-2* oncogenes. *Nature* **359,** 554–556.

Farber, E. (1994). Programmed cell death: Necrosis versus apoptosis. *Mod. Pathol.* **7,** 605–609.

Farinelli, S. E., Park, D. S., and Greene, L. A. (1996). Nitric oxide delays the death of trophic factor-deprived PC12 cells and sympathetic neurons by a cGMP-mediated mechanism. *J. Neurosci.* **16,** 2325–2334.

Feelisch, M., and Noack, N. A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* **139,** 19–30.

Feig, L. A., Urano, T., and Cantor, S. (1996). Evidence for a Ras/Ral signalling cascade. *Trends Biochem. Sci.* **21,** 438–441.

Filipski, J., LeBlanc, J., Youdale, T., Sikorski, M., and Walker, P. R. (1990). Periodicity of DNA folding in higher order chromatin structures. *EMBO J.* **9,** 1319–1327.

Fink, K. L., and Cross, R. W. (1984). Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds. *Circ. Res.* **55,** 585–594.

Firtel, R. A. (1995). Integration of signalling information in controlling cell-fate decitions in *Dictyostelium. Genes Dev.* **9,** 1427–1444.

Florin-Christensen, J., Florin-Christensen, M., Rasmussen, L., and Knudsen, J. (1986). An acid phospholipase C from *Tetrahymena* culture medium. *Comp. Biochem. Physiol. B* **85,** 143–148.

Friis, J., Szablewski, J., Christensen, S. T., Schousboe, P., and Rasmussen, L. (1994). Physiological studies on the effect of Ca^{2+} on the duration of the lag phase of *Saccharomyces cerevisiae. FEMS Microbiol. Lett.* **123,** 33–36.

Fujimoto, M., and Okabayashi, T. (1975). Proposed mechanisms of stimulation and inhibition of guanylate cyclase with reference to the action of chlorpromazine, phospholipases and Triton X-100. *Biochem. Biophys. Res. Commun.* **67,** 1332–1336.

Fujino, M., Wakimasu, M., Taketomi, S., and Iwatsuka, H. (1977). Insulin-activities and insulin-potentiating actions of a modified insulin $B_{21\text{-}26}$ fragment: β-Ala-Arg-Gly-Phe-Phe-Tyr-NH_2. *Endocrinology* **101,** 360–364.

Fukui, Y. (1985). Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium. Nature* **314,** 194–196.

Fukuto, J. M. (1995). Chemistry of nitric oxide: Biologically relevant aspects. *In* Nitric Oxide. Biochemistry, Molecular Biology, and Therapeutic Implications (L. Ignarro and F. Murad, eds.). Academic Press, San Diego.

Funder, J. W. (1996). Mineralocorticoid receptors in the central nervous system. *J. Steroid Biochem. Mol. Biol.* **56,** 179–183.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994). Quorum sensing in bacteria: The *luxR-luxl family of cell density-responsive transcriptional regulators. J. Bacteriol.* **176,** 269–275.

Gale, M., Jr., Carter, V., and Parsons, M. (1994). Cell cycle-specific induction of an 89 kDa serine/threonine protein kinase activity in *Trypanosoma brucei. J. Cell Sci.* **107,** 1825–1832.

Gammeltoft, S. (1984). Insulin receptors: Binding kinetics and structure relationships of insulin. *Physiol. Rev.* **64,** 1321–1378.

Garbers, D. L. (1992). Guanylyl cyclase receptors and their endocrine, paracrine and autocrine ligands. *Cell* **17,** 1–4.

Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Mayer, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.* **48,** 184–188.

Gaskins, C., Maeda, M., and Firtel, R. (1994). Identification and functional analysis of a developmentally regulated extracellular signal-regulated kinase gene in *Dictyostelium discoideum. Mol. Cell. Biol.* **14,** 6996–7012.

Gaskins, C., Clark, A. M., Aubry, L., Segall, J., and Firtel, R. A. (1996). The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways. *Genes Dev.* **10,** 118–128.

Gerisch, G. (1986). Interrelation of cell adhesion and differentiation in *Dictyostelium discoideum. J. Cell Sci. Suppl.* **4,** 201–219.

Gerisch, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium. Annu. Rev. Biochem.* **56,** 853–879.

Gerzer, R., Hamet, P., Ross, A. H., Lawson, J.A., and Hardman, J.G. (1983). Calcium-induced release from platelet membranes of fatty acids that modulate soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.* **226,** 280–286.

Gerzer, R., Brash, A. R., and Hardman, J. G. (1986). Activation of soluble guanylate cyclase by arachidonic acid and 15-lipooxygenase products. *Biochim. Biophys. Acta* **886,** 383–389.

Gold, M. E., Wood, K. S., Byrns, R. E., Fukuto, J., and Ignarro, L. J. (1990). N^G-methyl-L-arginine causes endothelium-dependent contraction and inhibition of cyclic GMP formation in artery and vein. *Proc. Natl. Acad. Sci. USA* **87,** 4430–4434.

Goldberg, D., Segal. M., and Levitzki, A. (1994). Cdc25 is not the signal receiver for glucose induced cAMP response in *S. cerevisiae. FEBS Lett.* **356,** 249–254.

Gomer, R. H. (1994). Knowing that you're among friends. *Curr. Biol.* **4,** 734–735.

Gomer, R. H., and Firtel, R. A. (1987). Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. *Science* **237,** 758–762.

Gomer, R. H., Yuen, I. S., and Firtel, R. A. (1991). A secreted 80 × 10^3 M_r protein mediates sensing of cell density and the onset of development in *Dictyostelium. Development* **112,** 269–278.

Gomez, M. L., Erijman, L., Arauzo, S., Torres, H. N., and Tellez-Inon, M. T. (1989). Protein kinase C in *T. cruzi* epimastigote forms: Partial purification and characterization. *Mol. Biochem. Parasitol.* **36,** 101–108.

Grabel, L., and Loomis, W. F. (1978). Effector controlling accumulation of *N*-acetylglucosaminidase during development of *Dictyostelium discoideum. Dev. Biol.* **64,** 203–209.

Graves, M. G., Dickinson, J. R., and Swoboda, B. E. P. (1976). Cyclic GMP and cyclic GMP phosphodiesterase in the cell cycle of *Tetrahymena pyriformis. FEBS Lett.* **69,** 165–166.

Greenhalf, W., Stephan, C., and Chaudhuri, B. (1996). Role of mitochondria and C-terminal anchor of Bcl-2 in Bax-induced growth arrest and mortality in *Saccharomyces cerevisiae. FEBS Lett.* **380,** 169–175.

Griffiths, A. J. F. (1992). Fungal senescence. *Annu. Rev. Genet.* **26,** 351–372.

Häcker, G., and Vaux, D. L. (1995). The medical significance of physiological cell death. *Med. Res. Rev.* **15,** 299–311.

Hagemeister, J. J., Grave, M., Kristiansen, T. B., and Hellung-Larsen, P. (1996). New forms of cell death in *Tetrahymena thermophila.* The effect of the constituents of the chemically defined medium. *J. Euk. Microbiol. Abstr.* **44,** 44A.

Hall, B. F., and Joiner, K. A. (1993). Developmentally-regulated virulence factors of T. cruzi and their relationship to evasion of host defences. *J. Euk. Microbiol.* **40,** 207–213.

Hall, R. P., and Loefer, J. B. (1940). Effects of culture filtrates and old medium on growth of the ciliate, *Colpidium campylum. Proc. Soc. Exp. Biol. Med.* **43,** 128–133.

Hanada, M., Aimé-Sempé, C., Sato, T., and Reed, J. C. (1995). Structure–function analysis of bcl-2 protein. Identification of conserved domains important for homodimerization with bcl-2 and heterodimerization with *Bax. J. Biol. Chem.* **270,** 11962–11969.

Hardie, D. G. (1991). "Biochemical Messengers. Hormones, Neurotransmitters and Growth Factors." Chapman & Hall, London.

Harrington, E. A., Bennet, M. R., Fanidi, A., and Evan, G. I. (1994). C-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.* **13,** 3286–3295.

Haselbacher, G., Custer, M., Lutz, U., and Humbel, R. E. (1992). The N-terminal hexapeptide fragment of IGF II stimulates thymidine incorporation into fibroblasts. *Biochem. Biophys. Res. Commun.* **187,** 279–286.

Hegyesi, H., and Csaba, G. (1992a). Specific insulin binding by, and imprintability of, the nuclear membrane of *Tetrahymena. Cytobios* **72,** 153–157.

Hegyesi, H., and Csaba, G. (1992b). Effects of pretreatments (imprinting) with different concentrations of insulin on insulin binding to the nuclear envelope and plasma membrane in *Tetrahymena. Cytobios* **72,** 191–196.

Hegyesi, H., and Csaba, G. (1994a). A calcium-dependent protein kinase is present in *Tetrahymena. Cell Biochem. Funct.* **12,** 221–226.

Hegyesi, H., and Csaba, G. (1994b). Change in insulin binding capacity of plasma membrane and nuclear envelope of *Tetrahymena* following a single long-term pretreatment and several short-term pretreatments (imprinting) with insulin. *Cytobios* **79,** 189–194.

Hellingwerf, K. J., Postma, P. W., Tommassen, J., and Westerhoff, H. V. (1995). Signal transduction in bacteria: Phospho-neural network(s) in *Escherichia coli. FEMS Microbiol. Rev.* **16,** 309–321.

Hermeking, H., and Eick, D. (1994). Mediation of c-Myc-induced apoptosis by p53. *Science* **265,** 2091–2093.

Hermeking, H., Wolf, D. A., Kohlhuber, F., Funk, J. O., Reichert, M., Dickmanns, A., Fanning, E., and Eick, D. (1995). p53 mediates c-Myc-induced apoptosis. *Cell Biol. Int.* **19,** 705–706.

Herskowitz, I. (1995). MAP kinase pathways in yeast: For mating and more. Cell **80,** 187–197.

Hide, G., Gray, A., Harrison, C. M., and Tait, A. (1989). Identification of an epidermal growth factor receptor homologue in trypanosomes. *Mol. Biochem. Parasitol.* **36,** 51–60.

Hide, G., Graham, T., Buchanan, N., Tait, A., and Keith, K. (1994). *Trypanosoma brucei:* Characterization of protein kinases that are capable of autophosphorylation *in vitro. Parasitology* **108,** 161–166.

Hii, C. S. T., Ferrante, A., Edwards, Y. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1995). Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C-dependent mechanism. *J. Biol. Chem.* **270,** 4201–4204.

Hinrichsen, R. D., and Blackshear, P. J. (1993). Regulation of peptide–calmodulin complexes by protein kinase C *in vivo. Proc. Natl. Acad. Sci. USA* **90,** 1585–1589.

Hirano, H., Kagawa, H., and Okubo, K. (1992). Characterization of proteins released from legume seeds in hot water. *Phytochemistry* **31,** 731–735.

Hirano-Ohnishi, J., and Watanabe, Y. (1989). Ca^{2+}/calmodulin-dependent phosphorylation of ciliary beta-tubulin in *Tetrahymena. J. Biochem.* **105,** 858–860.

Hirsch, J. P., and Cross, F. R. (1992). Pheromone response in yeast. *BioEssays* **14,** 367–373.

Holz, G. G., Jr., Erwin, J., Rosenbaum, N., and Aaronson, S. (1962a). Triparanol inhibition of *Tetrahymena* and its prevention by lipids. *Arch. Biochem. Biophys.* **98,** 312–322.

Holz, G. G., Jr., Erwin, J., Wagner, B., and Rosenbaum, N. (1962b). The nutrition of *Tetrahymena setifera* HZ-1: Sterol and alcohol requirements. *J. Protozool.* **9,** 359–363.

Honda, H., and Miyake, A. (1975). Taxis to a conjugation-inducing substance in the ciliate *Blepharisma. Nature* **257,** 678–680.

Housley, M. D., Wakelam, M. J. O., Murphy, G. J., Gawler, D. J., and Pyne, N. J. (1987). Glucagon stimulates adenylate cyclase through GR2 glucagon receptors: A process which can be attenuated by glucagon stimulating inositol phospholipid metabolism through GR1 glucagon receptors. *Biochem. Soc. Trans.* **15,** 21–24.

Huisman, G. W., and Roberto, K. (1994). Sensing starvation: A homoserine lactone-dependent signalling pathway in *Escherichia coli. Science* **265,** 537–539.

Hünseler, P., Scheidgen-Kleyboldt, G., and Tiedtke, A. (1987). Isolation and characterization of a mutant of *Tetrahymena thermophila* blocked in secretion of lysosomal enzymes. *J. Cell Sci.* **88,** 47–55.

Ignarro, L. J. (1992). Haem-dependent activation of cytosolic guanylate cyclase by nitric oxide: A widespread signal transduction mechanism. *Biochem. Soc. Trans.* **20,** 465–469.

Ignarro, L. J., Lippton, H. L., Edwards, J. C., Barricos, W. H., and Hyman, A. L. (1981). Mechanisms of vascular smooth muscle cell relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* **218,** 739–749.

Ignarro, L. J., Wood, K. S., and Wolin, M. S. (1984). Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: A unifying concept. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17,** 267–274.

Ishizaki, Y., Vouvodic, J. T., Burne, J. F., and Raff, M. C. (1993). Control of lens epithelial cell survival. *J. Cell Biol.* **121,** 899–908.

Ishizaki, Y., Burne, J. F., and Raff, M. C. (1994). Autocrine signals enable chondrocytes to survive in culture. *J. Cell Biol.* **126,** 1069–1077.

Iwai, T., Fuyisawa, N., Ogita, K., and Kikkawa, U. J. (1992). Catalytic properties of yeast protein kinase C: Difference between the yeast and mammalian enzyme. *J. Biochem.* **112,** 7–10.

Jain, R., Yuen, I. S., Taphouse, C. R., and Gomer, R. H. (1992). A density-sensing factor controls development in *Dictyostelium. Genes Dev.* **6,** 390–400.

Janakidevi, K., Devey, V. C., and Kidder, G. W. (1966). The biosynthesis of catecholamines in two genera of protozoa. *J. Biol. Chem.* **241,** 2576–2578.

Janssens, P. M. (1988). The evolutionary origin of eukaryotic transmembrane signal transduction. *Comp. Biochem. Physiol.* A **90,** 209–223.

Jensen, R. B., and Gerdes, K. (1995). Programmed cell death in bacteria: Proteic plasmid stabilization systems. *Mol. Microbiol.* **17,** 205–210.

Kaprelyants, A. S., and Kell, D. B. (1996). Do bacteria need to communicate with each other for growth? *Trends Microbiol.* **4,** 237–242.

Kardami, E., Pearson, T. W., Beecroft, R. P., and Fandrich, R. R. (1991). Identification of basic fibroblast growth factor-like proteins in African trypanosomes and *Leishmania. Mol. Biochem. Parasitol.* **51,** 171–182.

Kashishian, A., and Cooper, J. A. (1993). Phosphorylation sites at the C-terminus of the platelet-derived growth factor receptor bind phospholipase C-gamma 1. *Mol. Biol. Cell* **4,** 49–57.

Kawata, T., Early, A., and Williams, J. (1996). Evidence that a combined activator–repressor protein regulates *Dictyostelium* stalk cell differentiation. *EMBO J.* **15,** 3085–3092.

Kay, R. R., and Jermyn, K. A. (1983). A possible morphogen controlling differentiation in *Dictyostelium. Nature* **303,** 242–244.

Keith, K., Hide, G., and Tait, A. (1990). Characterization of protein kinase C-like activities in *Trypanosoma brucei. Mol. Biochem. Parasitol.* **43,** 107–116.

Kerr, J. F. R. (1971). Shrinkage necrosis: A distinct mode of cellular death. *J. Pathol.* **105,** 13–20.

Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis is a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.

Kidder, G. W. (1939). The effect of biologically conditioned medium on the growth rate and population yield of certain ciliated protozoa. *Science* **90,** 405–406.

Kidder, G. W. (1941). Growth studies of ciliates. V. The acceleration and inhibition of ciliate growth in biologically conditioned medium. *Physiol. Zool.* **14,** 209–226.

Kidder, G. W., and Dewey, V. C. (1951). The biochemistry of ciliates in pure culture. In "Biochemistry and Physiology of Ciliates 1" (A. Lwoff, ed.), pp. 323–400. Academic Press, New York.

Kidder, G. W., and Dutta, B. N. (1958). The growth and nutrition of *Crithidia fasciculata. J. Gen. Microbiol.* **18,** 621–638.

Kikuchi, K., Larner, J., Freer, R. J., Day, A. R., Morris, H., and Dell, A. (1980). Studies on the biological activity of degraded insulins and insulin fragments. *J. Biol. Chem.* **255,** 9281–9288.

Kikuchi, K., Larner, J., Freer, R. J., and Day, A. R. (1981a). Supplemental material to effect of insulin fragment on biological activity of insulin and desoctapeptide insulin. I. Potentiation of biological activities. *J. Biol. Chem.* **256,** 9443–9446.

Kikuchi, K., Larner, J., Freer, R. J., and Day, A. R. (1981b). Supplemental material to effect of insulin fragment on biological activity of insulin and desoctapeptide insulin. II. Potentiation of biological activities. *J. Biol. Chem.* **256,** 9447–9449.

Knoebl, I., Fitzpatrick, M. S., and Schreck, C. B. (1996). Characterization of a glucocorticoid receptor in the brains of chinook salmon, *Oncorhynchos tshawytscha. Gen. Comp. Endocrinol.* **101,** 195–204.

Köhidai, L., and Csaba, G. (1996). Differential and selective chemotactic responses of *Tetrahymena pyriformis.* Two families of signal molecules: Lectins and peptide hormones. *Acta Microbiol. Immunol. Hung.* **43,** 83–91.

Köhidai, L., Barsony, J., Roth, J., and Marx, S. J. (1992). Rapid effects of insulin on cyclic GMP location in an intact protozoon. *Experientia* **40,** 476–481.

Köhidai, L., Lovas, B., and Csaba, G. (1995). Effect of adrenocorticotropic hormone (ACTH) and insulin on the phagocytic capacity of *Tetrahymena. Zool. Sci.* **12,** 277–281.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D., and Rapp, U. R. (1993). Protein kinase C-alpha activates RAF-1 by direct phosphorylation. *Nature* **364**, 249–252.

Komatsu, S., and Hirano, H. (1991). Plant basic 7S globulin-like proteins have insulin and insulin-like growth factor binding activity. *FEBS Lett.* **294**, 210–212.

Komatsu, S., Koshio, O., and Hirano, H. (1994). Protein kinase activity and insulin-binding activity in plant basic 7S globulin. *Biosci. Biotechnol. Biochem.* **58**, 1705–1706.

Komori, Y., Chiang, K. T., and Fukuto, J. M. (1993). The effect of non-ionic detergents on the activity and/or stability of rat brain nitric oxide synthase. *Arch. Biochem. Biophys.* **307**, 311–315.

Koppelhus, U., Hellung-Larsen, P., and Leick, V. (1994). An improved quantitative assay for chemokinesis in *Tetrahymena. Biol. Bull.* **187**, 8–15.

Koshland, D. E. (1988). Chemotaxis as a model second-messenger system. *Biochemistry* **27**, 5829–5834.

Kothe, E. (1996). Tetrapolar fungal mating types: Sexes by the thousands. *FEMS Microbiol. Lett.* **18**, 65–87.

Kovács, P. (1996). Cell-surface GPI expression in Protozoa. The connection with the PI system. In "Progress in Molecular and Subcellular Biology. Signaling Mechanisms in Protozoa and Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 105–118. Springer Verlag, Berlin/New York.

Kovács, P., and Csaba, G. (1987). Cytochemical investigation into the Ca-dependence of positive and negative hormonal imprinting in *Tetrahymena. Histochemistry* **87**, 619–622.

Kovács, P., and Csaba, G. (1990). Evidence of the receptor nature of the binding sites induced in *Tetrahymena* by insulin treatment. A quantitative cytofluoremetric technique for the study of binding kinetics. *Cell Biochem. Funct.* **8**, 49–56.

Kovács, P., Csaba, G., Nagao, S., and Nozawa, Y. (1989). The regulatory role of calmodulin-dependent guanylate cyclase in association with hormonal imprinting in *Tetrahymena. Microbios* **59**, 123–128.

Kovács, P., Csaba, G., Ito, Y., and Nozawa, Y. (1996). Effect of insulin on the phospholipase-D activity of untreated and insulin-pretreated (hormonally imprinted) *Tetrahymena. Biochem. Biophys. Res. Commun.* **222**, 359–361.

Kristiansen, T. B., Hagemeister, J. J., Grave, M., and Hellung-Larsen, P. (1996). Surface mediated death of unconditioned *Tetrahymena* cells: Effect of physical parameters, growth factors, hormones and surfactants. *J. Cell. Physiol.* **169**, 139–148.

Kuo, W. N., Jones, D. L., Ku, T. W., Weeks, K. D., Jordon, P. M., and Dopson, N. C. (1995a). Immunoreactivity of PKC gammalambda and RACK 1 in baker's yeast, lobster and wheat germ. *Biochem. Mol. Biol. Int.* **36**, 957–963.

Kuo, W. N., Ku, T. W., Jones, D. L., Jn-Baptiste, J. B., and Bradshaw, C. J. (1995b). Immunoreactivities of PKC delta, eta, zeta in baker's yeast. *Biochem. Mol. Biol. Int.* **37**, 423–430.

Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* **260**, 315–319.

Leick, V. (1992). Chemotactic properties, cellular binding and uptake of peptide derivates: Studies with *Tetrahymena thermophila. J. Cell Sci.* **103**, 565–570.

Leick, V., and Hellung-Larsen, P. (1985). Chemosensory responses in *Tetrahymena:* The involvement of peptides and other signal substances. *J. Protozool.* **32**, 550–553.

Leick, V., Koppelhus, U., and Rosenberg, J. (1995). Cilia-mediated oriented chemokinesis in *Tetrahymena thermophila. J. Euk. Microbiol.* **41**, 546–553.

Leick, V., Bøg-Hansen, T., Christensen, S. T., and Kaufman, S. (1996a). Concanavalin A receptors and the chemosensory behaviour of *Tetrahymena thermophila. Exp. Biol. Online* **1**, 5.

Leick, V., Grave, M., and Hellung-Larsen, P. (1996b). Signal peptide-induced sensory behaviour in free ciliates: Bioassays and cellular mechanisms. In "Progress in Molecular and Subcellular Biology. Signaling Mechanisms in Protozoa and Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 61–79. Springer-Vellag, Berlin/New York.

Lenard, J. (1992). Mammalian hormones in microbial cells. *Trends Biochem. Sci.* **17,** 147–150.

LeRoith, D., Shiloach, J., Roth, J., and Lesniak, M. A. (1980). Evolutionary origins of vertebrate hormones: Substances similar to mammalian insulins are native to unicellular organisms. *Proc. Natl. Acad. Sci. USA* **77,** 6184–6188.

LeRoith, D., Liotta, A. S., Roth, J., Shiloach, J., Lewis, M. E., Pert, C. B., and Krieger, D. T. (1982). Corticotrophin and beta-endorphin-like material are native to unicellular organisms. *Proc. Natl. Acad. Sci. USA* **79,** 2086–2090.

LeRoith, D., Roberts, C., Jr., Lesniak, M.A., and Roth, J. (1987). Receptors for intercellular messenger molecules in microbes: Similarities to vertebrate receptors and possible implications for diseases in man. In "Development of Hormone Receptors" (G. Csaba, ed.), pp. 167–180. Birkhäuser, Basel.

Levandowsky, M., and Hauser, D. C. R. (1978). Chemosensory responses of swimming algae and protozoa. *Int. Rev. Cytol.* **53,** 145–210.

Levandowsky, M., Cheng, T., Kehr, A., Kim, J., Gardner, L., Tsang, L., Lai, G., Chung, C., and Prakash, E. (1984). Chemosensory responses to amino acids and certain amines by the ciliate *Tetrahymena:* A flat capillary assay. *Biol. Bull.* **167,** 322–330.

Levin, D. E., and Errede, B. (1995). The proliferation of MAP kinase signaling pathways in yeast. *Curr. Opin. Cell Biol.* **7,** 197–202.

Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1990). A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. *Cell* **6,** 213–224.

Levy, M. R., and Elliot, A. M. (1968). Biochemical and ultrastructural changes in *Tetrahymena pyriformis* during starvation. *J. Protozool.* **15,** 208–222.

Lilly, D. M., and Stillwell, R. H. (1965). Probiotics: Growth-promoting factors produced by microorganisms. *Science* **147,** 747–748.

Liscovitch, M., and Cantley, L. C. (1994). Lipid second messengers. *Cell* **77,** 329–334.

Liu, G., and Newell, P. C. (1994). Regulation of myosin regulatory light chain phosphorylation via cyclic GMP during chemotaxis of *Dictyostium. J. Cell Sci.* **107,** 1737–1743.

Loomis, W. F. (ed.) (1982). "Development of *Dictyostelium discoideum.*" Academic Press, New York.

Loomis, W. F. (1987). Genetic tools for *Dictyostelium.* In "Methods in Cell Biology" (J. A. Spudich, ed.), Vol. 28, pp. 39–40. Academic Press, New York.

Losick, R., and Kaiser, D. (1997). Why and how bacteria communicate. *Sci. Am.* **276 (Feb.),** 52–57.

Lucas, R., Magez, S., De Leys, R., Fransen, L., Scheelinck, J. P., Rampelberg, E. S., and De Baetselier, P. (1994). Mapping the lectin-like activity of tumour necrosis factor. *Science* **263,** 814–817.

Luginbühl, P., Ottiger, M., Mronga, S., and Wüthrich, K. (1994). Structure comparison of the pheromones E*r*-1, E*r*-10 and E*r*-2 from *Euplotes raikovi. Protein Sci.* **3,** 1537–1547.

Luo, D., Das, S., and Vincent, S. R. (1995). Effects of methylene blue and LY83583 on neuronal nitric oxide synthase and NADPH-diaphorase. *Eur. J. Pharmacol.* **290,** 247–251.

Luporini, P., and Miceli, C. (1986). Mating pheromones. In "Molecular Biology of Ciliated Protozoa" (J. G. Gall, ed.), pp. 263–299. Academic Press, New York.

Luporini, P., Miceli, C., Ortenzi, C., and Vallesi, A. (1992). Development analysis of the cell recognition mechanism in the ciliate *Euplotes raikovi. Dev. Genet.* **13,** 9–15.

Luporini, P., Vallesi, A., Miceli, C., and Bradshaw, R. A. (1994). Ciliate pheromones as early growth factors and cytokines. *Ann. NY Acad. Sci.* **712,** 195–205.

Luporini, P., Miceli, C., Ortenzi, C., and Vallesi, A. (1996). Ciliate pheromones. In "Progress in Molecular and Subcellular Biology. Signaling Mechanisms in Protozoa and Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 80–104. Springer-Verlag, Berlin/New York.

Lynch, S. E., Nixon, J. C., Colvin, R. B., and Antoniades, H. N. (1987). Role of platelet-derived growth factor in wound healing: Synergistic effects with other growth factors. *Proc. Natl. Acad. Sci. USA* **84,** 7696–7700.

Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996). Seven helix chemoattractant receptors transiently stimulate mitogen-activated protein kinase in *Dictyostelium. J. Biol. Chem.* **271,** 3351–3354.

Maghazachi, A. A. (1992). Cholera toxin inhibits interleukin-2-induced, but enhances pertussis toxin-induced T-cell proliferation: Regulation by cyclic nucleotides. *Immunology* **75,** 103–107.

Maihle, N. J., and Satir, B. H. (1985). Protein secretion in *Tetrahymena thermophila:* Characterization of the secretory mutant strain SB 281. *J. Cell Sci.* **78,** 49–65.

Marín, J., and Govantes, C. (1995). Angiotensin-converting enzyme inhibitors and the role of nitric oxide and excitatory amino acids in improvement of cognition and memory. *J. Auton. Pharmacol.* **15,** 129–149.

Marshall, C. J. (1996). Raf gets it together. *Nature* **383,** 127–128.

Martin, W., Villani, G. M., Jothianandan, D., and Furchgott, R. F. (1985). Selective blockade of endothelium-dependent and glycerol trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.* **232,** 708–716.

Massagué, J. (1990). Transforming growth factor-alpha. A model for membrane-anchored growth factors. *J. Biol. Chem.* **265,** 21393–21396.

Matsuda, N., Lin, W.-L., Kumar, N. M., Cho, M. I., and Genco, R. J. (1992). Mitogenic, chemotactic, and synthetic responses of rat peridontal ligament fibroblastic cells to polypeptide growth factors in vitro. *J. Periodontol.* **63,** 515–525.

Matthews, K. R., and Gull, K. (1994). Cycles within cycles—The interplay between differentiation and cell division in *Trypanosoma brucei. Parasitol. Today* **10,** 473–476.

Maudlin, I., and Welburn, S. C. (1987). Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina* morsitans. *Trop.Med. Parasitol.* **38,** 167–170.

Mayer, B., Brunner, F., and Schmidt, K. (1993). Inhibition of nitric oxide synthesis by methylene blue. *Biochem. Pharmacol.* **45,** 367–374.

Mehdy, M. C., and Firtel, R. A. (1985). A secreted factor and cAMP jointly regulate cell-type specific gene expression. *Mol. Cell. Biol.* **5,** 705–713.

Menuelle, P., Plas, C., and Taketomi, S. (1989). Effects of modified insulin B21–B26 fragments on glycogenesis and on insulin-receptor complex fate in cultured fetal hepatocytes. *Mol. Cell. Endocrinol.* **66,** 143–151.

Miceli, C., Concetti, A., and Luporini, P. (1983). Isolation of the mating inducing factor of the ciliate *Euplotes. Exp. Cell Res.* **149,** 593–598.

Miceli, C., La Terza, A., Bradshaw, R. A., and Luporini, P. (1991). Structural characterization of mating pheromone precursors of the ciliate protozoan *Euplotes raikovi. Eur. J. Biochem.* **202,** 759–764.

Miceli, C., La Terza, A., Bradshaw, R. A., and Luporini, P. (1992). Identification and structural characterization of a cDNA clone encoding a membrane-bound form of the polypeptide pheromone Er-1 in the ciliate protozoan *Euplotes raikovi. Proc. Natl. Acad. Sci. USA* **89,** 1988–1992.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* **266,** 1719–1723.

Mirmira, R. G., and Tager, H. S. (1989). Role of the phenylalanine B24 side chain in directing insulin intercations with its receptor. *J. Biol. Chem.* **264,** 6349–6354.

Mirmira, R. G., Nakagawa, S. H., and Tager, H. S. (1991). Importance of the character and configuration of residues B24, B25 and B26 in insulin–receptor interactions. *J. Biol. Chem.* **266,** 1428–1436.

Mirsky, N., and Berdicevski, I. (1994). Effects of insulin and glucose tolerance factor on glucose uptake by yeast cells. *Biol. Signals* **3,** 271–277.

Mitev, V., Le Panse, R., Coulomb, B., Miteva, L., and Houdebine, L.-M. (1995). Epidermal growth factor stimulates mitogen-activated protein kinase by a PKC-dependent pathway in human keratinocytes. *Biochem. Biophys. Res. Commun.* **208,** 245–252.

Miyake, A. (1981). Cell interaction by gamones in Blepharisma. In "Sexual Interactions in Eukaryotic Microbes" (D. H. O'Day and P. A. Horgen, eds.), pp. 95–129. Academic Press, New York.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: Physiology, patophysiology and pharmacology. *Pharmacol. Rev.* **4,** 109–142.

Mordret, G. (1993). MAP kinase kinase: A node connecting multiple pathways. *Biol. Cell* **79,** 193–207.

Moreira, M. E. C., Del Portillo, H. A., Milder, R. V., Balanco, J. M. F., and Barcinski, M. A. (1996). Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania (Leishmania) amazonensis. J. Cell. Physiol.* **167,** 305–313.

Morimoto, B. H., and Koshland, D. E., Jr. (1991). Short-term and long-term memory in single cells. *FASEB J.* **5,** 2061–2067.

Morris, H. R., Taylor, G. W., Masento, M. S., Jermyn, K. A., and Kay, R. R. (1987). Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum. Nature* **328,** 811–814.

Motizuki, M., Yokota, S., and Tsurugi, K. (1995). Autophagic death after cell cycle arrest at the restrictive temperature in temperature-sensitive cell division cycle and secretory mutants of the yeast *Saccharomyces cerevisiae. Eur. J. Cell Biol.* **68,** 275–287.

Mpoke, S., and Wolfe, J. (1996). DNA digestion and chromatin condensation during nuclear death in *Tetrahymena. Exp. Cell Res.* **225,** 357–365.

Mulsch, A., Busse, R., Liebau, S., and Forstermann, U. (1988). LY83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.* **247,** 283–288.

Murofushi, H. (1973). Purification and characterization of a protein kinase in *Tetrahymena thermophila. Biochem. Biophys. Acta* **327,** 354–364.

Murofushi, H. (1974). Protein kinases in *Tetrahymena* cilia. II. Partial purification and characterization of adenosine 3'5'-monophosphate-dependent and guanosine 3'5'-monophosphate-dependent protein kinases. *Biochem. Biophys. Acta* **370,** 130–139.

Mutzel, R. (1995). Molecular biology, growth and development of the cellular slime mold *Dictyostelium discoideum. Experientia* **51,** 1103–1109.

Nagawa, S. H., and Tager, H. S. (1987). Role of the COOH-terminal B-chain domain in insulin–receptor interactions. *J. Biol. Chem.* **262,** 12,054–12,058.

Nanney, D. L., and McCoy, J. W. (1976). Characterization of species of the *Tetrahymena pyriformis* complex. *Trans. Am. Microsc. Soc.* **95,** 664–682.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051–3064.

Newell, P. C., Malchow, D., and Gross, J. D. (1995). The role of calcium in aggregation and development of *Dictyostelium. Experientia* **51,** 1155–1165.

Ng, F. M., Zhu, S. Q., Cui, D. F., Fan, L., Huang, Y. D., and Zhang, Y. S. (1989). Structure and activity of the B-chain of insulin. *Biochem. Int.* **18,** 373–381.

Nielsen, O., and Davey, J. (1995). Pheromone communication in the fission yeast *Schizosaccharomyces pombe. Semin. Cell Biol.* **6,** 95–104.

Nilsson, J. R. (1970a). Cytolysosomes in *Tetrahymena pyriformis* GL. I. Synchronized cells dividing in inorganic salt medium. *C. R. Trav. Lab. Carlsberg* **38,** 87–106.

Nilsson, J. R. (1970b). Cytolysosomes in *Tetrahymena pyriformis* GL. II. Reversible degeneration. *C. R. Trav. Lab. Carlsberg* **38,** 107–121.

Nilsson, J. R. (1976). Physiological and structural studies on *Tetrahymena pyriformis* GL. With special reference to endocytosis and to structural changes during the cell cycle and in response to alterations in the cellular environment. *C. R. Trav. Lab. Carlsberg* **40,** 215–355.

Nilsson, J. R., and Leick, V. (1970). Nucleolar organization and ribosome formation in *Tetrahymena pyriformis* GL. *Exp. Cell Res.* **60,** 361–372.

Nishizawa, N. K., Mori, S., Watanabe, Y., and Hirano, H. (1994). Ultrastructural localization of the basic 7S globulin in soybean (*Glycine max*) cotyledons. *Plant Cell Physiol.* **35,** 1079–1085.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258,** 607–614.

Nishizuka, Y. (1995). Protein kinase C and lipid signalling for sustained cellular responses. *FASEB J.* **9,** 484–496.

Noegel, A. A., and Luna, J. E. (1995). The *Dictyostelium* cytoskeleton. *Experientia* **51,** 1135–1143.

Nogueira, N., Bionco, C., and Cohn, Z. (1975). Studies on the selective lysis and purification of *T. cruzi. J. Exp. Med.* **142,** 225–229.

Ogita, K., Miyamoto, S.-I., Koide, H., Iwai, T., Oka, M., Ando, K., Kishimoto, A., Ikeda, K., Fukami, Y., and Nishizuka, Y. (1990). Protein kinase C in *Saccharomyces cerevisiae:* Comparison with the mammalian enzyme. *Proc. Natl. Acad. Sci. USA* **87,** 5011–5015.

Oh, S. (1995). The generation of nitric oxide and its roles in neurotransmission and neurotoxicity. *Keio J. Med.* **44,** 53–61.

Oishi, K., Raynor, R. L., Charp, P. A., and Kuo, J. F. (1988). Regulation of protein kinase C by lysolipids: Potential role for signal transduction. *J. Biol. Chem.* **263,** 6865–6971.

Olsson, T., Bakheit, M., and Kristensson, K. (1992). Interactions between *Trypanosoma brucei* and $CD8^+$ T cells. *Parasitol. Today* **79,** 202–205.

O'Neill, J. B., Pert, C. B., Ruff, M. R., Smith, C. C., Higgins, W. J., and Zipser, B. (1988). Identification and characterization of the opiate receptor in the ciliated protozoan, *Tetrahymena. Brain Res.* **450,** 303–315.

Orias, E. (1986). Ciliate conjugation. In "The Molecular Biology of Ciliated Protozoa" (J. G. Gall, ed.), pp. 45–84. Academic Press, San Diego.

Orias, E., and Bruns, P. J. (1976). Induction and isolation of mutants in *Tetrahymena. In* "Methods in Cell Biology" (D. M. Prescott, ed.), pp. 263–300. Academic Press, New York.

Orias, E., and Rasmussen, L. (1976). Dual capacity for nutrient uptake in *Tetrahymena.* IV. Growth without food vacuoles and its implications. *Exp. Cell Res.* **102,** 127–137.

Ortenzi, D., Miceli, C., Bradshaw, R. A., and Luporini, P. (1990). Identification and initial characterization of an autocrine pheromone receptor in the protozoan ciliate *Euplotes raikovi. J. Cell Biol.* **111,** 607–614.

Overgaard, A. K., Friis, J., Christensen, L., Christiansen, H., and Rasmussen, L. (1995). Effects of glucose, tetrapyrroles and protein kinase C activators on cell proliferation in cultures of *Saccharomyces cerevisiae. FEMS Microbiol. Lett.* **132,** 159–163.

Overgaard, A. K., Kierklo, H., Christiansen, P., Chrois, L., Rasmussen, L., and Friis, J. (1997). Mechanisms involved in the transition from the lag phase to the cell division cycle in *Saccharomyces cerevisiae.* Possible involvement of PKA, PKC and PKG. Submitted for publication.

Pandey, S., and Wang, E. (1995). Cells en route to apoptosis are characterized by the upregulation of *c-fos, c-myc, c-jun, cdc2,* and RB phosphorylation, resembling events of early cell-cycle traverse. *J. Cell. Biochem.* **58,** 135–150.

Parfenova, E. V., Afon'kin, S. Y., Yodin, A. L., and Etingof, R. N. (1989). Characterization and partial purification of mating pheromone excreted by mating type II cells of the ciliate *Dileptus anser. Acta Protozool.* **28,** 11–21.

Park, D. S., Stefanis, L., Yan, C. Y. I., Farinelli, S. E., and Greene, L. A. (1996). Ordering the cell death pathway. Differential effects of BCL2, an interleukin-1-converting enzyme

family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth factor-deprived PC12 cells. *J. Biol. Chem.* **271,** 21898–21905.

Parkinson, J. S. (1993). Signal transduction schemes in bacteria. *Cell* **73,** 857–871.

Parsons, M., Valentine, M., Deans, J., Schieven, G. L., and Ledbetter, J. A. (1991). Distinct patterns of tyrosine phosphorylation during the life cycle of *Trypanosoma brucei. Mol. Biochem. Parasitol.* **45,** 241–248.

Parsons, M., Valentine, M., and Carter, V. (1993). Protein kinases in divergent eukaryotes: Identification of protein kinase activities regulated during trypanosome development. *Proc. Natl. Acad. Sci. USA* **90,** 2656–2660.

Partanen, J., Vainikka, S., Korhonen, J., Armstrong, E., and Alitalo, K. (1992). Diverse receptors for fibroblast growth factors. *Prog. Growth Factor Res.* **4,** 69–83.

Pasternak, C., Flicker, P. F., Ravid, S., and Spudich, J. A. (1989). Intermolecular versus intramolecular interactions of *Dictyostelium* myosin: Possible regulation by heavy chain phosphorylation. *J. Cell Biol.* **109,** 203–210.

Paveto, C., Pereira, C., Espinosa, J., Montagna, A. E., Farber, M., Esteva, M., Flawiá, M. M., and Torres, H. N. (1995). The nitric oxide pathway in *Trypanosoma cruzi. J. Biol. Chem.* **270,** 16,576–16,579.

Pepe, G. J., and Albrecht, E. D. (1995). Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr. Rev.* **16,** 608–648.

Pertseva, M. (1991). The evolution of hormonal signalling. *Comp. Biochem. Physiol. A* **100,** 775–785.

Raff, M. C. (1992). Social controls on cell survival and cell death. *Nature* **356,** 397–400.

Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y., and Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: Lessons from the nervous system. *Science* **262,** 695–700.

Raffioni, S., Miceli, C., Concetti, A., Barra, D., and Luporini, P. (1987). Purification and characterization of new mating pheromones of the ciliate *Euplotes raikovi. Exp. Cell Res.* **172,** 417–424.

Raffioni, S., Luporini, P., Chait, B. T., Disper, S. S., and Bradshaw, R. A. (1988). The primary structure of the mating pheromone Er-1 of the ciliate *Euplotes raikovi. J. Biol. Chem.* **263,** 18, 152–18, 159.

Raffioni, S., Luporini, P., and Bradshaw, R. A. (1989). Purification, characterization and amino acid sequence of the mating pheromone Er-10 of the ciliate *Euplotes raikovi. Biochemistry* **28,** 5250–5256.

Raffioni, S., Miceli, C., Vallesi, A., Chowdhury, S. K., Chait, B. T., Luporini, P., and Bradshaw, R. A. (1992). Primary structure of *Euplotes raikovi* pheromones: Comparison of five sequences of pheromones with variable mating interactions. *Proc. Natl. Acad. Sci. USA* **89,** 2071–2075.

Rahmsdorf, H. J., Malchow, D., and Gerisch, G. (1978). Cyclic AMP-induced phosphorylation in *Dictyostelium* of a polypeptide comigrating with myosin heavy chain. *FEBS Lett.* **88,** 322–326.

Rahti, A., and Clarke, M. (1992). Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine-dependent mechanism. *Mech. Dev.* **36,** 173–182.

Rahti, A., Kayman, S. C., and Clarke, M. (1991). Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. *Dev. Genet.* **12,** 82–87.

Ralston, S. H., Todd, D., Helfrich, M., Benjamin, N., and Grabowski, P. S. (1994). Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. *Endocrinology* **135,** 330–336.

Randriamampita, C., Ciapi, B., and Trautmann, A. (1991). Cyclic GMP-dependent refilling of calcium stores in macrophages. *Eur. J. Physiol.* **417,** 633–637.

Rasmussen, L., and Dive, D. (1980). Phospholipids and cell generation times in cultures of *Tetrahymena. Naturwissenschaften* **67,** 98.

Rasmussen, L., and Modeweg-Hansen, L. (1973). Cell multiplication in *Tetrahymena* cultures after addition of particulate materials. *J. Cell Sci.* **12,** 275–286.

Rasmussen, L., Christensen, S. T., Schousboe, P., and Wheatley, D. N. (1996). Cell survival and multiplication. The overriding need for signals: From unicellular to multicellular systems. *FEMS Microbiol. Lett.* **137,** 123–128.

Ravid, S., and Spudich, J. A. (1989). Myosin heavy chain kinase from developed Dictyostelium cells. Purification and characterization. *J. Biol. Chem.* **264,** 15144–15150.

Ravid, S., and Spudich, J. A. (1992). Membrane-bound *Dictyostelium* myosin heavy chain kinase: A developmentally regulated substrate-specific member of the protein kinase C family. *Proc. Natl. Acad. Sci. USA* **89,** 5877–5881.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124,** 1–6.

Renaud, F. L., Chiesa, R., Rodríguez, F., Tomassini, N., and Marino, M. (1996). Studies on the opioid mechanism in *Tetrahymena.* In "Progress in Molecular and Subcellular Biology. Signaling Mechanisms in Protozoa and Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 29–39. Springer-Verlag, Berlin/New York.

Reymond, C. D., Schaap, P., Véron, M., and Williams, J.G. (1995). Dual role of cAMP during *Dictyostelium* development. *Experientia* **51,** 1166–1174.

Robbins, S. M., Williams, J. G., Jermyn, K. A., Spiegelman, G. B., and Weeks, G. (1989). Growing and developing *Dictyostelium* cells express different ras genes. *Proc. Natl. Acad. Sci. USA* **86,** 938–942.

Robertson, T. B. (1924). CLXII. Allellocatalytic effect in cultures of *Colpodium* in hay-infusion and in synthetic media. *Biochem. J.* **18,** 1240–1247.

Rönnstrand, L., Mori, S., Arvidsson, A. K., Eriksson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L., and Heldin, C.-H. (1992). Identification of two C-terminal autophosphorylation sites in the PDGF beta-receptor: Involvement in the interaction with phospholipase Cgamma. *EMBO J.* **11,** 3911–3919.

Russo, F. D., and Silhavy, T. J. (1993). The essential tension: Opposed reactions in bacterial two-component regulatory systems. *Trends Microbiol.* **1,** 306–310.

Sabit, I., Couldwell, W. T., Hinton, D. R., He, S., Weiss, M. H., and Law, R. (1994). Induction of apoptosis (programmed cell death) in human established glioma cell line by protein kinase C inhibitors. *Clin. Res.* **42,** 115–120.

Salamán, A., Román, M., Renaud, F. L., and Silva, W. I. (1990). Effect of chronic opioid treatment on phagocytosis in *Tetrahymena. Neuropeptides* **16,** 115–120.

Salesse, R., Garnier, J., and Daveloose, D. (1982). Modulation of adenylate cyclase activity by the physical state of pigeon erythrocyte membrane. II. Fluidity controlled coupling between the subunits of the adenylate cyclase system. *Biochemistry* **21,** 1587–1590.

Sanchez, V., Lucas, M., Sanz, A., and Goberna, R. (1992). Decreased protein kinase C activity is associated with programmed cell death (apoptosis) in freshly isolated rat hepatocytes. *Biosci. Rep.* **12,** 199–206.

Sano, M., and Kitajima, S. (1992). Activation of microtubule-associated protein kinase in PC12D cells in response to both fibroblast growth factor and epidermal growth factor and concomittant stimulation of the outgrowth of neurites. *J. Neurochem.* **58,** 837–844.

Sato, T., Hanada, M., Bodrug, S. Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H.-G., and Reed, J. C. (1994). Interactions among members of the bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. USA* **91,** 9238–9242.

Saunders, D. J., Brandenburg, D., Shang-chuan, C., and Chih-chen, W. (1982). Insulin chemistry—Pathway to understand insulin action. *Endeavour* **6,** 146–152.

Schatzle, J., Rathi, A., Clarke, M., and Cardelli, J.A. (1991). Developmental regulation of the alpha-mannosidase gene in *Dictyostelium discoideum*—Control is at the level of transcription and is affected by cell density. *Mol. Cell. Biol.* **11,** 3339–3347.

Schaub, G. A. (1994). Pathogenicity of Trypanosomatids on insects. *Parasitol. Today* **10,** 463–468.

Schmidt, M. J., Sawyer, B. D., Truex, L. L., Marshall, W. S., and Fleish, J. H. (1985). LY83583: An agent that lowers intracellular levels of cyclic guanosine 3′5′-monophosphate. *J. Pharmacol. Exp. Ther.* **232,** 764–769.

Schousboe, P., and Rasmussen, L. (1994). Survival of *Tetrahymena thermophila* at low initial cell densities. Effects of lipids and long-chained alcohols. *J. Euk. Microbiol.* **41,** 195–199.

Schousboe, P., Christensen, S. T., Ghiladi, M., and Rasmussen, L. (1992). Porphyrin rings and phospholipids: Stimulators of cloning efficiency in certain species of *Tetrahymena. J. Protozool.* **39,** 343–345.

Schousboe, P., Wheatley, D. N., and Rasmussen, L. (1997). Autocrine/paracrine activators and inhibitors of cell proliferation: Purification of a 7–8 kDa compound with growth factor-like effects in *Tetrahymena thermophila.* Submitted for publication.

Schultz, J. E., and Klumpp, S. (1984). Calcium/calmodulin-regulated guanylate cyclase in the ciliary membranes of *Paramecium* and *Tetrahymena. Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17,** 275–283.

Schultz, J. E., and Klumpp, S. (1994). Cyclic GMP in lower forms. *Adv. Pharmacol.* **26,** 285–303.

Schwartz, L. M., Kosz, L., and Kay, B. K. (1990). Gene actiivation is required for developmentally programmed cell death. *Proc. Natl. Acad. Sci. USA* **87,** 6594–6598.

Schwartz, L. M., Smith, S. W., Jones, M. E. E., and Osborne, B. A. (1993). Do all programmed cell deaths occur via apoptosis? *Proc. Natl. Acad. Sci. USA* **90,** 980–984.

Segall, J., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R., and Loomis, W. (1995). A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium. J. Cell Biol.* **128,** 405–413.

Sengeløv, H., Boulay F., Kjeldsen, L., and Borregaard, N. (1994). Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem. J.* **299,** 473–479.

Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton, R. T., and Thanassi, N. M. (1976). Regulation of guanylate and adenylate cyclase activities by lysolecithin. *Proc. Natl. Acad. Sci. USA* **73,** 1586–1590.

Shiloach, J., Rubinovitz, C., and LeRoith, D. (1985). Extraction of insulin-related material and other peptide-hormones from *Tetrahymena pyriformis. ACS Symp. Ser.* **271,** 175–191.

Simon, A. J., Milner, Y., Saville, S. P., Dvir, A., Mochley-Rosen, D., and Orr, E. (1991). The identification and purification of a mammalian-like protein kinase C in the yeast *Saccharomyces cerevisiae. Proc. R. Soc. Lond. B* **243,** 165–171.

Smeyne, R. J., Vendrel, M., Hayward, M., Baker, S. J., Mlao, G. G., Schilling, K., Robertson, L. M., Curran, T., and Morgan, J. I. (1993). Continuous c-fos expression preceeds programmed cell death in vivo. *Nature* **363,** 166–169.

Smith-Somerville, H. E., and Buhse, H. E., Jr. (1984). Changes in the oral apparatus structure accompanying vacuolar formation in the macrostomal form of *Tetrahymena vorax.* A model for the formation of food vacuoles in *Tetrahymena. J. Protozool.* **31,** 373–380.

Sözeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G. E., III, and Stabel, S. (1992). Activation of the c-Raf protein kinase by protein kinase C phosphorylation. *Oncogene* **7,** 2259–2262.

Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science* **258,** 1898–1902.

Stein, J. M., Kornberg, H. L., and Martin, B. R. (1985). Effects of GTP, GDP (βS) and glucose on adenylate cyclase activity in *E. coli* B. *FEBS Lett.* **182,** 429–434.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267,** 1445–1449.

Sternberg, J. M., and McGuigan, F. (1994). *Trypanosoma brucei:* Mammalian epidermal growth factor promotes the growth of the african trypanosome bloodstream form. *Exp. Parasitol.* **78,** 422–424.

Stewart, A. E., Raffioni, S., Chaudhary, T., Chait, B. T., Luporini, P., and Bradshaw, R. A. (1992). The disulfide bond pairing of the pheromones Er-1 and Er-2 of the ciliated protozoan *Euplotes raikovi. Protein Sci.* **1,** 777–785.
Stock, J. B., Sock, A. M., and Mottonen, J. M. (1990). Signal transduction in bacteria. *Nature* **344,** 395–400.
Straarup, E. M., Schousboe, P., Christensen, S. T., Quie, H., Kristiansen, K., Hoffmann, E., and Rasmussen, L. (1997). Effects of protein kinase C activators and staurosporine on cell survival and protein kinase activity in *Tetrahymena thermophila.* Submitted for publication.
Swift, S., Bainton, N. J., and Winson, M. K. (1994). Gram-negative bacterial communication by N-acyl homoserine lactones: A universal language? *Trends Microbiol.* **2,** 193–198.
Swift, S., Throup, J. P., Williams, P., Salmond, G. P. C., and Stewart, G. S. A. B. (1996). Quorum sensing: A population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* **21,** 214–219.
Szablewski, L., Andresen, P. H., Florin-Christensen, J., Florin-Christensen, M., and Rasmussen, L. (1991). *Tetrahymena thermophila:* Growth in synthetic medium in the presence and in the absence of glucose. *J. Protozool.* **38,** 62–65.
Takagi, Y., Suzuki, T., and Shimada, C. (1987). Isolation of a *Paramecium tetraurelia* mutant with short clonal life-span and with novel life-cycle features. *Zool. Sci.* **4,** 73–80.
Takagi, Y., Nimura, K., Kinoshita, H., Yamada, T., Kaji, K., and Tanabe, H. (1989). Identification of a gene that shortens clonal life span of *Paramecium tetraurelia. Genetics* **123,** 749–754.
Takagi, Y., Nimura, K., Tokusumi, Y., Fujisawa, H., Kaji, K., and Tanabe, H. (1993). Secretion of mitogenic factor(s) from stocks of *Paramecium tetraurelia, P. caudatum* and *P. multimicronucleatum. Zool. Sci.* **10,** 53–56.
Tanabe, H., Nishi, N., Takagi, Y., Nada, F., Ahatmatsu, I., and Kaji, K. (1990). Purification and identification of a growth factor by *Paramecium tetraurelia. Biochem. Biophys. Res. Commun.* **170,** 786–792.
Tao, Y., Howlett, A., and Klein, C. (1992a). Nitric oxide-releasing compounds inhibit *Dictyostelium discoideum* aggregation without altering cGMP production. *FEBS Lett.* **314,** 49–52.
Tao, Y., Howlett, A., and Klein, C. (1992b). Nitric oxide stimulates the ADP-ribosylation of a 41-kDa cytosolic protein in *Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA* **89,** 5902–5906.
Thompson, H. L., Shiroo, M., and Saklatvala, J. (1993). The chemotactic factor N-formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein 2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leucocytes. *Biochem. J.* **290,** 483–488.
Tiedtke, A., Hünseler, P., and Rasmussen, L. (1988). Growth requirements of a new food-vacuole-less mutant of *Tetrahymena. Eur. J. Protistol.* **23,** 350–353.
Tippmer, S., Quitterer, U., Kolm, V., Faussner, A., Rosher, A., Mosthaf, L., Müller-Estnerl, W., and Häring, H. (1994). Bradykinin induces translocation of the protein kinase C isoforms alpha, epsilon, and zeta. *Eur. J. Biochem.* **225,** 297–304.
Tokusumi, Y., Nishi, N., and Takagi, Y. (1996). A substance secreted from *Tetrahymena* and mammalian sera acts as mitogens on *Paramecium tetraurelia. Zool. Sci.* **13,** 89–96.
Town, C. D., Gross, J. D., and Kay, R. R. (1976). Cell differentiation without morphogenesis in *Dictyostelium discoideum. Nature* **119,** 171–174.
Tsai, S. C., Adamik, R., Manganiello, V. C., and Vaughan, M. (1983). Regulation of activity of purified guanylate cyclase from liver that is unresponsive to nitric oxide. *Biochem. J.* **215,** 447–455.
Tschan, T., Hoerler, I., Houtze, Y., Winterhalter, K. H., Richter, C., and Bruckner, P. (1990). Resting chondrocytes in culture survive without growth factors, but are sensitive to toxic oxygen metabolites. *J. Cell Biol.* **111,** 257–260.
Umeki, S., and Nozawa, Y. (1996). Adenylate and guanylate cyclase in *Tetrahymena.* In "Progress in Molecular and Subcellular Biology. Signalling Mechanisms in Protozoa and

Invertebrates" (G. Csaba and W. E. G. Müller, eds.), Vol. 17, pp. 40–60. Springer-Verlag, New York.

Vallesi, A., Giovanni, G., Bradshaw, R. A., and Luporini, P. (1995). Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi. Nature* **376,** 522–524.

Van Aelst, L., Boy-Marcotte, E., Camonis, J. H., Thevelein, J. M., and Jacquet, M. (1990). The C-terminal part of the *CDC25* gene product plays a key role in signal transduction in the glucose-induced modulation of cAMP level in *Saccharomyces cerevisiae. Eur. J. Biochem.* **193,** 675–680.

Van den Bosch, H. B. (1980). Intracellular phospholipases A. *Biochim. Biophys. Acta* **604,** 191–246.

Van Haastert, P. J. M. (1987). Down regulation of cell surface cyclic AMP receptors and desensitization of cyclic AMP-stimulated adenylate cyclase by cyclic AMP in *Dictyostelium discoideum. J. Biol. Chem.* **262,** 7700–7704.

Van Haastert, P. J. M. (1991). Transmembrane signal transduction pathways in *Dictyostelium.* In "Advances in Second Messenger and Phosphoprotein Research" (P. Greegard and G. A. Robison, eds.), pp. 185–226. Academic Press, New York.

Van Haastert, P. J. M. (1995). Transduction of the chemotactic cAMP signal cross the plasma membrane of *Dictyostelium* cells. *Experientia* **51,** 1144–1154.

Van Haastert, P. J. M., Bishop, J. D., and Gomer, R. H. (1996). The cell density factor CMF regulates the chemoattractant receptor cAR1 in *Dictyostelium. J. Cell Biol.* **134,** 1543–1549.

Vaux, D. L. (1993). Toward an understanding of the molecular mechanisms of physiological cell death. *Proc. Natl. Acad. Sci. USA* **90,** 786–789.

Vaux, D. L., Haecker, G., and Strasser, A. (1994). An evolutionary perspective on apoptosis. *Cell* **76,** 777–779.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *Br. Med. Bull.* **41,** 105–114.

Wagner, A. J., Small, M. B., and Hay, N. (1993). Myc-mediated apoptosis is blocked by ectopic expression of Bcl-2. *Mol. Cell. Biol.* **13,** 2432–2440.

Wagner, A. J., Kokontis, J. M., and Hay, N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce $p21^{waf1/cip1}$. *Genes Dev.* **8,** 2817–2830.

Wang, A.-M. (1996). "Investigation of the "Signalling" Requirements for *Escherichia coli.*" Thesis, Univ. Aberdeen. M.Sc.

Ward, J. G., and Herrick, G. (1996). Effects of the transcription inhibitor actinomycin D on postzygotic development of *Tetrahymena thermophila* conjugants. *Dev. Biol.* **173,** 174–184.

Watanabe, M., Chen, C. Y., and Levin, D. E. (1994a). *Saccharomyces cerevisiae* PKC1 encodes a protein kinase C (PKC) homolog with a substrate specificity similar to that of mammalian PKC. *J. Biol. Chem.* **269,** 16829–16836.

Watanabe, Y., Barbashov, S. F., Komatsu, S., Hemmings, A. M., Miyagi, M., Tsunasawa, S., and Hirano, H. (1994b). A peptide that stimulates phosphorylation of the plant insulin-binding protein. Isolation, primary structure and cDNA cloning. *Eur. J. Biochem.* **224,** 167–172.

Weiske-Benner, A., and Eckert, W. A. (1987). Differentiation of nuclear structure during the sexual cycle in *Tetrahymena thermophila.* II. Degeneration and autolysis of macro- and micronuclei. *Differentiation* **34,** 1–12.

Weitzel, G., Eisele, K., Guglielmi, H., Stock, W., and Renner, R. (1971). Structure and activity of insulin. XI. Biologically active synthetic peptides of the insulin sequence B22–B25. *Hoppe-Seyler's Z. Physiol. Chem.* **352,** 1735–1738.

Weitzel, G., Eisele, K., Schultz, V., and Stock, W. (1973). Structure and activity of insulin. XII. Further studies on biologically active synthetic fragments of the B-chain. *Hoppe-Seyler's Z. Physiol. Chem.* **354,** 321–330.

Weitzel, G., Eisele, K., and Stock, W. (1975). Specificity of the arginine–guanidione group in biologically active tetrapeptides of the insulin sequence B22-B25 (Arg-Gly-Phe-Phe). *Hoppe-Seyler's Z. Physiol. Chem.* **365,** 583–590.

Weitzel, G., Bauer, F. U., and Eisele, K. (1976). Further studies on the three-step-increase in activity due to the aromatic amino acids (-Phe-Phe-Tyr-). *Hoppe-Seyler's Z. Physiol. Chem.* **357,** 187–200.

Welburn, S. C., Maudlin, I., and Ellis, D. S. (1989). Rate of trypanosome killing by lectins in midguts of different species and strains of *Glossina. Med. Vet. Entomol.* **3,** 77–82.

Welburn, S. C., Dale, C., Ellis, D., Beecroft, R., and Pearson, T. W. (1996). Apoptosis in procyclic *Trypanosoma brucei rhodesiense in vitro. Cell Death Differ.* **3,** 229–236.

Wenkert, D., and Allis, C. D. (1984). Timing of the appearance of macronuclear-specific histone variant hv1 and gene expression in developing new macronuclei of *Tetrahymena thermophila. J. Cell Biol.* **98,** 2107–2117.

Wertz, I. E., and Hanley, M. R. (1996). Diverse molecular provocation of programmed cell death. *Trends Biochem. Sci.* **21,** 359–364.

Wheatley, D. N., Christensen, S. T., Schousboe, P., and Rasmussen, L. (1993). Signalling in cell growth and death: Adequate nutrition alone may not be sufficient for ciliates. *Cell Biol. Int.* **17,** 817–823.

Wheatley, D. N., Rasmussen, L., and Tiedtke, A. (1994). *Tetrahymena:* A model for growth, cell cycle and nutritional studies, with biotechnological potential. *BioEssays* **16,** 367–372.

Wheeler-Alm, E., and Shapiro, S. Z. (1992). Evidence of tyrosine kinase activity in the protozoan parasite *Trypanosoms brucei. J. Protozool.* **39,** 413–416.

Whitbread, J. A., Sims, M., and Katz, E. R. (1991). Evidence for the presence of a growth factor in *Dictyostelum discoideum. Dev. Genet.* **12,** 78–81.

Wickerham, L. J. (1946). A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. *J. Bacteriol.* **52,** 293–301.

Wiesner, D. A., and Dawson, G. (1996). Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway. *J. Neurochem.* **66,** 1418–1425.

Wilden, P. A., and Kahn, C. R. (1994). The level of insulin receptor tyrosine kinase activity modulates the activities of phosphatidylinositol 3-kinase, microtubule-associated protein, and S6 kinases. *Mol. Endocrinol.* **8,** 558–567.

Wilkinson, P. C. (1987). Leucocyte locomotion. Behavioural mechanism for accumulation. *J. Cell Sci. Suppl.* **8,** 103–119.

Wilkinson, P. C. (1990). How do leucocytes perceive chemical gradients? *FEMS Microbiol. Immunol.* **64,** 303–312.

Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Early, A., Berks, M., and Jermyn, K. A. (1987). Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. *Cell* **49,** 185–192.

Wolin, M., Cherry, P. D., Rodenberg, J. M., Messina, E. J., and Kaley, G. (1990). Methylene blue inhibits vasodilation of skeletal muscle arterioles to acetylcholine and nitric oxide via the extracellular generation of superoxide anion. *J. Pharmacol. Exp. Ther.* **254,** 872–876.

Wyllie, A. H. (1992). Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: An overview. *Cancer Metastasis Rev.* **11,** 95–103.

Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980). Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68,** 251–306.

Wyllie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D. (1984). Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macronuclear synthesis. *J. Pathol.* **142,** 67–77.

Wyllie, A. H., Arends, M. J., Morris, R. G., Walker, S. W., and Evan, G. (1992). The apoptosis endonuclease and its regulation. *Semin. Immunol.* **4,** 389–397.

Yarmolinsky, M. B. (1995). Programmed cell death in bacterial populations. *Science* **267,** 836–837.

Yomura, S., and Kitanishi-Yomura, T. (1992). Release of myosin II from the membrane cytoskeleton of *Dictyostelium discoideum* mediated by heavy chain phosphorylation at the foci within the cortical actin network. *J. Cell Biol.* **117,** 1231–1239.

Yoshida, A., Pozdnyakov, N., Dang, L., Orselli, S. M., Reddy, V. N., and Sitaramayya, A. (1995). Nitric oxide synthesis in retinal photoreceptor cells. *Visual* Neurosci. **12,** 493–500.

Yuen, I. S., Taphouse, C. R., Halfant, K. A., and Gomer, R. H. (1991). Regulation and processing of a secreted protein that mediates sensing of cell density in *Dictyostelium. Genes Dev.* **113,** 1375–1385.

Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., Van Haastert, P. J. M., and Gomer, R. H. (1995). A density-sensing factor regulates signal transduction in *Dictyostelium. J. Cell Biol.* **129,** 1251–1262.

Zagon, I. S., and McLaughlin, P. J. (1992). An opioid growth factor regulates replication of microorganisms. *Life Sci.* **50,** 1179–1187.

Zakeri, Z., Quaglino, D., Latham, T., Woo, K., and Lockshin, R. A. (1996). Programmed cell death in the tobacco hornworm, *Manduca sexta:* Alteration in protein synthesis. *Microsc. Res. Technol.* **34,** 192–201.

Zakeri, Z. F., Quaglino, D., Latham, T., and Lockshin, R. (1993). Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB J.* **7,** 470–478.

Zipser, B., O'Neill, J. B., Ruff, M. R., Smith, C. C., Higgins, W. J., and Pert, C. B. (1988). The opiate receptor: A single 110 kDa recognition molecule appears to be conserved in *Tetrahymena,* leech and rat. *Brain Res.* **463,** 296–304.

Zwiller, J., Basset, P., Ulrich, G., and Mandel, P. (1982). Guanylate cyclase activators hemin and sodium nitroprusside stimulate growth in serum-free medium. *Exp. Cell Res.* **141,** 445–449.

This Page Intentionally Left Blank

Action of SO_2 on Plants and Metabolic Detoxification of SO_2

Ulrich Heber and Katja Hüve
Julius-von-Sachs-Institute of Biosciences, University of Würzburg, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany

SO_2 is a potentially toxic air pollutant which diffuses only slowly through the protecting layers of epidermal tissues of higher plants, but enters leaves rapidly through open stomata. Inside leaves, it dissolves in the aqueous phase of the apoplast, is hydrated to sulfurous acid, and is finally trapped as bisulfite and sulfite in the slightly alkaline cytoplasm of mesophyll cells, where it either reacts with cellular constituents, causing inhibition of metabolic reactions, or is metabolically detoxified. Oxidative detoxification produces sulfuric acid. In the light, this can occur via a radical-chain mechanism. Involved radicals are highly reactive and may themselves cause cellular damage by reacting with essential cell constituents. Reductive detoxification leads to sulfide. The incorporation of reduced sulfur into serine produces cysteine. From there, reduced sulfur is passed on to glutathione, methionine, proteins, and other sulfur-containing cellular constituents.

Since plants are equipped with powerful detoxification and pH-stabilizing mechanisms, and since inhibition of metabolic reactions, when it occurs, is largely reversible, SO_2 is, at low concentrations, unlikely to be directly and acutely harmful to plants. It can actually be a nutrient. Rather, chronic exposure to SO_2 causes damage preferentially by interfering with the cation nutrition of plants, particularly of trees which grow on poor soils. Cations are immobilized together with SO_2-derived sulfate in the vacuoles. Thus, SO_2 creates additional cation demands which are difficult to satisfy by uptake from impoverished soils. At ambient concentrations of SO_2, radical action during oxidative detoxification may contribute to damage, but is unlikely to be primarily responsible for it.

KEY WORDS: Air pollution, Forest damage, Oxidative detoxification, Radical action, Reductive detoxification, Sulfitolysis, Sulfur dioxide.

I. Introduction

During the last century, industrialization in England, central Europe, and the United States resulted in rapidly increasing energy demands. Coal

0074-7696/98 $25.00
Copyright © 1998 by Academic Press.
All rights of reproduction in any form reserved.

replaced wood as the main fuel. Where coal was used to roast sulfide-containing ore, vegetation suffered heavily in the surroundings of smelting plants (Stöckhardt, 1871). Close to the plants, complete loss of the vegetation was no rare event. When it occured, it led to extensive erosion (Haselhoff and Lindau, 1903; Wieler, 1905). During the second half of the 19th century, processes were introduced for the production of sulfuric acid from large amounts of sulfur dioxide which earlier had escaped into the atmosphere. This resulted in partial recovery of damaged vegetation. However, much more wide-spread than the damage caused in the immediate surroundings of foundries was the damage produced by chronic exposure of forests to the gases produced by the burning of sulfur-containing coal in the households and small plants of industrial centers with a large population (Wislicenus, 1908).

In 1849, Adolph Stöckhardt, Professor at the reputed Forest Academy at Tharandt, not far from Dresden in Saxony, was requested by the Saxon Ministry of Finances to investigate damaging influences of the smoke emitted by smelting plants at Freiberg on the vegetation of nearby fields, meadows, and forests (Stöckhardt, 1871). It appears that this was the beginning of systematic research on damage caused to plants by industrial emissions. Initially it was unclear which components of the emissions caused the damage. However, exposure of plants in glass houses to sulfur dioxide, one of the components, produced symptoms similar to those observed close to the source of the emissions which also contained arsenics and lead. Thus, SO_2 was identified as the main culprit.

Smoke research, as it was called, was intense in Germany, particularly in the decades before World War I. During the European wars and in the time between the First and the Second World War, research on the action of air pollutants stagnated even though emission of SO_2 by industrial emittents continued to increase until about 1973 (Fig. 1). This increase was interrupted in Germany only briefly during and after the First and the Second World War and during times of economical crisis. Starting with the recovery of economy after the Second World War and initiated by growing air pollution caused also by large coal-burning power plants particularly in the industrial zones of the Ruhr area, new investigations were performed (Guderian *et al.*, 1960; Guderian, 1977). However, the public, more concerned with prosperity than with environmental protection, showed little interest initially. This situation changed gradually. The "Landesanstalt für Immissions- und Bodennutzungsschutz" was founded by the country of Northrhine-Westphalia already in 1963. However, public concern about increasing air pollution developed earlier in the United States than in Germany. With some delay, it resulted in political action and led in 1970 to the creation of the U.S. Environmetal Protection Agency, which was followed in Germany by the foundation of the federal Umweltbundesamt in 1974. The

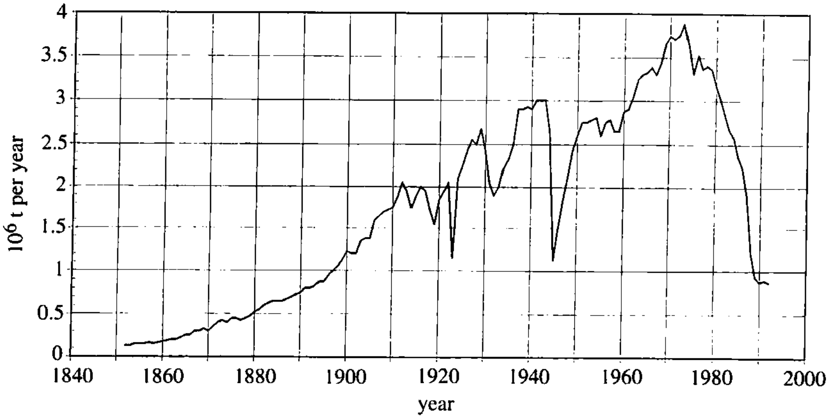


FIG. 1 Sulfur dioxide emissions in Germany between 1852 and 1992. Data after World War II are for the Federal Republic of Germany without the new countries of the former German Democratic Republic (from Elling, 1993, with permission).

German public became strongly involved, and air pollution turned finally into a political issue in the 1970s and 1980s when the press circulated reports on the imminent death of our cherished forests. Measures were taken to set limits to tolerable pollution and to force industry to clean emissions. A federal law on protection against immissions became effective in the Federal Republic of Germany in 1974. Alterations were introduced in 1976, 1982, 1985, 1991, and 1993. Within the European Community, agreements were reached in 1988 to reduce immissions in different countries. These measures proved effective. In Europe (including the former Soviet Union) the annual emission of sulfur dioxide fell from about 52 million tons in 1980 to about 36 million tons in 1990 [Federal Ministry of Nutrition, Agriculture and Forestry (FMNAF), 1993]. Starting with 1973, SO_2 emissions were sharply reduced in the Federal Republic of Germany (Fig. 1). By the end of the 1980s, the decrease amounted to about 70% of the maximum that was reached in the early seventies. Ironically, even though public concern in the United States preceded similar awareness in Germany, output of SO_2 in the United States was not much reduced during the same period [U.S. Environmental Protection Agency (USEPA), 1989, 1992]. It was about 18 million tons annually between 1985 and 1992 (USEPA, 1994).

Much of the research done to understand the deleterious effects of SO_2 on plants has employed concentrations of SO_2 far above the levels presently encountered in the atmosphere after large smoke stacks have reduced local deposition and have spread SO_2 over wide areas. They have become responsible for the distribution of SO_2 beyond national boundaries at re-

duced and, according to national or international convention, often considered tolerable concentrations. Most of the numerous reviews dealing with plant damage by SO_2 have considered acute damage caused by high concentrations of this air pollutant (Ziegler, 1975; Petering, 1977; Heath, 1980; Alscher, 1984; Rennenberg, 1984; Winner *et al.*, 1985; Alscher *et al.*, 1987; Lange *et al.*, 1989; Rennenberg and Polle, 1994; De Kok, 1995). In this review, attention is focused on the mode of action of SO_2 on plants at ambient levels of SO_2. For obvious reasons, effects produced only after years of exposure to low levels of SO_2 are much more difficult to understand on a molecular basis than acute effects produced by short periods of exposure to high concentrations of SO_2. Therefore, deduction needs to be used to analyze the cause of chronic effects exerted by low levels of SO_2.

II. Emissions and Immissions

Atmospheric SO_2 is of natural and anthropogenic origin. Volcanic eruptions release 10 to 20 million tons of SO_2 annually. When the El Chichon erupted in 1982, about 20 million tons was estimated to be released, whereas up to to 60 million tons was thrown out when the Pinatubo exploded in 1991. Anthropogenic SO_2 production is mainly based on the burning of sulfur-containing fossil fuels. Worldwide, it may amount to 160 to 240 million tons annually. Table I lists emissions from different countries in 1990 or the preceding years. In these countries, anthropogenic sources of SO_2 account for more than 95% of emitted SO_2. In the atmosphere, SO_2 is oxidized to sulfuric acid by gaseous phase oxidation with hydroxyl radicals (Calvert and Stockwell, 1983) and by aqueous phase oxidation by H_2O_2 and ozone (National Academy of Sciences, 1983). The residence time of SO_2 in a midlatitude atmosphere is only about 25 h (Rohde, 1978). Chemical processes leading to atmospheric acidity and rate laws are discussed by Pienaar and Helas (1996).

Obviously, atmospheric concentrations of SO_2 can vary within wide limits. When weather conditions permit the metastable existence of so-called inversion layers (cold layers close to the surface below warm air), SO_2 may accumulate close to the ground level and reach concentrations which are not only damaging to plants but hazardous also for humans. During the winter time, levels approaching or exceeding 200 nPa Pa^{-1} (about 200 ppb, or 0.2 ppm) SO_2 in air are of fairly frequent occurrence locally when the weather situation does not permit convective mixing of air. When averaged over a year, however, SO_2 concentrations are far lower. Figure 2 shows annual averages of SO_2 immissions measured up to 1992 or 1993 at different measuring stations within Germany (Slovik *et al.*, 1996a). Even though

TABLE I

SO_2 Emissions in Different Countries in 1988,[a] 1989,[b] or 1990[c] (FMNAF, 1993)

Country	SO_2 emission (million tons per year)	Change since 1980 (%)
Austria	0.09[c]	−77
Belgium	0.443[c]	−46
Belorussia	0.6[b]	−19
Bulgaria	1.27[b]	+22
Canada	3.7[c]	−20
Former CSFR	2.44[c]	−21
Denmark	0.18[c]	−60
Federal Republic of Germany, before reunification	0.98[c]	−71
Former German Democratic Republic	4.75[c]	+10
Finland	0.26[c]	−55
France	1.26[c]	−62
Great Britain	3.77[c]	−23
Hungary	1.0[c]	−38
Ireland	0.17[c]	−24
Italy	2.18[b]	−43
The Netherlands	0.21[c]	−55
Norway	0.05[c]	−61
Poland	3.21[c]	−22
Portugal	0.2[a]	−23
Romania	1.8[b]	No change
Russia, european part	4.46[c]	−38
Spain	2.3[c]	−30
Sweden	0.17[c]	−67
Switzerland	0.06[c]	−51
United States	21.2[c]	− 9
Ukraine	2.8[c]	−28
Yugoslavia	1.48[c]	+14

Germany is a small country compared to the United States, average concentrations measured in different parts of the country differ considerably. The highest average values were recorded for the Erzgebirge in Saxony, not far from the sites in Czechia where large power plants burn sulfur-containing brown coal. At the upper reaches of the Erzgebirge, older spruce stands are heavily damaged (Emde, 1993). On the Czech side, deforestation is wide-spread.

Table II shows limits to tolerable SO_2 immissions as recommended by different national or international commissions between 1978 and 1988. As the years went by, recommended tolerance levels decreased. A comparison of Table I with Fig. 2 reveals that in 1992 annual averages of SO_2 concentrations are above officially accepted limits in Germany only in the Erzgebirge.

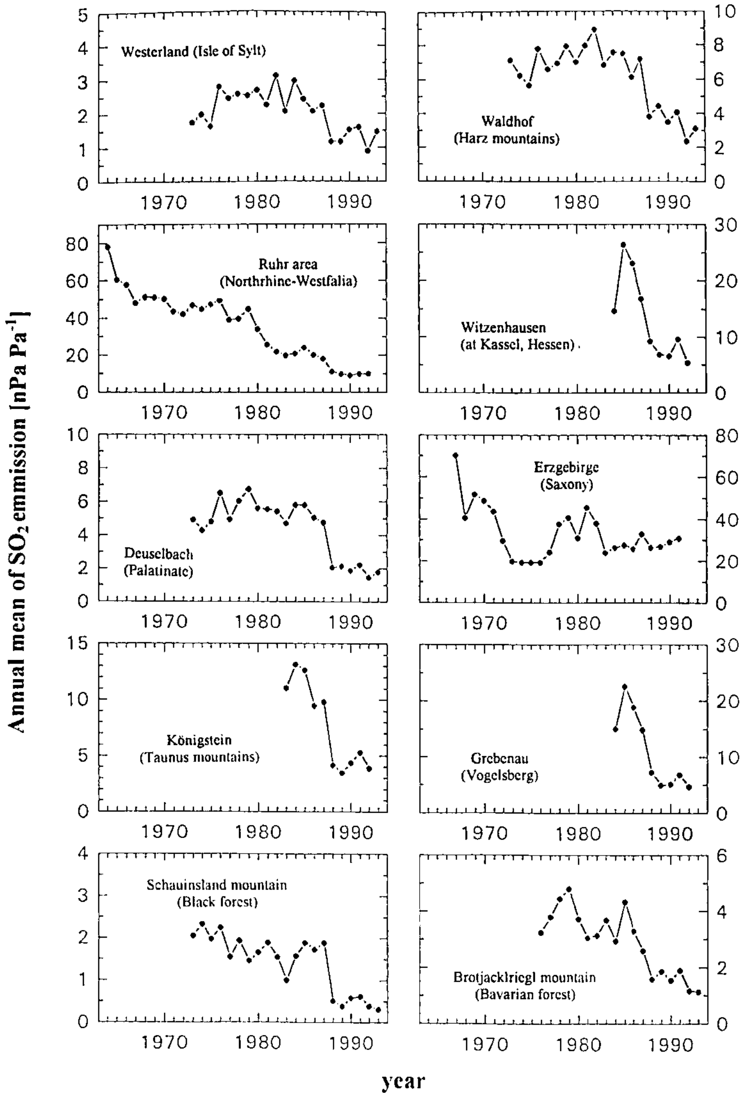


FIG. 2 Annual averages of SO_2 immissions at different measuring stations within the Federal Republic of Germany, including the former German Democratic Republic (Erzgebirge, Saxony) (from Slovik *et al.*, 1996a, with permission).

However, it must be pointed out that damage to plants by SO_2 does not follow a dosis law according to which damage is determined by the product of concentration and exposure time. Rather, damage increases with concentration when the product of concentration and exposure time is kept con-

TABLE II

Limits to Tolerable SO_2 Immissions as Recommended by Different Organizations

Organization	Year	Comment	So_2(μg m^{-3})	SO_2(nPa Pa^{-1})
VDI	1978	Insensitive plants	120	42
		Sensitive plants	80	28
		Very sensitive plants	50	17.5
IUFRO	1978	Forests, optimum locations	50	17.5
		Forests, extreme locations	25	8.7
TA-Luft	1986	Plants	50	17.5
WHO	1987	Terrestrial vegetation	30	10.5
UNECE	1988	Forests, crop plants	30	10.5
		Sensitive plants	20	7

Note. VDI, Verein Deutscher Ingenieure, recommendation 2310 (Düsseldorf); IUFRO, International Union of Forest Research Organizations (Vienna); TA-Luft, Technische Anleitung Luft, March 1st, 1986, Federal Government, Bonn; WHO, World Health Organization (New York); UNECE, United Nations Economic Commission for Europe (Brüssels). VDI recommendations consider a 7-month vegetation period only. All other recommendations are 24 h/annual averages. According to Jäger (1989).

stant. These old findings are a simple consequence of the existence of detoxification mechanisms in plants as will be shown below.

III. Deposition

A. Dry Deposition

The loss of SO_2 to a surface is termed dry deposition. Being effective gas exchange systems, plants have large surfaces. However, gas exchange occurs predominantly through the stomata which can be regulated. It was early recognized that spruce is much more sensitive to SO_2 when assimilation is active, i.e., when stomata are open rather than when they are closed (Wislicenus 1898). Uptake of SO_2 through the epidermis of leaves or needles is negligible compared to diffusion of SO_2 through the open stomata. The cuticular resistance to SO_2 flux has been calculated from published permeability coefficients (Lendzian, 1984) to be about 50,000 s cm^{-1} in citrus leaves, whereas the combined boundary layer and stomatal resistances to SO_2 flux may be as low as 2 to 5 s cm^{-1} when stomata are open. As humidity in the leaf interior is close to 100% and cell walls are water-saturated, SO_2 is hydrated in the leaf interior to sulfurous acid which dissociates with pK values of 1.8 and 7.2, releasing protons and forming bisulfite and sulfite

anions. The Henry coefficient of SO_2 is 29 at 25°C compared to 0.76 for CO_2. A simple calculation performed on the basis of the Henderson/Hasselbalch equation reveals that the combined aqueous concentrations of bisulfite and sulfite in equilibrium with 30 nPa Pa^{-1} SO_2 in air (see SO_2 levels in the Erzgebirge, Fig. 2) are at pH 5.5, the pH of the aqueous cell wall phase, 180 m*M*, and at pH 7.2, the cytosolic pH, even 2.7 *M*. It is important to realize that in view of the high reactivity of the anions, cellular life would not be able to exist at equilibrium concentrations. Rather, equilibrium is never attained. Cellular life is maintained in a flux situation. SO_2 diffuses continuously through the stomata into leaves where it enters into metabolic transformations which safeguard cellular life. Competition between detoxifying and potentially damaging reactions decide survival or damage of the cellular system.

By approximation, the rates of entry of SO_2 into leaves can be calculated (Heath, 1980) using a simplified version of Fick's law

$$\Phi = (c_o - c_i)/R,$$

where Φ is the flux of SO_2 in nmol cm^{-2} s^{-1}, c_o and c_i are the concentrations of SO_2 in nmol cm^{-3} outside and inside the leaves, and R is the sum of boundary layer and stomatal resistences in s cm^{-1}. Since c_i is, owing to the high solubility of SO_2 in aqueous phases and the large surface of mesophyll cell walls, close to zero, only c_o and R need to be known. Both can be measured, the former directly and the latter via the transpirational loss of water vapor through the stomata (Nobel, 1983). In most plants, stomata close at night and open during the day. For unstressed illuminated conifer needles, the sum of stomatal and boundary layer resistances to diffusional water loss is often about 12 s cm^{-1} or, by another way of expression (Cowan and Farquhar, 1977), 0.03 m^2 s $mmol^{-1}$ (Wiese *et al.*, 1996). As SO_2 is larger than H_2O, its diffusion is slower. The factor to apply is 1.88 (Taylor and Tingey, 1983). Thus, at an SO_2 concentration in air of 30 nPa Pa^{-1} (see Fig. 2), SO_2 flux into exposed needles is at R = 12 s cm^{-1} about 2 μmol m^{-2} leaf area h^{-1} or about 20 μmol m^{-2} during daytime. If stomata are more widely open, fluxes will be larger, and when they are closed, smaller.

Stomatal conductances vary not only during the day, but also with the season. They are larger in the summer than in the winter in evergreens. Average water vapor conductances during the course of a year are shown for canopies of *Picea abies* in Fig. 3. Low conductances during the winter time decrease SO_2 influx when SO_2 concentrations in air are high (Fig. 4), and low temperatures slow metabolic detoxification of SO_2 down, whereas high conductances in the summer tend to facilitate influx, but SO_2 concentrations are low and metabolic detoxification is more efficient.

Slovik *et al.* (1996a), using data from different measuring stations distributed over the federal state of Hessen and considering a canopy situation,

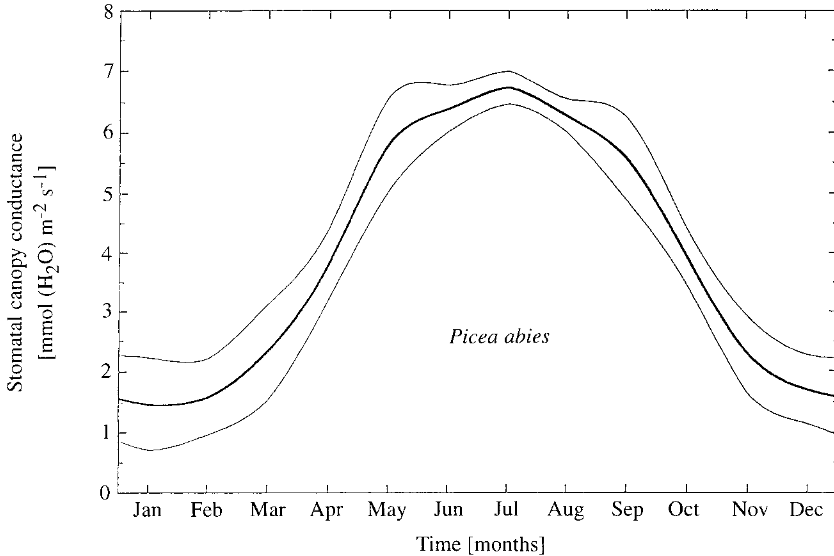


FIG. 3 Mean effective stomatal canopy conductances g_{H2O} of Norway spruce (*Picea abies* L.) during the course of a year (from Slovik *et al.*, 1996a, with permission). Mean conductances for SO_2 are lower by a factor of 1.88 (Taylor and Tingey, 1983).

have calculated average fluxes of SO_2 into spruce needles as a function of measured SO_2 concentrations in air (Fig. 5). Not unexpectedly, calculated fluxes are lower than in the example calculation given above and probably

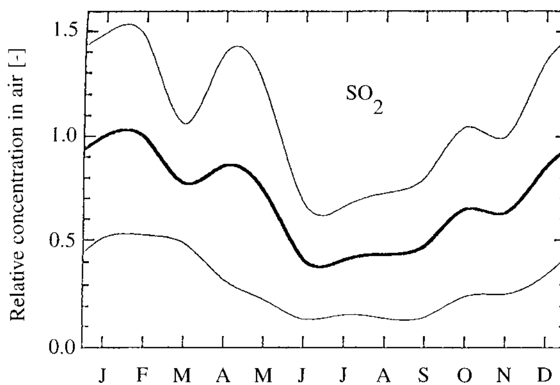


FIG. 4 Relative concentrations of SO_2 in air as a function during the course of a year. Thin lines indicate standard deviations. Data from six measuring stations in Germany (17,520 field measurements per site per year). From Slovik *et al.*, 1996a, with permission.

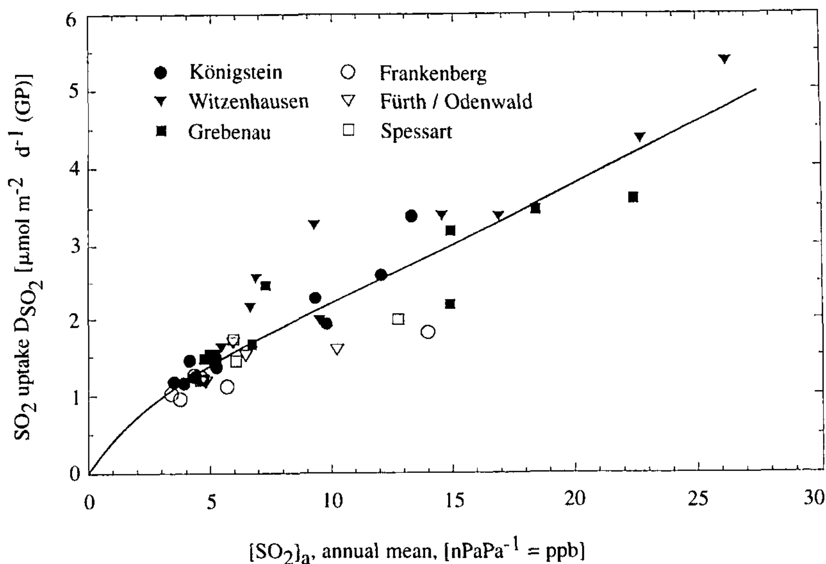


FIG. 5 SO_2 uptake of spruce needles calculated from mean effective stomatal conductances (Fig. 3) and field data on SO_2 concentrations in air at six different measuring stations in Germany (from Slovik *et al.*, 1996a, with permission). GP, growth period.

more realistic in regard to a field situation. The data illustrate the detoxification burden that must be borne by spruce forests exposed to SO_2 in the field. Obviously, the reactive anions formed inside the leaves from entering SO_2 must be constantly metabolized to prevent their accumulation to toxic levels. In fact, sulfite concentrations are usually too low to be measurable in leaves or needles when plants are exposed to ambient SO_2 concentrations. Concentrations in the micromolar range were found in extracellular fluids only when subzero temperatures slowed metabolic SO_2 detoxification down (Wolfenden *et al.*, 1991).

Since the intercellular concentration of SO_2 is close to zero at average, it is obvious that the guard cells of the stomata are exposed to higher concentrations of SO_2 than other cells. Reports on effects of SO_2 on stomatal opening are both numerous and inconsistent (Black, 1985). Perhaps, the common denominator is that if the energy-requiring opening mechanism is damaged by high concentrations of SO_2, stomata will close. If, at averaged ambient concentrations as listed in Fig. 2, no acute damage occurs, there is no measurable effect on stomatal conductance, or stomata may actually open somewhat (Hällgren, 1978; Atkinson and Winner, 1989), possibly owing to a stimulation of ATPases (Larson and Jagendorf, 1989).

B. Wet Deposition

SO_2 converted to sulfurous acid in moist or foggy air and then oxidized to sulfuric acid contributes to the so-called acid rain, when not neutralized in the atmosphere by ammonia or dust particles containing calcium carbonate or other neutralizing components of particulate atmospheric matter. In acid rain, sulfuric acid contributes to soil acidification (Ulrich *et al.*, 1980; Likens *et al.*, 1996). Even though it is known that fertilizers may relieve deficiency symptoms when applied to leaves by spraying, the cuticle of the epidermis is a highly effective barrier to the penetration of strong polar acids such as sulfuric acid. Surface tension prevents acidic solutions from entering through the stomata. Thus, at the acidity commonly encountered in acid rain, only epidermal tissues such as bark are likely to be affected by acid rain. Such plant surfaces can be considered as cation exchangers. Since Hofmeister has published his famous power series in 1888 and the following years (Abernethy, 1967), protons are known to be effective in liberating cations from cation exchangers. The order

$$H^+ > Ca^{2+} > Mg^{2+} > K^+ > Na^+$$

indicates that K^+ can replace Na^+, Mg^{2+} can replace K^+, Na^+, etc. and that H^+ is the strongest ion in this order. However, neither leaching of nutrients from tissues by acid rain nor effects of acidity on soil or roots are topics of this review.

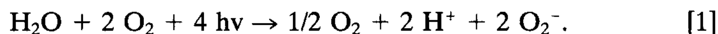
IV. Toxicity of SO_2 and Its Hydration Products

Paracelsus (1493–1541) has stated that the dose defines a poison. Once SO_2 has entered cells either by diffusion across the plasmalemma or by uptake of the anions formed from it outside the cells, SO_2 exists in the cytoplasm in quasi-equilibrium with bisulfite and sulfite. Compared to the concentration of these anions, which is determined by pK values and pH, its concentration is negligible. Thus, the reactivity of the anions is important for a consideration of the toxicity of SO_2 rather than the reactivity of SO_2 by itself. As nucleophilic agents, sulfites can attack many different substrates (Petering, 1977; Heath, 1980). Several enzymes are inhibited when plants are exposed to high concentrations of SO_2 (Tanaka *et al.*, 1982) or enzymes are incubated with sulfite solutions (1 mM or more; Ziegler, 1974; Ziegler *et al.*, 1976). It is known since long and used by wine producers that sulfites inhibit fermentation of yeast. Not only inhibition, but also stimulation of enzyme activity has been observed in the presence of sulfite (Zakharov *et al.*, 1983; Larson and Jagendorf, 1989). However, the significance of these

facts in relation to the action of ambient concentrations of SO_2 on plants remains obscure. Anions formed from SO_2 do not accumulate in plants exposed to ambient or even high levels of SO_2. Sophisticated methods are required to measure the low steady-state concentrations which are maintained in SO_2-polluted air (Wellburn, 1985; Wolfenden *et al.*, 1991).

A. Radical Formation

When brightly illuminated, thylakoid membranes isolated from leaves oxidize sulfite to sulfate at rates surpassing maximum rates of light-dependent electron transport. The high rates are due to a radical chain reaction (Asada, 1980). The reaction is sensitive to the electron transport inhibitor DCMU and insensitive to cyanide (Asada and Kiso, 1973; Asada, 1980). In contrast, slow sulfite oxidation in the dark is sensitive to cyanide and insensitive to DCMU. The light-dependent radical chain reaction is initiated by oxygen radicals which are formed by the univalent reduction of oxygen in the Mehler reaction (Mehler, 1951)



$$H_2O + 2\,O_2 + 4\,h\nu \rightarrow 1/2\,O_2 + 2\,H^+ + 2\,O_2^{-}. \quad [1]$$

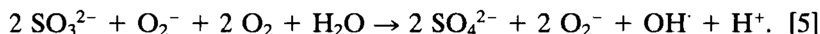
During the reaction, not only sulfite radicals but also highly reactive hydroxyl radicals are generated:

$$SO_3^{2-} + O_2^- + 3\,H^+ \rightarrow HSO_3^{\cdot} + 2\,OH^{\cdot} \quad [2]$$

$$SO_3^{2-} + OH^{\cdot} + 2\,H^+ \rightarrow HSO_3^{\cdot} + H_2O \quad [3]$$

$$HSO_3^{\cdot} + O_2 + H_2O \rightarrow H_2SO_4 + O_2^- + H^+. \quad [4]$$

Reactions [2] to [4] may be summarized as



$$2\,SO_3^{2-} + O_2^- + 2\,O_2 + H_2O \rightarrow 2\,SO_4^{2-} + 2\,O_2^- + OH^{\cdot} + H^+. \quad [5]$$

This sequence of reactions may also occur *in vivo* at high SO_2 concentrations (Madamanchi and Alscher, 1991; Okpodu *et al.*, 1996). Liberated radicals not only accelerate sulfite oxidation but also cause damage to components of the photosynthetic apparatus by reacting with essential catalysts. However, leaves possess effective mechanisms to scavenge radicals. Radical chain propagation will occur only if radical-scavenging systems are overtaxed. Figure 6 shows an experiment in which a leaf of *Pelargonium zonale* was repeatedly exposed to 4000 nPa Pa^{-1} SO_2. This level of SO_2 is almost 400 times above the maximum permissible concentration in air (see Table II). The first exposure to SO_2 (5 min) was in air. After a brief lag phase, it caused a 20% inhibition of photosynthetic carbon uptake. This inhibition

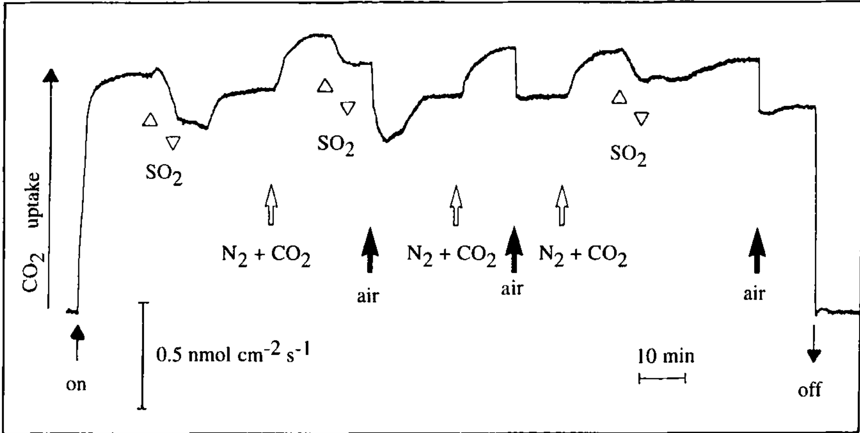
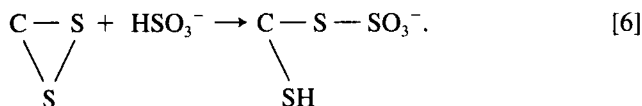


FIG. 6 Effects on photosynthesis of brief periods of exposure of a leaf of *Pelargonium zonale* to a high concentration of SO_2 (4000 nPa Pa^{-1}) either in air or in nitrogen. Note that carbon assimilation is increased in nitrogen owing to the inhibition of photorespiration. This increase is rapidly reversed in air. The photon flux density was 480 $\mu E\ m^{-2}\ s^{-1}$, the CO_2 concentration 500 $\mu Pa\ Pa^{-1}$.

was a direct effect on photosynthesis. Simultaneously performed measurements of transpiration showed that it was not caused by the closing of stomata. After a while, photosynthesis recovered. Obviously, little permanent damage had occured. Then, air was replaced by nitrogen. CO_2 uptake increased because CO_2 was no longer released by photorespiration. A second exposure to SO_2 in nitrogen decreased photosynthesis again. Since the radical chain oxidation of SO_2 requires oxygen (Eq. [1]), this inhibition is unlikely to be a consequence of the reaction sequence [2] to [4]. However, when nitrogen was replaced by air after fumigation with SO_2, CO_2 uptake decreased more than could be accounted for by photorespiration. This is clearly seen by a subsequent control experiment, in which air replaced nitrogen in the absence of prior exposure to SO_2. In this case, inhibition by photorespiration alone can be seen. The extra inhibition seen after exposure to SO_2 is oxygen-dependent and can be explained as a consequence of radical production as shown in Eqs. [2] to [5] (see also Madamanchi and Alscher, 1991). Even oxygen-dependent inhibition of photosynthesis was rapidly repaired in air, more rapidly than the inhibition produced in nitrogen in the third fumigation experiment of Fig. 5. Apparently, inhibition produced by radical reactions was largely reversible even when the concentration of SO_2 that caused inhibition was manyfold higher than ambient concentrations normally encountered in the field.

B. Sulfitolysis and Unspecified Sulfite Addition Compounds

Sulfite can add to oxidized disulfide compounds including proteins opening S-S bridges (Pechere *et al.,* 1958; Alscher, 1984):



$$C-S + HSO_3^- \rightarrow C-S-SO_3^-. \quad [6]$$

Thioredoxins are proteins with exposed disulfide groups. They are involved in cellular redox regulation, in particular in the regulation of photosynthetic enzymes (Buchanan, 1980). When oxidized, thioredoxins react with sulfites. Sulfitolysis causes their inactivation (Würfel *et al.,* 1990). It has been suggested that this is the basis of the phytotoxicity of SO_2 to plants (Häberlein and Follmann, 1991). However, sulfitolysis requires appreciable sulfite concentrations *in vitro.* Moreover, it is reversible. Nevertheless, photosynthetic enzymes known to be sensitive to SO_2 or sulfite, such as alkaline fructose bisphosphatase and phosphoribulokinase (Tanaka *et al.,* 1982), glyceraldehydephosphate dehydrogenase (Ziegler *et al.,* 1976), and NADP-dependent malic dehydrogenase (Ziegler, 1974), are light-regulated by thioredoxin and contain oxidizable SH groups. The sensitivity of photosynthesis to high concentrations of SO_2 (Ziegler, 1975; Darrell, 1989) is suggestive. Sulfitolysis may be involved in reversible SO_2-dependent photosynthesis inhibition (Fig. 6).

Sulfite is also known to add to aldehydes forming α-hydroxysulfonates which are metabolic inhibitors (Zelitch, 1957). Its reaction with other cellular solutes has been considered by Peiser and Yang (1985). In intact chloroplasts which have retained radical-scavenging enzymes and solutes such as ascorbate, sulfite oxidation was slow. Sulfite reduction and the formation of unspecified addition compounds competed with oxidation (Dittrich *et al.,* 1992). Formation of the addition compounds was light-dependent. Electron transport was involved as shown by the sensitivity of the reaction to the electron transport inhibitor DCMU. Accumulation of addition compounds was suppressed when the presence of *O*-acetylserine favored the reductive detoxification of sulfite which led to the formation of cysteine.

C. Acidification

Both sulfurous acid and the sulfuric acid formed from it by oxidation are trapped in the slightly alkaline cytoplasm of leaf cells which has a pH close to 7.2. The extent of resultant cytoplasmic acidification depends on the cytoplasmic buffering capacity which has been estimated to be between 20

and 40 mM pH-unit^{-1} (Guern *et al.*, 1986). Chloroplasts occupy about 70% of the cytoplasmic space in the mesophyll of leaves (Winter *et al.*, 1993). Within the physiological pH range (pH 7 to 8), intact isolated chloroplasts had buffering capacities close to 35 mM pH-unit^{-1} (Pfanz and Heber, 1986). Somewhat higher values were measured in chloroplasts *in vivo* (Hauser *et al.*, 1995a). In the Erzgebirge (for atmospheric SO_2 pollution there see Fig. 2), 4-year-old spruce needles contained 60 to 80 mM sulfate (Fig. 7, lower panels, Hüve *et al.*, 1995). Not rarely, however, more than 100 mM (Polle

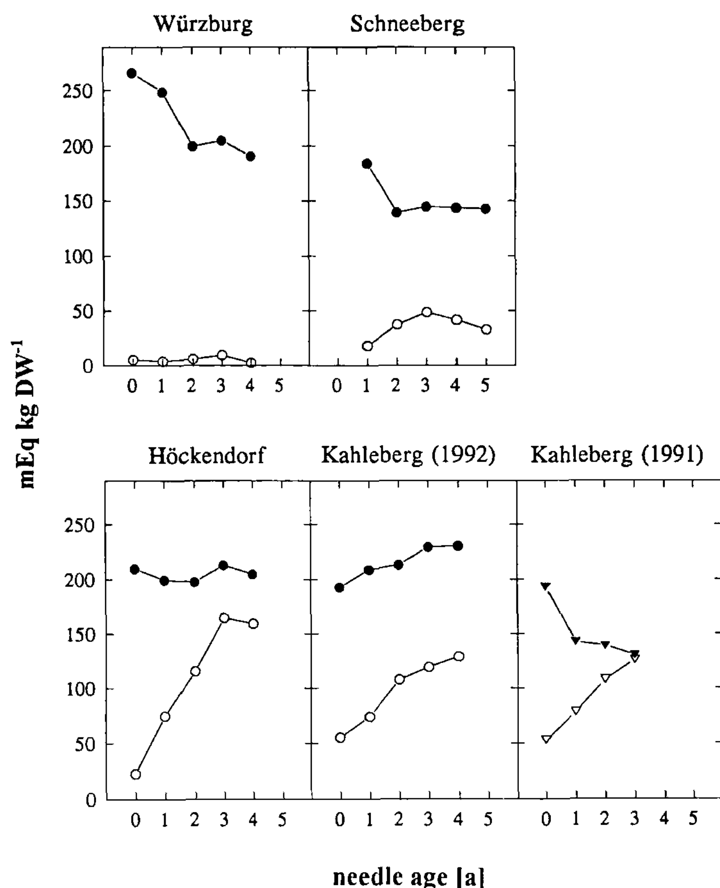


FIG. 7 Average contents of sulfate (open symbols) and the sum of potassium and magnesium (filled symbols) in meq (kg dry weight)$^{-1}$ in needles of Norway spruce as a function of needle age. 2 meq SO_4^{2-} or Mg^{2+} = 1 mmol; 1 meq K^+ = 1 mmol. Trees were from Würzburg (about 5 nPa Pa^{-1} SO_2), the Schneeberg (about 15 nPa Pa^{-1} SO_2), and Höckendorf and the Kahleberg in the Erzgebirge (about 30 nPa Pa^{-1} SO_2, see Fig. 2).

et al., 1994), and occasionally even up to 200 m*M* (Pfanz and Beyschlag, 1993), were measured. At Würzburg in Frankonia, where air pollution by SO_2 is low, sulfate in spruce needles was below 5 m*M* (Fig. 7).

Sulfate derived from SO_2 releases two protons per anion. Thus, maximum sulfate contents in needles of spruce exposed to ambient SO_2 concentrations in the Erzgebirge indicate that from 120 up to 400 m*M* H^+ had acted on mesophyll cells over time periods ranging from 1 to 4 years. Given a cytoplasmic buffering capacity of about 40 m*M* per pH-unit, strong cytoplasmic acidification should have been expected. In fact, none has been observed in the field (Kaiser *et al.*, 1993a). However, after fumigation of young spruce trees with 600–1000 nPa Pa^{-1} SO_2 for 2 weeks, the pH values of needle homogenates indicated modest acidification (Kaiser *et al.*, 1993a). In intact leaves of *Pelargonium zonale*, fluorescence of the the pH-indicating fluorescent dye pyranine responded to fumigation with SO_2 after a lag phase of about a minute (Fig. 8). Since pyranine records the cytosolic pH (Yin *et al.*, 1990), the lag may be taken as evidence of a hydration of SO_2 in the apoplast outside the cytoplasm and of transport of protons and anions derived from SO_2 across the plasmalemma into the cytoplasm. In line with this explanation, acidification indicated by the decrease in fluorescence emission continued to increase for a few minutes even after the SO_2

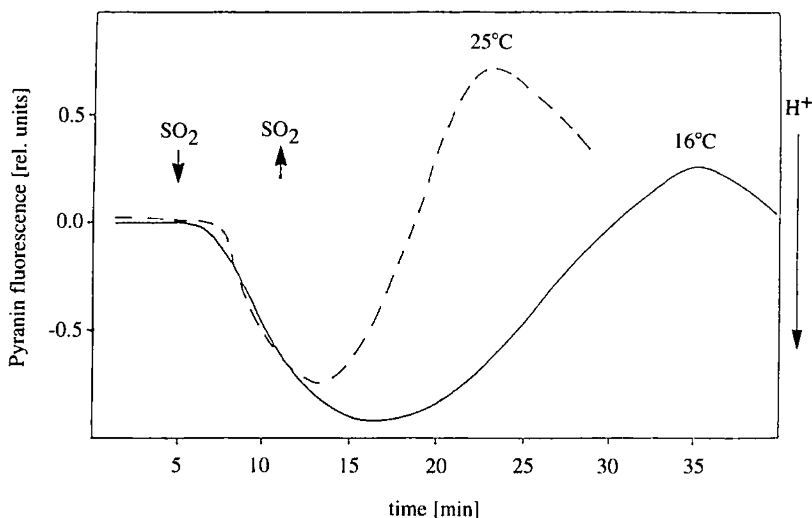


FIG. 8 Effect of a massive dose of SO_2 (85 μmol m^{-2} leaf area) on the cytosolic pH in a leaf of *Pelargonium zonale* as shown by fluorescence changes of pyranine which had been taken up by the leaf. A fluorescence decrease indicates acidification, an increase alkalinization. The experiment was performed at 16 and at 25°C. Pyranine is a pH sensitive fluorescent pH indicator (Yin *et al.*, 1990).

had been removed from the atmosphere. Importantly, the fluorescence trace then reversed direction finally reestablishing the original pH in an oscillatory mode. This return was faster at 25°C than at 16°C, indicating temperature-dependent metabolic control of the cytoplasmic pH (Gout *et al.*, 1992; Veljovic-Jovanovic *et al.*, 1993; Heber *et al.*, 1994).

V. Metabolic Detoxification of SO_2 and Its Hydration Products

A. Oxidative Detoxification

Detoxification of SO_2 by oxidation to sulfate would be most effective, if it occured in the apoplast before SO_2, or the reactive anions formed from SO_2, reach the cytoplasm (Rennenberg and Polle, 1994). Pfanz *et al.* (1990) have reported oxidation of sulfite by apoplastic peroxidase in the presence of H_2O_2. It appears that the first step in the reactions sequence is the oxidation of an apoplastic phenolic compound by H_2O_2. The resulting phenoxy radical oxidizes sulfite. However, ascorbate reduces phenoxy radicals effectively inhibiting apoplastic sulfite oxidation (Takahama *et al.*, 1992). As long as ascorbate is present in the apoplast, apoplastic sulfite oxidation is unlikely to occur.

Cytoplasmic oxidation of sulfite anions to sulfate does not need to follow a fast light-dependent radical chain mechanism. A slower oxidation of sulfite appears to be catalyzed by sulfite oxidase (Ballantyne, 1977; Jolivet *et al.*, 1995). Whereas in isolated and washed thylakoid membranes from chloroplasts maximum light-dependent rates of radical-mediated sulfite oxidation exceeded 1000 μmol (mg chl h)$^{-1}$, they were only 2 μmol (mg chl h)$^{-1}$ in intact chloroplasts, when *O*-acetylserine was absent, and 1 μmol (mg chl h)$^{-1}$, when in the presence of *O*-acetylserine sulfite reduction competed with sulfite oxidation. In the dark, sulfite oxidation was, at about 0.5 μmol (mg chl h)$^{-1}$, still appreciable. These rates were observed in the presence of about 1.5 m*M* sulfite (Dittrich *et al.*, 1992).

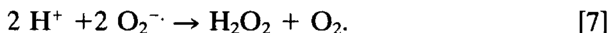
It is informative to compare oxidation rates as observed *in vitro* with SO_2 fluxes into leaves. According to Slovik *et al.* (1996a), SO_2 fluxes into canopies of Norway spruce increase almost linearly with ambient SO_2 concentrations in air. They are about 0.16 μmol m^{-2} day^{-1}/(nPa SO_2 Pa^{-1}) or about 5 μmol m^{-2} day^{-1}, if the average SO_2 concentration in air is as high as 30 nPa Pa^{-1} (Fig. 2). A 1-m^2 needle surface area contains about 600 to 700 mg chlorophyll. Thus, the maximum capacity of chloroplasts for oxidative SO_2 detoxification is more than 300 μmol m^{-2} h^{-1} even in the dark. It is considerably higher in the light. Obviously, maximum capacities for

oxidative SO_2 detoxification are by orders of magnitude higher than fluxes on the borderline of SO_2 toxicity (Table II). However, this comparison does not consider the large differences between the very low concentrations of sulfites *in vivo* and the much higher concentrations used *in vitro* to measure detoxification capacities. Sulfite concentrations far below Michaelis constants of the enzymes involved in detoxification *in vivo* obviously decrease the effectiveness of enzymic detoxification.

B. Radical Scavengers and Antioxidants

Leaves are equipped with complex radical-scavenging enzymic systems (Asada and Takahashi, 1987; Foyer *et al.*, 1991). Even simple cellular constituents such as sugars can serve as radical scavengers. Particularly important as an antioxidant and radical scavenger is ascorbate, which is oxidized by radicals to monodehydroascorbate.

Radicals are not only produced as a consequence of reactions of air pollutants such as SO_2 or ozone. They are also unavoidable and occasionally necessary products of metabolism. In photosynthesis, the univalent reduction of oxygen in the Mehler reaction results in the formation of the superoxide radical $O_2^{-\cdot}$, which can initiate a radical chain reaction with sulfite radicals as intermediates (Eqs. [2] to [5]). Superoxide dismutase removes $O_2^{-\cdot}$, producing H_2O_2



$$2\ H^+ + 2\ O_2^{-\cdot} \rightarrow H_2O_2 + O_2. \qquad [7]$$

When H_2O_2 is detoxified in the chloroplasts by ascorbate peroxidase, the monodehydroascorbate radical is formed (Asada and Takahashi, 1987). It is either rapidly reduced by a ferredoxin-dependent reductase in the chloroplasts or reacts to dehydroascorbate and ascorbate. Mainly by regenerating ascorbate from dehydroascorbate, glutathione serves as an antioxidant in cellular metabolism. Its concentration is much lower than that of ascorbate, but increases in response to exposure to SO_2. Polle *et al.* (1994) have reported increased contents of glutathione, cysteine, glutamylcysteine, and even soluble proteins in needles of Scots pine from SO_2-polluted sites, whereas activities of superoxide dismutase and peroxidases did not increase in response to exposure to SO_2. According to Willekens *et al.* (1994), increases in mRNA levels suggested increased expression of catalase and glutathione peroxidase genes as a result of SO_2 fumigation.

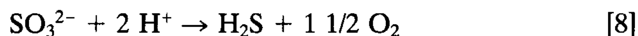
C. Sulfate Sequestration

Sulfate, usually the main product of SO_2 detoxification (Rennenberg, 1984; Maas *et al.*, 1987; Wiese *et al.*, 1996), accumulates in leaves or needles.

Concentrations up to 240 mmol (kg dry wt)$^{-1}$, corresponding to about 200 m*M* in cellular solution, have been reported (Pfanz and Beyschlag, 1993; but see Figs. 7 and 9 for lower values). As an inhibitor of ribulose bisphosphate carboxylase (Paulsen and Lane, 1966) and of photophosphorylation (Ryrie and Jagendorf, 1971), it inhibits photosynthesis of isolated chloroplasts (Baldry *et al.*, 1968). In leaves, it is transported from the chloroplasts through the phosphate translocator of the chloroplast envelope (Hampp and Ziegler, 1977) into the cytosol and from there in an energy-dependent reaction into the vacuoles (Kaiser *et al.*, 1989) where it is immobilized. A small part (0.3 to 1%) of the total amount has been found in the apoplast of leaves (Polle *et al.*, 1994) from which it is distributed to other locations within the plant. After Garsed and Read (1977) reported loss of sulfate from the roots, further attempts to demonstrate excretion of sulfate from the plants through the root system were unsuccessful (Kaiser *et al.*, 1993b; Wiese *et al.*, 1996).

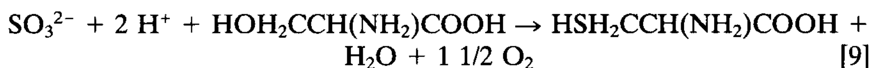
D. Reductive Detoxification

In the light, chloroplasts can either oxidize or reduce SO_2. They contain sulfite reductase. The reduction of sulfite is ferredoxin-dependent (Schwenn, 1994). Whereas oxidation actually increases the acidification produced by the formation of sulfurous acid because sulfuric acid is a stronger acid, reduction alleviates acidification. During light-dependent reduction, hydrogen sulfide is an intermediate:



$$SO_3^{2-} + 2\,H^+ \rightarrow H_2S + 1\,1/2\,O_2 \qquad [8]$$

It can escape into the atmosphere. However, only a small percentage of the SO_2 that has entered leaves is re-emitted as H_2S (Hällgren and Fredriksson, 1982; Sekiya *et al.*, 1982; Kindermann *et al.*, 1995; Rennenberg and Herschbach, 1996). Most of the reduced sulfur is, via *O*-acetylserine, incorporated into serine. The overall reaction may be simplified as



$$SO_3^{2-} + 2\,H^+ + HOH_2CCH(NH_2)COOH \rightarrow HSH_2CCH(NH_2)COOH + H_2O + 1\,1/2\,O_2 \qquad [9]$$

From cysteine, the sulfur is transfered to methionine, glutathione, and other sulfur-containing compounds. The sulfur-containing amino acids are incorporated into newly synthesized protein which contains approximately 1% sulfur.

In intact chloroplasts, maximum rates of light-dependent reductive sulfite detoxification were, in the presence of *O*-acetylserine as sulfide acceptor, about 2.5 μmol (mg chl h)$^{-1}$. Thus, reduction in intact chloroplasts can be even faster than sulfite oxidation (Dittrich *et al.*, 1992). Also, maximum

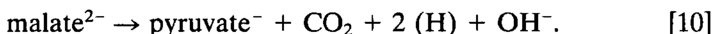
reduction rates observed at a sulfite concentration of 1.5 m*M* are manyfold higher than SO_2 fluxes from moderately polluted air into leaves (Fig. 2). However, it must be stressed once again that sulfite never accumulates in leaves. Also, sulfite reduction is strongly depressed in the absence of *O*-acetylserine. Thus, the availability of sulfide acceptors and of sulfite will limit rates of reduction *in vivo*.

A main problem in reductive SO_2 detoxification is storage of reduced sulfur. Glutathione levels are known to increase when plants are exposed to SO_2 (Grill *et al.*, 1982; Soldatini *et al.*, 1992; Rennenberg *et al.*, 1996). However, when compared to the storage capacity of leaves for sulfate, storage of reduced sulfur in solutes of low molecular weight is negligible. Most of the reduced sulfur goes into protein, but net protein synthesis in leaves or needles of trees is largely restricted to periods of leaf development or to the appearance and development of the new flush in conifers. It is absent during the winter time. In spruce growing at sites where SO_2 immission was low and periods of active metabolism were extended, SO_2 reduction surpassed oxidation, whereas at exposed sites and increased SO_2 immission oxidation was the predominant detoxification mechanism (Slovik *et al.*, 1995; Wiese *et al.*, 1996).

E. pH Stat Mechanisms

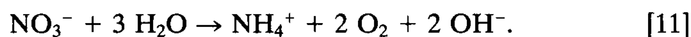
The accumulation of high concentrations of SO_2-derived sulfate in needles of spruce (Kaiser *et al.*, 1993a; Pfanz and Beyschlag, 1993) is evidence of acidification by sulfuric acid. Nevertheless, pH values of needle homogenates from trees growing at SO_2-polluted sites were comparable to pH values of homogenates of unstressed material. Obviously, neutralization of sulfuric acid had occured *in vivo*.

Base for neutralization may be mobilized by decarboxylation of endogenous acids such as malate:



$$\text{malate}^{2-} \rightarrow \text{pyruvate}^{-} + CO_2 + 2\,(H) + OH^{-}. \qquad [10]$$

Alternatively, nitrate may be reduced in the light:



$$NO_3^{-} + 3\,H_2O \rightarrow NH_4^{+} + 2\,O_2 + 2\,OH^{-}. \qquad [11]$$

Base in the form of NH_3 is also liberated during the oxidation of proteins.

When leaves were incubated with 15% CO_2 as a potential acid instead of SO_2 to avoid the high toxicity of the sulfite anions, NH_3 accumulated slowly, within hours, in the leaf tissue. It disappeared slowly after the acid stress was alleviated (Heber *et al.*, 1994). Malate was degraded under acid stress in sycamore cells (Guern *et al.*, 1986; Mathieu *et al.*, 1986) and SO_2 fumigation decreased the content of organic anions in leaves of oak

(Thomas and Runge, 1992). However, in contrast to these slow responses to acid stress, the inhibition of photosynthesis by CO_2 was rapidly, within several minutes, relieved while CO_2 was present in air at concentrations of up to 25% or more (Heber *et al.*, 1994). Photosynthesis is known to be highly sensitive to acidification of the chloroplast stroma (Werdan *et al.*, 1975). The rapid reversal of inhibition indicates the presence of a powerful mechanism of pH regulation in the chloroplasts of leaves which is faster than the slow biochemical production of base under acid stress.

In sycamore cells, acid stress triggered the rapid pumping of protons across the plasmalemma into the medium (Gout *et al.*, 1992), whereas in leaves energy-dependent transport of protons into the vacuoles of leaf cells appeared to be the predominant mechanism of rapid pH regulation (Wagner *et al.*, 1990; Hauser *et al.*, 1995b; Heber *et al.*, 1995).

Transport of protons together with sulfate into the vacuoles may only be the first stage of pH regulation in leaves when SO_2 is the source of acid stress (Kaiser *et al.*, 1989). Slow subsequent proton/cation exchange across the tonoplast finally appears to adjust also the vacuolar pH to controlled values. This must be concluded from the observation that sulfate accumulation in the vacuoles of mesophyll cells of spruce from field sites which are exposed to SO_2-polluted air is not accompanied by corresponding vacuolar acidification (Kaiser *et al.*, 1993a). From the cytoplasm, protons are pumped into the apoplast while cations enter the cytoplasm. The further path of the protons is unclear, but they finally appear to leave the plant through the roots in exchange against cations (Thomas and Runge, 1992; Kaiser *et al.*, 1993b). Thus, there is evidence that even dry deposition of SO_2 results finally in soil acidification.

VI. Effects of SO_2

A. Interference with Cation Nutrition

As long-term acid import in the form of SO_2 into needles resulted in sulfate accumulation but not in acidification, it is obvious that protons were exported from the needles while sulfate accumulated. To maintain electroneutrality, proton export must either be accompanied by the export of anions or by import of cations. Since sulfate is sequestered in the leaf cell vacuoles, cations are imported in exchange for protons which left the leaves. At observed levels of sulfate accumulation, base production by biochemical pH stat mechanisms is insufficient to liberate the cations needed as counterions for the sulfate that is sequestered in the vacuoles (Slovik *et al.*, 1992a,b; Pfanz and Beyschlag, 1993).

Cations sequestered and immobilized in the vacuoles are unavailable for the metabolic requirements of growth (Slovik, 1996). In extreme cases, cation deficiency symptoms are expected to appear. Magnesium deficiency is characterized in spruce by needle chlorosis. Extensive yellowing of needles has been reported for the Fichtelgebirge (Zech *et al.*, 1985; Lange *et al.*, 1987; Weikert *et al.*, 1989) on the Western side of the Eger valley in Czechia where brown coal is burnt in large power plants. It is spreading into the Erzgebirge north of the Eger valley. Removal of buds prevented the yellowing of last year's needles, apparently by preventing the translocation of magnesium to the new flush (Lange *et al.*, 1987; Weikert *et al.*, 1989). Also, fertilization with magnesium salts led to the recovery from chlorosis (Kaupenjohann and Zech, 1989; Zöttl and Hüttl, 1986). However, in vacuoles, potassium rather than magnesium is the dominant cation. Calcium appears to play a minor role (Slovik *et al.*, 1996b). Potassium deficiency was observed by Rehfuss (1983) and Tomlinson (1987). Fertilization with potassium has been shown to revitalize Norway spruce in regions chronically exposed to SO_2 (Ranft, 1982; Zöttl *et al.*, 1989). In Fig. 7, the sum of K^+ and Mg^{2+}, expressed in meq (kg dry wt)$^{-1}$, is compared with needle contents of sulfate, again on a meq kg^{-1} basis to permit direct comparison of mono- and divalent ions. The needles were from spruce growing in Bavaria and Saxony. The difference between the sum of the cations and sulfate became smaller with needle age. Importantly, it was still large even in 4-year-old needles from the Würzburg area where the SO_2 content of air is very low. The gap narrowed with increasing atmospheric SO_2 concentrations. Measurements at the Kahleberg, Erzgebirge, indicated increased cation availability in 1992 compared with 1991. Inquiries revealed that the site had been fertilized.

The data indicate that atmospheric SO_2 can produce cation deficiencies which are not revealed by usual analysis. Conventional measurements do not differentiate between the cations available for the needs of metabolism and those immobilized as counterions of sequestered sulfate. Obviously, long-term resistance to SO_2 requires the mobilization of cations by the root system of plants on a proton/cation exchange basis. Simultaneously, this solves problems of acidification and of availability of essential cations. Cation availability of the soil thus becomes an important aspect of plant tolerance to SO_2. By leaching cations from the soil, wet deposition of SO_2 aggrevates the problem of nutrient availability. In this situation, reduced growth must be expected. Tree ring studies have indeed revealed a clear relationship between SO_2 immissions and tree productivity (Elling, 1992, 1993; see also Wentzel, 1982).

B. Interference with Stress Tolerance

It has long been known that prolonged exposure to SO_2-polluted air decreases frost tolerance (Wentzel, 1956; Davison *et al.*, 1988). Frost hardening is a complex process. It involves the accumulation in the cells of cryoprotectants such as soluble sugars which decrease the extent of cellular dehydration during extracellular ice formation at subzero temperatures (Levitt, 1980) and the formation of specific proteins which are capable of preventing membrane rupture during freeze–thaw cycles (Sieg *et al.*, 1996). Perhaps, the increase in frost sensitivity is related to the lowering of carbohydrate contents and to a decrease in the activity of sucrose synthase and sucrose-6-phosphate phosphatase observed as a result of the exposure of *Picea abies* and *Pinus sylvestris* to low concentrations of SO_2 (Miller, 1987; Peace *et al.*, 1995).

C. Differential Sensitivity of Plants to SO_2

Reductive detoxification of SO_2 avoids not only problems associated with acidification and the necessity to provide cations as counterions for accumulated sulfate but also radical damage which may occur during light-dependent sulfite oxidation. It is therefore to be expected that herbaceous plants such as maize, with a ratio of carbon to reduced sulfur in the biomass of about 600/1, are less sensitive to SO_2 than trees with a much higher ratio. For spruce, the ratio of carbon to reduced sulfur in the total biomass has been estimated to be about 10,000/1 (Heber *et al.*, 1987). Obviously, for a comparable deposition of carbon in the biomass, maize can deposit much more SO_2 in the innocuous reduced form than spruce. Also, maize grows on well fertilized soil and spruce usually on unfertilized soil.

However, SO_2 tolerance is very different even among trees. An extensive list on the order of tolerance to "smoke gas" has been published as early as 1883 (von Schröder and Reuss, 1883). Very sensitive to SO_2 are the conifers *Abies alba* (white fir), *Picea abies* (Norway spruce), *Picea omorica* (Serbian spruce), and *Pinus sylvestris* (Scots pine) and insensitive are *Picea pungens* (Colorado spruce), *Pinus nigra* (Austrian spruce), and *Pinus mugo* (dwarf pine; Dässler, 1991). Interestingly, this order of tolerance is valid for field conditions only, not for fumigation experiments with concentrations of SO_2 well above those experienced in the field (Wiese *et al.*, 1996). In Fig. 9, sulfate contents are compared as a function of needle age in needles of Norway spruce, Scots pine, and Colorado spruce from an SO_2-exposed site at the Kahleberg and an unpolluted site at Würzburg. Only the Norway spruce from the

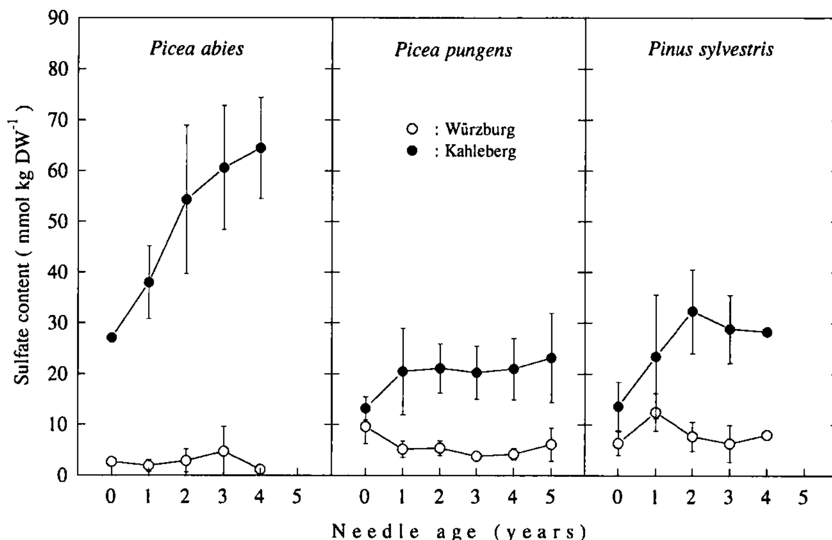


FIG. 9 Contents of sulfate (mmol (kg dry weight)$^{-1}$) in needles of three conifer species growing next to one another at the Kahleberg (●) and at Würzburg (o) as a function of needle age. SO_2 pollution of air is negligible at Würzburg (about 5 nPa Pa^{-1} SO_2) and considerable at the Kahleberg, Erzgebirge (about 30 nPa Pa^{-1} SO_2, see Fig. 2). Lines indicate standard deviation. From *Planta*, Detoxification of SO_2 in conifers differing in SO_2 tolerance. Hüve *et al.* **195,** 578–585, fig. 4 (1995), © Springer-Verlag, with permission.

Kahleberg exhibited clear signs of damage, whereas Colorado spruce had a healthy appearance. Even though the different species had been growing side by side, only needles of Norway spruce from the exposed site accumulated sulfate to high levels, whereas not much accumulation was seen in the needles of Colorado spruce from the same location. Scots pine occupied an intermediate position, both in appearance and sulfate accumulation. The differences in sulfate accumulation cannot be explained by stomatal closure (Atkinson and Winner, 1989), as rates of photosynthesis had been comparable. Either Colorado spruce is capable of increased reductive detoxification of SO_2, or, rather than being stored in leaf cell vacuoles, sulfate is exported and distributed within the plant. Interestingly, when exposed to high concentrations of SO_2, even those conifers that were tolerant to ambient levels showed symptoms of damage comparable to the damage suffered by sensitive species. Moreover, sensitivity of one particular species to high concentrations of SO_2 changed during the year. It was higher in May when new twigs developed than earlier or later in the year (Wiese *et al.,* 1996).

VII. Conclusions

In general, plants are admirably equipped with mechanisms to detoxify reactive molecules once they are introduced into leaves from the atmosphere through open stomata. Leaves are effective gas exchange systems constructed to balance entry of carbon dioxide, the substrate of photosynthesis, against the escape of cellular water by transpiration. Once air pollutants have entered through open stomata, they are detoxified in the mesophyll by reactions which are highly competitive in relation to uncontrolled reactions that are potentially damaging. Since legislation and international agreements have reduced SO_2 emissions in many countries (Tables I and II), most higher plants appear to be capable of detoxifying SO_2 sufficiently fast either by reduction to the sulfide level or by oxidation to sulfuric acid to avoid direct damage by uncontrolled reactions of sulfites (Fig. 10). Insofaras oxidation occurs in the light, reactive radicals might be formed to some extent during detoxification even under field conditions. However, radical chain propagation is effectively prevented by radical scavengers and antioxidants. Most of the oxidation of oxidation-sensitive cellular catalysts that cannot be avoided is reversed by cellular reduction. At ambient concentrations of SO_2 in air (Fig. 2), radical production may perhaps be a contribut-

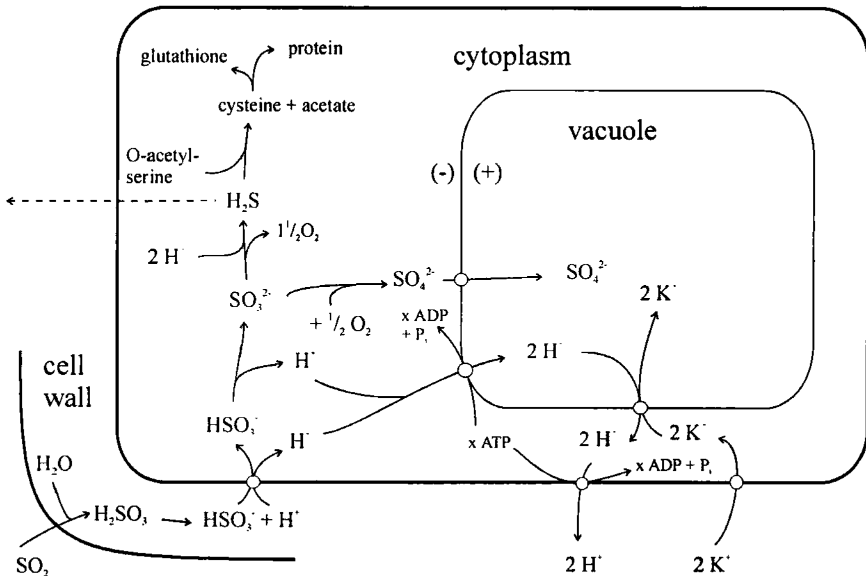


FIG. 10 Pathways of SO_2 detoxification in leaf cells. In the light, chloroplasts (not shown) of the mesophyll are main sites both of reductive and of oxidative detoxification.

ing factor in shortening the life span of long-living plants such as trees. However, the main threat to cellular balance seems to be the acidification accompanying the oxidative detoxification of SO_2. In the short run, cellular pH regulation can cope with considerable acid loads: protons are pumped together with sulfate into vacuoles. Why this is not effective in the long run is presently unknown. However, it appears that if cations can no longer balance the sulfate slowly accumulating in the vacuoles during chronic exposure to low levels of SO_2, leaves or needles are dropped. This leads to the well-known phenomenon of crown thinning. Neutralization of acid introduced from the atmosphere requires base. As biochemical base mobilization from cellular resources is insufficient to neutralize invading acid, the only recourse is export of protons in exchange for cations. Since, finally, the protons are exported into the soil and cations need to be mobilized in exchange, a main problem related to SO_2 toxicity becomes soil quality. In many cases, canopy thinning, slow growth, and premature ageing of forest trees exposed chronically to low levels of SO_2 can be traced back to damage caused by inadequate cation nutrition.

From 1984 to 1992, 16,000 km^2 forest area have been limed or fertilized in Germany (FMNAF, 1993), not in recognition of these relations, but rather in an attempt to remedy soil acidification caused by wet deposition. It now seems that it is less important to counter soil acidification than to increase the availability of essential cations in the soil, particularly those of potassium and magnesium.

References

Abernethy, J. L. (1967). Franz Hofmeister. The impact of his life and research on chemistry. *J. Chem. Educ.* **44,** 177–180.

Alscher, R. (1984). Effect of sulfur dioxide on light-modulated enzyme reactions. *In* "Gaseous Air Pollutants and Plant Metabolism" (M. J. Koziol and F. R. Whatley, eds.), pp. 181–200. Butterworths, London.

Alscher, R., Franz, M., and Jeske, C. W. (1987). Sulfur dioxide and chloroplast metabolism. *Recent Adv. Phytochem.* **21,** 1–28.

Asada, K. (1980). Formation and scavenging of superoxide in chloroplasts, with relation to injury by sulfur dioxide. *Res. Rep. Natl. Inst. Environ. Stud. (Jpn.)* **11,** 165–179.

Asada, K., and Kiso, K. (1973). Initiation of aerobic oxidation of sulfite by illuminated spinach chloroplasts. *Eur. J. Biochem.* **33,** 253–257.

Asada, K., and Takahashi, M. (1987). Production and scavenging of active oxygen in photosynthesis. *In* "Photoinhibition" (D. J. Kyle, C. B. Osmond, and C. J. Arntzen, eds.), pp. 227–287. Elsevier, Amsterdam.

Atkinson, C. J., and Winner, W. E. (1989). Modification of stomatal conductance by sulphur dioxide. *J. Exp. Bot.* **40,** 461–467.

Baldry, C. W., Cockburn, W., and Walker, D. A. (1968). Inhibition, by sulphate, of the oxygen evolution associated with photosynthetic carbon assimilation. *Biochim. Biophys. Acta* **153,** 476–483.

Ballantyne, D. J. (1977). Sulphite oxidation by mitochondria from green and etiolated peas. *Phytochemistry* **16,** 49–50.

Black, V. J. (1985). SO_2 effects on stomatal behavior. *In* "Sulphur Dioxide and Vegetation" (W. E. Winner, H. A. Mooney, and R. A. Goldstein, eds.), pp. 96–117. Stanford University Press, Stanford, CA.

Buchanan, B. B. (1980). Role of light in the regulation of chloroplast enzymes. *Annu. Rev. Plant Physiol.* **31,** 341–374.

Calvert, J. G., and Stockwell, W. R. (1983). Acid generation in the troposphere by gas-phase chemistry. *Environ. Sci. Technol.* **17,** 428A-443A.

Cowan, I. R., and Farquhar, G. D. (1977). Stomatal function in relation to leaf metabolism and environment. *In* "Integration of Activity in the Higher Plant" (D. H. Jennings, ed.), pp. 471–505. Cambridge University Press, Cambridge, UR.

Darrell, N. M. (1989). The effect of air pollutants on physiological processes in plants. *Plant Cell Environ.* **12,** 1–30.

Dässler, H.-G. (1991). "Einfluss von Luftverunreinigungen auf die Vegetation." Fischer, Jena.

Davison, A. W., Barnes, J. D., and Renner, C. J. (1988). Interactions between air pollutants and cold stress. *In* "Air Pollution and Plant Metabolism" (S. Schulte-Hostede, N. M. Darrell, L. W. Blank, and A. R. Wellburn, eds.), pp. 307–328. Elsevier, London.

De Kok, L. J. (1995). Sulfur metabolism in plants exposed to atmospheric sulfur. *In* "Sulfur Nutrition and Sulfur Assimilation in Higher Plants." 2nd edition (H. Rennenberg, C. Brunold, L. J. De Kok, and I. Stulen, eds.), pp. 111–131. SPB Academic Publ., The Hague.

Dittrich, A. P. M., Pfanz, H., and Heber, U. (1992). Oxidation and reduction of sulfite by chloroplasts and formation of sulfite addition compounds. *Plant Physiol.* **98,** 738–744.

Elling, W. (1992). Waldschäden und Waldschadensforschung. *Naturwiss. Rundsch.* **45,** 184–189.

Elling, W. (1993). Immissionen im Ursachenkomplex von Tannenschädigung und Tannensterben. *Allg. Forst Z.* **48,** 87–95.

Emde, F. A. (1993). Waldschäden und grenzüberschreitende Luftverschmutzung. *Allg. Forst Z.* **48,** 1298–1299.

Federal Ministry of Nutrition, Agriculture and Forestry (FMNAF) (1993). "Waldzustandsbericht der Bundesregierung." FMNAF, Bonn.

Foyer, C. H., Lelandais, M., Edwards, E. A., and Mullineaux, P. M. (1991). The role of ascorbate in plants, interactions with photosynthesis and regulatory significance. *Curr. Top. Plant Physiol.* **6,** 131–145.

Garsed, S. G., and Read, D. J. (1977). Sulphur dioxide metabolism in soybean, *Glycine max* var. biloxi. I. The effects of light and dark on uptake and translocation of ^{35}S. *New Phytol.* **78,** 11–119.

Gout, E., Bligny, R., and Douce, R. (1992). Regulation on intracellular pH values in higher plant cells. *J. Biol. Chem.* **267,** 13903–13909.

Grill, D., Esterbauer, H., and Hellig, K. (1982). Further studies on the effect of SO_2 pollution on the sulphhydryl system of plants. *Phytopathol. Z.* **104,** 264–271.

Guderian, R. (1977). Air pollution, phytotoxicity of acidic gases and its significance in air pollution control. *Ecol. Stud.* **22.**

Guderian, R., van, Haut, H., and Stratmann, H. (1960). Probleme der Erfassung und Beurteilung von Wirkungen gasförmiger Luftverunreinigungen auf die Vegetation. *Z. Pflanzenkr. Pflanzenschutz* **67,** 257–264.

Guern, J., Mathieu, Y., Pean, M., Pasquier, C., Beloeil, J.-C., and Lallemand, J.-Y. (1986). Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. I. A ^{31}P NMR description of acid-load effects. *Plant Physiol.* **82,** 840–845.

Häberlein, I., and Follmann, H. (1991). Die Sulfitolyse von Thioredoxinen. *Naturwissenschaften* **78,** 259–265.

Hällgren, J. E. (1978). Physiological and biochemical effects of sulfur dioxide on plants. *In* "Sulfur in the Environment. II. Ecological Impacts" (J. O. Nriagu, ed.), pp. 164–209. Wiley, New York.

Hällgren, J. E., and Fredriksson, S. A. (1982). Emission of hydrogen sulfide from sulfur dioxide fumigated pine trees. *Plant Physiol.* **70,** 456–459.

Hampp, R., and Ziegler, I. (1977). Sulfate and sulfite translocation via the phosphate translocator of the inner envelope membrane of chloroplasts. *Planta* **137,** 309–312.

Haselhoff, E., and Lindau, G. (1903). "Die Beschädigung der Vegetation durch Rauch." Borntraeger, Leipzig.

Hauser, M., Eichelmann, H., Heber, U., and Laisk, A. (1995a). Chloroplast pH values and buffer capacities in darkened leaves as revealed by CO_2 solubilization *in vivo*. *Planta* **196,** 199–204.

Hauser, M., Eichelmann, H., Oja, V., Heber, U., and Laisk, A. (1995b). Stimulation by light of rapid pH regulation in the chloroplast stroma *in vivo* as indicated by the titration of leaves with CO_2. *Plant Physiol.* **108,** 1059–1066.

Heath, R. L. (1980). Initial events in injury to plants by air pollutants. *Annu. Rev. Plant Physiol.* **31,** 395–431.

Heber, U., Laisk, A., Pfanz, H., and Lange, O. L. (1987). Wann ist SO_2 Nährstoff und wann Schadstoff? *Allg. Forst Z.* **27/28/29,** 700–705.

Heber, U., Wagner, U., Neimanis, S., Bailey, K., and Walker, D. (1994). Fast cytoplasmic pH regulation in acid-stressed leaves. *Plant Cell Physiol.* **35,** 479–488.

Heber, U., Hauser, M., Oja, V., Laisk, A., Bligny, R., and Douce, R. (1995). Photosynthesis and pH regulation in leaves. *In* "Photosynthesis: from Light to Biosphere" (P., Mathis, ed.), Proc. 10th Int. Photosynth. Congr., Vol. 5, pp. 441–446. Klüwer Academic Publishers, Dordrecht, The Netherlands.

Hüve, K., Dittrich, A., Kindermann, G., Slovik, S., and Heber, U. (1995). Detoxification of SO_2 in conifers differing in SO_2 tolerance. A comparison of *Picea abies, Picea pungens* and *Pinus sylvestris*. *Planta* **195,** 578–585.

Jäger, H. J. (1989). Stand der Diskussion über Richtwerte für Schadstoffkonzentrationen in der Luft. *In* "International Congress on Forest Decline Research: State of Knowledge and Perspectives" (B., Ulrich, ed.), Vol. 2, pp. 717–731. Friedrichshafen.

Jolivet, P., Bergeron, E., and Meunier, J.-C. (1995). Evidence for sulphite oxidase activity in spinach leaves. *Phytochemistry* **40,** 667–672.

Kaiser, G., Martinoia, E., Schröppel-Meier, G., and Heber, U. (1989). Active transport of sulfate into the vacuole of plant cells provides halotolerance and can detoxify SO_2. *J. Plant Physiol.* **133,** 756–763.

Kaiser, W. M., Dittrich, A., and Heber, U. (1993a). Sulfate concentrations in Norway spruce needles in relation to atmosphric SO_2: A comparison of trees from various forests in Germany with trees fumigated with SO_2 in growth chambers. *Tree Physiol.* **12,** 1–13.

Kaiser, W. M., Höfler, M., and Heber, U. (1993b). Can plants exposed to SO_2 excrete sulfuric acid through the roots? *Physiol. Plant.* **87,** 61–67.

Kaupenjohann, M., and Zech, W. (1989). Waldschäden und Düngung. *Allg. Forst Z.* **37,** 1002–1008.

Kindermann, G., Hüve, K., Slovik, S., Lux, H., and Rennenberg, H. (1995). Emission of hydrogen sulfide by twigs of conifers — a comparison of Norway spruce (*Picea abies* L.), Scotch pine (*Pinus sylvestris L.*) and blue spruce (*Picea pungens* Engelm.) in the ore mountains. *Plant Soil* **168–169,** 421-423.

Lange, O. L, Zellner, H., Gebel, J., Schramel, P., Köstner, B., and Czygan, F.-C. (1987). Photosynthetic capacity, chloroplast pigments and mineral content of the previous year's spruce needles with and without the new flush: Analysis of the forest decline phenomenon of needle bleaching. *Oecologia* **73,** 351–357.

Lange, O. L., Heber, U., Schulze, E.-D., and Ziegler, H. (1989). Atmospheric pollutants and plant metabolism. *Ecol. Stud.* **77,** 238–273.

Larson, E. M., and Jagendorf, A. T. (1989). Sulfite stimulating chloroplast coupling factor ATPase. *Biochim. Biophys. Acta* **973,** 67–77.

Lendzian, K. J. (1984). Permeability of plant cuticles to gaseous air pollutants. *In* "Gaseous Air Pollutants and Plant Metabolism" (M. J. Koziol and F. R. Whatley, eds.), pp. 77–82. Butterworth, London.

Levitt, J. (1980). "Responses of Plants to Environmental Stresses, "Vol. I. Academic Press, New York.

Likens, G. E., Driscoll, C. T., and Buso D. C. (1996). Long-term effects of acid rain: Response and recovery of a forest ecosystem. *Science* **272,** 244–246.

Maas, F. M., De Kok, L. J., Peters, J. L., and Kuiper, P. J. C. (1987). A comparative study on the effects of H_2S and SO_2 fumigation on the growth and accumulation of sulphate and sulphhydryl compounds in *Trifolium pratense* L., *Glycine max* Merr. and *Phaseolus vulgaris* L. *J. Exp. Bot.* **38,** 1459–1469.

Madamanchi, N. R., and Alscher, R. G. (1991). Metabolic bases for differences in sensitivity of two pea cultivars to sulfur dioxide. *Plant Physiol.* **97,** 88–93.

Mathieu, Y., Guern, J., Pean, M., Pasquier, C., Beloeil, J.-C., and Lallemand, J.-Y. (1986). Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. II. Possible mechanisms involved in pH regulation during acid-load. *Plant Physiol.* **82,** 846–852.

Mehler, A. H. (1951). Studies on the reaction of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. *Arch. Biochem. Biophys.* **34,** 339–351.

Miller, J. E. (1987). Effects of ozone and sulfur dioxide stress on growth and carbon allocation in plants. *Recent Adv. Phytochem.* **21,** 55–100.

National Academy of Sciences (1983). "Acid Deposition Atmospheric Processes in Eastern North America." National Academy of Sciences, National Academy Press, Washington, DC.

Nobel, P. S. (1983). "Biophysical Plant Physiology and Ecology." Freeman, San Francisco.

Okpodu, C. M., Alscher, R. G., Grabau, E. A., and Cramer, C. L. (1996). Physiological, biochemical and molecular effects of sulfur dioxide. *J. Plant Physiol.* **148,** 309–316.

Paulsen, J. M., and Lane, M. D. (1966). Spinach ribulose biphosphate carboxylase. I. Purification and properties of the enzyme. *Biochem.* **5,** 2350–2357.

Peace, E. A., Lea, P. J., and Darrall, N. M. (1995). The effect of open-air fumigation with SO_2 and O_3 on carbohydrate metabolism in Scots Pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). *Plant, Cell Environ.* **18,** 277–283.

Pechere, J. P., Dixon, H., Maybury, R. H., and Neurath, H. (1958). Cleavage of disulfide bonds in trypsinogen and α-chymotrypsinogen. *J. Biol. Chem.* **233,** 1364–1372.

Peiser, G., and Yang, S. F. (1985). Biochemical and physiological effects of SO_2 on nonphotosynthetic processes in plants. *In* "Sulfur Dioxide and Vegetation" (W. E. Winner H. A. Mooney, and R. A. Goldstein, eds.), pp. 148–161. Stanford University Press, Stanford, CA.

Petering, D. H. (1977). Sulfur dioxide: A view of its reactions with biomolecules. *In* "Biochemical Effects of Environmental Pollution" (S. D. Lee, ed.), pp. 293–306. Ann Arbor Books, Ann Arbor, MI.

Pfanz, H., and Beyschlag, W. (1993). Photosynthetic performance and nutrient status of Norway spruce (*Picea abies* (L.) Karst.) at forest sites in the Ore Mountains (Erzgebirge). *Trees* **7,** 115–122.

Pfanz, H., and Heber, U. (1986). Buffer capacities of leaves, leaf cells and leaf cell organelles in relation to fluxes of potentially acidic gases. *Plant Physiol.* **81,** 597–602.

Pfanz, H., Dietz, K.-J., Weinerth, I., and Oppmann, B. (1990). Detoxification of sulfur dioxide by apoplastic peroxidases. *In* "Sulfur Nutrition and Sulfur Assimilation in Higher Plants: Fundamental, Environmental and Agricultural Aspects" (H. Rennenberg, C. Brunold, L. J. De Kok, and I. Stulen, eds.), pp. 229–233. SPB Academic Publishing, The Hague.

Pienaar, J. J., and Helas, G. (1996). The kinetics of chemical processes affecting acidity in the atmosphere. *S. Afr. J. Sci.* **92,** 128–132.

Polle, A., Eiblmeier, M., and Rennenberg, H. (1994). Sulphate and antioxidants in needles of Scots pine (*Pinus sylvestris* L.) from three SO_2-polluted field sites in eastern Germany. *New Phytol.* **127,** 571–577.

Ranft, H. (1982). Düngung als Anpassungsmassnahme im Immissionsschadgebiet Oberes Erzgebirge. *Beitr. Forstwirtsch.* **3,** 119–122.

Rehfuss, K.-E. (1983). Walderkrankungen und Immissionen—eine Zwischenbilanz. *Allg. Forst Z.* **21,** 601–610.

Rennenberg, H. (1984). The fate of excess sulfur in higher plants. *Annu. Rev. Plant Physiol.* **35,** 121–153.

Rennenberg, H., and Herschbach, C. (1996). Responses of plants to atmospheric sulfur. *In* "Plant Response to Air Pollution" (M. Yunus and M. Iqbal, eds.) pp. 285–293. Wiley, New York.

Rennenberg, H., and Polle, A. (1994). Metabolic consequences of atmospheric sulfur influx into plants. *In* "Plant Response to Gaseous Environment" (R. Alscher and A. Wellburn, eds.), pp. 165–180. Chapman & Hall, London.

Rennenberg, H., Herschbach, C., and Polle, A. (1996). Consequences of air pollution on shoot-root interactions. *J. Plant Physiol.* **148,** 296–301.

Rohde, H. (1978). Budget and turn-over times of atmospheric sulfur compounds. *Atmos. Environ.* **12,** 671–680.

Ryrie, I. J., and Jagendorf, A. T. (1971). Inhibition of photophosphorylation in spinach chloroplasts by inorganic sulfate. *J. Biol. Chem.* **246,** 582–588.

Schwenn, J. D. (1994). Photosynthetic sulphate reduction. *Z. Naturforsch., C: Biosci.* **49C,** 531–539.

Sekiya, J., Wilson, L. G., and Filner, P. (1982). Resistance to injury by sulfur dioxide. Correlation with its reduction to, and emission of, hydrogen sulfide in Cucurbitaceae. *Plant Physiol.* **70,** 437–441.

Sieg, F., Schröder, W., Schmitt, J. M., and Hincha, D. K. (1996). Purification and characterization of a cryoprotective protein (cryoprotectin) from the leaves of cold-acclimated cabbage. *Plant Physiol.* **111,** 215–221.

Slovik, S. (1996). Chronic SO_2- and NO_x-pollution interferes with the K^+ and Mg^{2+} budget of Norway spruce trees. *J. Plant Physiol.* **148,** 276-286

Slovik, S., Kaiser, W. M., Körner, C., Kindermann, G., and Heber, U. (1992a). Quantifizierung der physiologischen Kausalkette von SO_2-Immssionsschäden für Rotfichten (*Picea abies* (L.) Karst.). I. Ableitung von SO_2-Immissionsgrenzwerten für akute Schäden an Fichte. *Allg. Forst Z.* **15,** 800–805.

Slovik, S., Kaiser, W. M., Körner, C., Kindermann, G., and Heber, U. (1992b). Quantifizierung der physiologischen Kausalkette von SO_2-Immssionsschäden für Rotfichten (*Picea abies* (L.) Karst.). II. Ableitung von SO_2-Immissionsgrenzwerten für chronische Schäden. *Allg. Forst Z.* **17,** 913–920.

Slovik, S., Siegmund, A., Kindermann, G., Riebeling, R., and Balacs, A. (1995). Stomatal SO_2 uptake and sulfate accumulation in needles of Norway spruce stands (*Picea abies*) in Central Europe. *Plant Soil* **168–169,** 405-419.

Slovik, S., Siegmund, A., Führer, H.-W., and Heber, U. (1996a). Stomatal uptake of SO_2, N_{ox} and O_3 by spruce crowns (*Picea abies*) and canopy damage in Central Europe. *New Phytol.* **132,** 661–676.

Slovik, S., Hüve, K., Kindermann, G., and Kaiser, W. M. (1996b). SO_2-dependent cation competition and compartmentation in Norway spruce needles. *Plant, Cell Environ.* **19,** 321–328.

Soldatini, G. F., Ranierei, A., Lencioni, L., and Lorenzini, G. (1992). Effects of continuous SO_2 fumigation on SH-containing compounds in two wheat cultivars of different sensitivities. *J. Exp. Bot.* **43,** 797-801.

Stöckhardt, J. A. (1871). Untersuchungen über die schädliche Einwirkung des Hütten- und Steinkohlenrauches auf das Wachsthum der Pflanzen, insbesondere der Fichte und Tanne. *Tharandter Forstl. Jahrb.* **21,** 218–254.

Takahama, U., Veljovic-Jovanovic, S., and Heber, U. (1992). Effects of the air pollutant SO_2 on leaves: Inhibition of sulfite oxidation in the apoplast by ascorbate and of apoplastic peroxidase by sulfite. *Plant Physiol.* **100,** 261-266.

Tanaka, K., Otsubo, T., and Kondo, N. (1982). Participation of hydrogen peroxide in the inactivation of Calvin-cycle SH enzymes in SO_2-fumigated spinach leaves. *Plant Cell Physiol.* **23** (6), 1009–1018.

Taylor, G. E. J., and Tingey, D. T. (1983). Sulfur dioxide flux into leaves of *Geranium carolinianum* L. *Plant Physiol.* **72,** 237–244.

Thomas, F. M., and Runge, M. (1992). Proton neutralization in the leaves of English oak (*Quercus robur* L.) exposed to sulfur dioxide. *J. Exp. Bot.* **43,** 803–809.

Tomlinson, G. H. (1987). Acid deposition, nutrient imbalance and tree decline: A commentary. *In* "Effects of Atmospheric Pollutants on Forests, Wetlands and Agricultural Ecosystems" (T. C. Hutchinson and K. M. Meema, eds.), pp. 189–199. Springer, Berlin.

Ulrich, B., Mayer, R., and Khanna, P. K. (1980). Chemical changes due to acid precipitation in a loess-derived soil in Central Europe. *Soil Sci.* **30,** 193–199.

U.S. Environmental Protection Agency (USEPA) (1989). "National Air Quality and Emissions Trends Report, 1987," EPA-450/4-89-001. Office of Air Quality Planníng and Standards, Research Triangle Part, NC.

U.S. Environmental Protection Agency (USEPA) (1992). "National Air Quality and Emissions Trends Report, 1991," EPA-450/4-89-001. Office of Air Quality Planníng and Standards, Research Triangle Part, NC.

U.S. Environmental Protection Agency (USEPA) (1994). "A Clean Air Act Exposure and Effects Assessment 1993–1994," EPA 600/X-94-020. Office of Research and Development, Washington, DC.

Veljovic-Jovanovic, S., Bilger, W., and Heber, U. (1993). Inhibition of photosynthesis, stimulation of zeaxanthin formation and acidification in leaves by SO_2 and reversal of these effects. *Planta* **191;** 365–376.

von Schröder, J., and Reuss, C. (1883). "Die Beschädigung der Vegetation durch Rauch und die Oberharzer Hüttenrauchschäden." Parey, Berlin.

Wagner, U., Kolbowski, J., Oja, V., Laisk, A., and Heber, U. (1990). pH homeostasis of the chloroplast stroma can protect photosynthesis of leaves during the influx of potentially acidic gases. *Biochim. Biophys. Acta* **1016,** 115–120.

Weikert, R. M., Wedler, M., Lippert, M., Schramel, P., and Lange, O. L. (1989). Photosynthetic performance, chloroplast pigments, and mineral content of various needle age classes of spruce (*Picea abies*) with and without the new flush: An experimental approach for analysing forest decline phenomena. *Trees* **3,** 161–172.

Wellburn, A. R. (1985). Ion chromatographic determination of anions in plastids from fumigated and non-fumigated barley seedlings. *New Phytol.* **100,** 329–339.

Wentzel, K.-F. (1956). Winterfrost 1956 und Rauchschäden. *Allg. Forst Z.* **11,** 541–543.

Wentzel, K.-F. (1982). Ursachen des Waldsterbens in Mitteleuropa. *Allg. Forst Z.* **45,** 1365–1368.

Werdan, K., Heldt, H. W., and Milovancev, M. (1975). The role of pH in the regulation of carbon fixation in the chloroplast stroma. *Biochim. Biophys. Acta* **396,** 276–292.

Wieler, A. (1905). "Untersuchungen über die Einwirkung schwefliger Säure auf die Pflanzen." Borntraeger, Berlin.

Wiese, C., Slovik, S., and Kaiser, W. M. (1996). Wirkungen hoher SO_2-Konzentrationen auf Fichte, Stechfichte, Waldkiefer und Latsche. *Allg. Forst Z.* **10**, 570–572.

Willekens, H., Vancamp, W., Vanmontagu, M., Inze, D., Langebartels, C., and Sandermann, H. (1994). Ozone, sulfur dioxide, and ultraviolet b have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumpaginifolia. Plant Physiol.* **106,** 1007–1014.

Winner, W. E., Mooney, H. A., and Goldstein, R. A., eds. (1985). "Sulfur Dioxide and Vegetation." Stanford University Press, Stanford, CA.

Winter, H., Robinson, D. G., and Heldt, H. W. (1993). Subcellular volumes and metabolite concentrations in barley leaves. *Planta* **191,** 180–190.

Wislicenus, H. (1898). Resistenz der Fichte gegen saure Rauchgase bei ruhender und thätiger Assimilation. *Tharandter Forstl. Jahrb.* **48,** 152–172.

Wislicenus, H. (1908). Über die Grundlagen technischer und gesetzlicher Massnahmen gegen Rauchschäden. *In* "Wislicenus, H. Waldsterben im 19. Jahrhundert." VDI-Verlag, Düsseldorf (reprint 1985).

Wolfenden, J., Pearson, M., and Francis, B. J. (1991). Effects of over-winter fumigation with sulphur and nitrogen dioxides on biochemical parameters and spring growth in red spruce (*Picea rubens* Sarg.). *Plant, Cell Environ.* **14,** 35–45.

Würfel, M., Häberlein, I., and Follmann, H. (1990). Inactivation of thioredoxin by sulfite ions. *FEBS Lett.* **268,** 146–148.

Yin, Z. H., Neimanis, S., Wagner, U., and Heber, U. (1990). Light-dependent pH changes in leaves of C3 plants. I. Recording pH changes in different cellular compartments by fluorescent probes. *Planta* **182,** 244–252.

Zakharov, S. D., Bronnikov, D. E., and Maylan, A. N. (1983). Effect of sulfite on the Pi-ATP exchange activity of chloroplast and mitochondrial proton-ATPase proteoliposomes. *Biochem. Int.* **6,** 805–811.

Zech, W., Suttner, T. H., and Popp, E. (1985). Elemental analysis and physiological response of forest trees in SO_2 polluted areas of N.E. Bavaria. *Water, Air, Soil Pollut.* **25,** 175–183.

Zelitch, I. (1957). α-Hydroxysulfonates as inhibitors of the enzymatic oxidation of glycolic and lactic acids. *J. Biol. Chem.* **224,** 251–260.

Ziegler, I. (1974). Action of sulphite on plant malate dehydrogenase. *Phytochemistry* **13,** 2411–2416.

Ziegler, I. (1975). The effect of SO_2 pollution on plant metabolism. *Residue Rev.* **56,** 79–105.

Ziegler, I., Marewa, A., and Schoepe, E. (1976). Action of sulphite on the substrate kinetics of chloroplastic NADP-dependent gyceraldehyde-3-phosphate dehydrogenase. *Phytochemistry* **15,** 1627–1632.

Zöttl, H. W., and Hüttl, R. F. (1986). Nutrient supply and forest decline in southwest Germany. *Water, Air, Soil Pollut.* **31,** 449–462.

Zöttl, H. W., Hüttl, R. F., Fink, S., Tomlinson, G. H., and Wisniewski, G. (1989). Nutritional disturbances and histological changes in declining forests. *Water, Air, Soil Pollut.* 48, 87–109.

INDEX

A

Abnormalities, genetic, cytogenetic and molecular types, 20–21
Acidification, by sulfurous acid and sulfuric acid, 268–271
Adaptation, in tumor progression, 29–30
Amino acid sequence, zona pellucida, 65–70
Amplification, genes, 23–24
Antigens, *see* Autoantigens
Apoptosis, in parasitic flagellates, 191–193
Arabidopsis
  chm mutants, 125
  cuel mutants, 129–130
  morphology, effect of light, 131
  pac mutants, 129
Autoantigens, rabbit sperm, role in interaction with zona pellucida, 84–85
Autocrine signals, in cell survival and proliferation
  associated molecules, 201–202
  cell-to-cell interactions, 199–201
  complex media, 202–203
  nutrient media, 199

B

Behavior, tumor, variability, 2–3
Biogenesis, *Chlamydomonas reinhardtii* cytochromes, 157–158
Biological systems, chemosensory behavior, 219–220
Bradykinin, effect on *Saccharomyces cerevisiae* cell proliferation, 226–227

C

Cancer, *see also* Tumors
  clonal evolution, 12–14
  experimental and clinical research, 32–33
  human, cellular heterogeneity, 10–12
  treatment, tumor heterogeneity in, 34–36
Cations, nutrition, effect of sulfur dioxide, 275–276
Cell communication, *see* Signal transduction
Cell cultures, *Tetrahymena thermophila*, death mechanisms, 212–215
Cell death
  programmed, in metazoa, 186–188
  in *Saccharomyces cerevisiae*, 228–229
  Tetrahymena pyriformis, 217
  Tetrahymena setosa, 217–218
  Tetrahymena thermophila, mechanisms, 211–212
    death induction by staurosporine, 215–216
    in low-density cultures, 212–215
Cell fusion, and tumor heterogeneity, 31–32
Cell populations, normal, heterogeneity, 37–38
Cell proliferation
  autocrine signals
    associated molecules, 201–202
    cell-to-cell interactions, 199–201
    complex media, 202–203
    nutrient media, 199
  control in *Saccharomyces cerevisiae*, 224–225
    effect of compounds affecting cyclic GMP systems, 226

Cell proliferation (*continued*)
effect of insulin, 226
effect of protein kinase C, 226–227
effect of senescence factors, 228
intercellular and intracellular signal molecules, 227–228
nutrient media for, 225–226
overlap with other signals, 223–224
Tetrahymena
intercellular activation, 218–219
intracellular activation, 219
role of signal molecules, 196
receptors, 197–198
signal compound homologies, 197
signal imprinting and receptor induction, 198–199
Tetrahymena pyriformis, 217
Tetrahymena setosa, 217–218
Tetrahymena vorax, 218
Cell survival
autocrine signals
associated molecules, 201–202
cell-to-cell interactions, 199–201
complex media, 202–203
nutrient media, 199
overlap with other signals, 223–224
Tetrahymena
intercellular activation, 218–219
intracellular activation, 219
role of signal molecules, 196
receptors, 197–198
signal compound homologies, 197
signal imprinting and receptor induction, 198–199
Tetrahymena pyriformis, 217
Tetrahymena setosa, 217–218
Tetrahymena vorax, 218
Cell-to-cell interactions, in *Tetrahymena,* 199–201
Cellular heterogeneity
animal tumors, 9–10
as basis of tumor progression, Nowell hypothesis, 7–9
clonal evolution of cancer, 12–14
human cancers, 10–12
origins
epigenetic mechanisms in tumor progression
alterations in DNA methylation, 28–29
tumors and cell fusion, 31–32
genetic instability
cytogenetic and molecular genetic abnormalities, 20–21
gene amplification, 23–24
genetic damage and reactive oxygen species, 25
mutation frequency, 21–23
mutator phenotypes and DNA repair genes, 24–25
and tumor environment, 25–27
Chemoattractants, effect in *Tetrahymena* cells, 221
Chemosensory behavior
in biological systems, 219–220
in *Tetrahymena,* 220–221
effect of chemoattractants, 221
intracellular messenger systems, 222–223
overlap with other signals, 223–224
receptors, 221–222
Chlamydomonas reinhardtii
application of molecular genetics techniques, 121–122
chloroplast genes, 119
cytochrome biogenesis, 157–158
mitochondrial mutants, 125–126
Chloroplasts
assembly, subsequent degradation, 155–157
differentiation in C4 plants, 134–135
genes, in *Chlamydomonas reinhardtii,* 119
RNA polymerases, transcription, 136–138
Coalescence factors, in *Dictyostelium discoideum,* 193–195
Compatibility, nuclear–plastid interactions, 123–124
Conditioned medium factor, in *Dictyostelium discoideum,* 193–195
Cyclic AMP, in *Dictyostelium discoideum,* 193–195
Cyclic GMP
compounds affecting, effect on yeast cell proliferation, 226
role in metazoan cells, 207–208
role in unicellular eukaryotes, 208–210
Cyclophosphamide, susceptibility of tumor line 410.4, 16
Cytochrome b_6f, biogenesis, 157–158
Cytochrome c6, biogenesis, 157–158

D

Degradation
  chloroplast, after assembly, 155–157
  RNA, 141–142
Deposition
  dry, sulfur dioxide, 261–264
  wet, sulfur dioxide, 265
Detoxification, sulfur dioxide and hydration products
  oxidative detoxification, 271–272
  pH stat mechanisms, 274–275
  radical scavengers and antioxidants, 272
  reductive detoxification, 273–274
  sulfate sequestration, 272–273
Dictyostelium discoideum, coalescence factors, 193–195
Differentiation
  chloroplast in C4 plants, 134–135
  plastids, 128–130
  relationship to tumor progression, 30–31
Differentiation-inducing factor, in *Dictyostelium discoideum,* 193–195
DNA
  methylation, alterations, 28–29
  repair genes, and mutator phenotypes, 24–25
Dry deposition, sulfur dioxide, 261–264

E

Eggs
  mammalian
    extracellular matrix, 59
    gametic interaction with egg, 61–62
    interaction with sperm, 60–61
    ovulation, 58–59
    somatic cell layer, 59–60
    zona pellucida, 59
  plasma membrane, interaction with sperm, 85–87, 91–92
    candidate molecules, 91
    integrins, 88–90
    proteins, role in gamete interaction, 87–88
    role of DE protein, 92–94
    role of fertilin, 94–97
Emission, sulfur dioxide, 258–261
β-Endorphin-related receptor, in *Tetrahymena pyriformis,* 221–222
Environment, tumor, role in genetic instability, 25–27
Enzymes, effector, interaction with intercellular signal molecules, 227–228
Epidermal growth factor
  effect on *Tetrahymena,* 203
  in *Paramecium,* 190
Eukaryotes
  and prokaryote, signaling system comparison, 229–231
  unicellular
    cell communication in, 183
    role of cyclic GMP and nitric oxide, 208–210
    signaling systems, 189–190
      apoptosis in parasitic flagellates, 191–193
      coalescence factors in *Dictyostelium discoideum,* 193–195
      growth factors in paramecium, 190
      pheromones in *Euplotes raikovi,* 190–191
      Tetrahymena and yeast cells, 195
Euplotes raikovi, pheromones, 190–191
Evolution
  clonal, cancer, 12–14
  plastid mutations, 124
Extracellular matrix, in mammalian egg, 59

F

Fertilin, role in sperm–egg plasma membrane interaction, 94–97
Flagellates, parasitic, apoptosis in, 191–193
Fould's definition, tumor progression, 4–6
Fould's rules
  examples, 6–7
  tumor progression, 4–6
Frequency, mutations, 21–23

G

Galactosyltransferase, role in binding to zona pellucida, 77–78

Gametes
  egg plasma membrane protein role in interaction, 87–88
  interactions, 61–62
Genes
  amplification, 23–24
  chm, in *Arabidopsis*, 125
  damage, role in genetic instability, 25
  DNA repair, and mutator phenotypes, 24–25
  pac, mutants of *Arabidopsis*, 129
  plastid, nuclear control of expression
    chloroplast assembly and degradation, 155–157
    chloroplast RNA polymerases, 136–138
    coupling of mRNA metabolism and translation, 149–151
    cytochrome biogenesis, 157–158
    defective nuclear mutants, 144–146
    dependencies, 135
    effect of mutations on translation, 153–155
    gene splicing, 146–149
    nucleus-encoded RNA polymerases, 138–141
    protein import and targeting, 158–160
    RNA-binding proteins and translation, 151–153
    RNA processing and stability, 142–144
    transcription mutants, 141
  rbcL, 120
  system in plasmids, purpose, 119–121
  zona pellucida proteins, 63–65
Genetic analysis
  nuclear mutants with altered plastids, 126–128
  photosynthetic mutants, 128
Glycoproteins, zona pellucida, role in gamete interactions, 61–62
Growth, tumor line 168
  alteration, 16
  depression, 15
Growth factors, in *Paramecium*, 190

H

Heterogeneity, *see* Cellular heterogeneity; Tumor heterogeneity
Hormones
  biological activity in *Tetrahymena thermophila*, 205–206
  effect on *Tetrahymena*, 203
Hydration, products from sulfur dioxide
  oxidative detoxification, 271–272
  pH stat mechanisms, 274–275
  radical scavengers and antioxidants, 272
  reductive detoxification, 273–274
  sulfate sequestration, 272–273
  toxicity, 265–266

I

Immission, sulfur dioxide, 258–261
Inheritance, in plastids, 122
Insulin
  biological activity of fragments, 205–206
  biphasic response of *Tetrahymena thermophila*, 203–205
  effect on *Saccharomyces cerevisiae* cell proliferation, 226
Integrins, egg, 88–90
Intercellular messenger systems, interaction with intracellular messenger, 227–228
Intracellular messenger systems
  interaction with intercellular signal molecules, 227–228
  role in cells
    cyclic GMP and nitric oxide, 207–211
    protein kinases, 210–211
  in *Tetrahymena thermophila*, 222–223

L

Leishmania, apoptosis, 191–193

M

Mammalian cells, signal compound homology, 197
Mammalian growth factors, effect on *Tetrahymena*, 203–207
MAPK, *see* Mitogen-activated protein kinase
Maturation
  mRNA, defective nuclear mutants, 144–146
  RNA, 141–142
Media
  complex, for *Tetrahymena*, 202–203

nutrient
for cell proliferation control, 225–226
for *Tetrahymena,* 199
Melphalan, sensitivity of tumor line 4T07, 16
Membranes, *see* Plasma membranes
Metabolism, mRNA, coupling with translation, 149–151
Metastases, tumor lines 67 and 168, 16
Metazoan cells
communication, by protein kinase C, 185–186
death, 186–188
role of cyclic GMP and nitric oxide, 207–208
signal transduction
by MAPK pathways, 185
role of signal molecule, 183
Methotrexate, sensitivity of tumor line 66, 16
Methylation, DNA, alterations, 28–29
Mitochondria–plastid interactions, mutations, 124–126
Mitogen-activated protein kinase, in signal transduction in metazoa, 185
Mitogens, action in *Tetrahymena thermophila,* 206
Molecular genetics, technical developments, 121–122
Mutations
in *Arabidopsis* genes
chm, 125
cuel, 129–130
pac, 129
frequency, 21–23
genetic analysis
nuclear mutants with altered plastids, 126–128
photosynthetic mutants, 128
mitochondrial, in *Chlamyodomonas reinhardtii,* 125–126
nuclear
defective in mRNA maturation and stability, 144–146
effect on plastid translation, 153–155
in plastid–mitochondria interactions, 124–126
plastids
evolution, 124
from photomorphogenesis, 131–134
from transcription, 141
Mutators, phenotype, and DNA repair genes, 24–25

N

Nitric oxide
role in metazoan cells, 207–208
role in unicellular eukaryotes, 208–210
Nowell hypothesis
mutation frequency, 21–23
tumor progression, 7–9
Nucleocytoplasm, and plastid, signaling, 160–163
Nucleus
control of plastid gene expression
chloroplast assembly and degradation, 155–157
chloroplast RNA polymerases, 136–138
coupling of mRNA metabolism and translation, 149–151
cytochrome biogenesis, 157–158
defective nuclear mutants, 144–146
dependencies, 135
effect of mutations on translation, 153–155
gene splicing, 146–149
nucleus-encoded RNA polymerases, 138–141
protein import and targeting, 158–160
RNA-binding proteins and translation, 151–153
RNA processing and stability, 142–144
transcription mutants, 141
–plastid interactions
compatibility, 123–124
plasmid inheritance, 122
Nutrients, minimal substrates, 188–189
Nutrition, cation, effect of sulfur dioxide, 275–276

O

Ovulation, mammalian eggs, 58–60
Oxygen, reactive species, role in genetic instability, 25

P

Paramecium, growth factors, 190
pH, stat mechanisms, 274–275

Phenotypes, mutator, and DNA repair genes, 24–25
Pheromones, in *Euplotes raikovi,* 190–191
Photomorphogenesis, plastids, 130–134
Plants
  C4, chloroplast differentiation, 134–135
  differential sensitivity to sulfur dioxide, 277–278
  photomorphogenesis, 130–134
Plasma membranes, egg, interaction with sperm
  candidate molecules, 91
  egg integrin, 88–90
  general process, 85–87
  molecules involved in, 91–92
  proteins, role in gamete interaction, 87–88
  role of DE protein, 92–94
  role of fertilin, 94–97
Plastids
  altered, in nuclear mutants, 126–128
  differentiation, 128–130
  gene expression, nuclear control
    chloroplast assembly and degradation, 155–157
    chloroplast RNA polymerases, 136–138
    coupling of mRNA metabolism and translation, 149–151
    cytochrome biogenesis, 157–158
    defective nuclear mutants, 144–146
    dependencies, 135
    effect of mutations on translation, 153–155
    gene splicing, 146–149
    nucleus-encoded RNA polymerases, 138–141
    protein import and targeting, 158–160
    RNA-binding proteins and translation, 151–153
    RNA processing and stability, 142–144
    transcription mutants, 141
  gene system, purpose, 119–121
  –mitochondria interaction, mutations, 124–126
  –nuclear interactions
    compatibility, 123–124
    plasmid inheritance, 122
  and nucleocytoplasm, signaling, 160–163
  photomorphogenesis, 130–134
Platelet-derived growth factor, effect on *Tetrahymena,* 203
Prestarvation factor, in *Dictyostelium discoideum,* 193–195
Proacrosin, role in sperm–zona pellucida interaction, 83
Progression, *see* Tumor progression
Prokaryotes, and eukaryote, signaling system comparison, 229–231
Proliferation, *see* Cell proliferation
Protein kinase, in signal transduction in metazoa, 185–186
Protein kinase C
  effect on yeast cell proliferation, 226–227
  related activities in cells, 211
  in signal transduction in metazoa, 185–186
Protein kinase G, role in cells, 211
Proteins
  DE, role in sperm–egg plasma membrane interaction, 92–94
  egg plasma membrane, role in gamete interaction, 87–88
  import and targeting, 158–160
  membrane, sp56, role in binding to zona pellucida, 78–79
  PH-20, role in sperm–zona pellucida interaction, 82–83
  recombinant, zona pellucida, 73–74
  RNA-binding, translation, 151–153
  Sp17, role in interaction with rabbit sperm, 84–85
  sperm
    interaction with zona pellucida, 74–76
    role in primary binding to zona pellucida, 76–81
    role in secondary binding to zona pellucida, 82–85
  zona pellucida, encoding genes, 63–65
Protein tyrosine kinase, role in *Tetrahymena thermophila,* 210–211

R

Radicals, sulfur dioxide, toxicity, 266–267
Receptor
  induction in *Tetrahymena,* 198–199
  in *Tetrahymena,* 197–198
Ribulose-bisphosphate carboxylase oxygenase, *rbcL* gene, 120
RNA
  editing, 149

maturation and degradation, 141–142
messenger
maturation and stability, defective nuclear mutants, 144–146
metabolism and translation, coupling, 149–151
processing and stability, 142–144
RNA polymerase, transcription
chloroplast polymerase, 136–138
nucleus-encoded polymerase, 138–141

S

Saccharomyces cerevisiae
cell death, 228–229
cell proliferation control, 224–225
effect of compounds affecting cyclic GMP systems, 226
effect of insulin, 226
effect of protein kinase C, 226–227
effect of senescence factors, 228
intercellular and intracellular signal molecules, 227–228
nutrient media for, 225–226
signaling systems, 195
Senescence factors, effect on yeast cell proliferation, 228
Sensitivity, differential, plants to sulfur dioxide, 277–278
Sequestration, sulfates, 272–273
Signal imprinting, in *Tetrahymena,* 198–199
Signal molecules
autocrine, in *Tetrahymena thermophila,* 201–202
role in metazoa communication, 183
role in *Tetrahymena* survival and proliferation, 196
receptors, 197–198
signal compound homologies, 197
signal imprinting and receptor induction, 198–199
Signal transduction
mechanisms, 220
in metazoa
by protein kinase, 185–186
role of signal molecules, 183
between plastid and nucleocytoplasm, 160–163
prokaryote and eukaryote, comparison, 229–231
in unicellular eukaryotes, 183, 189–190
apoptosis in parasitic flagellates, 191–193
coalescence factors in *Dictyostelium discoideum,* 193–195
growth factors in paramecium, 190
pheromones in *Euplotes raikovi,* 190–191
Tetrahymena and yeast cells, 195
Somatic cells, in mammalian egg, 59–60
Sperm
gametic interaction with egg, 61–62
interaction with egg, 60–61
interaction with egg plasma membrane, 85–87, 91–92
candidate molecules, 91
egg integrins, 88–90
role of DE protein, 92–94
role of fertilin, 94–97
role of proteins in gamete interaction, 87–88
interaction with zona pellucida
galactosyltransferase, 77–78
proteins, 74–76
encoding genes, 63–65
sequence analysis, 65–70
role of PH-20, 82–83
role of proacrosin, 83
role of rabbit sperm autoantigens, 84–85
role of spermadhesins, 81
sp56 protein, 78–79
structural considerations, 62–63
ZP1, 70, 72–73
ZP2, 70, 72
ZP3, 70–72
Spermadhesins, role in sperm–zona pellucida interaction, 81
Splicing, plastid genes, 146–149
Stability
mRNA, defective nuclear mutants, 144–146
RNA, 142–144
Staurosporine, induction of *Tetrahymena thermophila* cell death, 215–216
Stress tolerance, interference of sulfur dioxide, 277
Sulfate, sequestration, 272–273
Sulfite, addition compounds, 268
Sulfitolysis, thioredoxins, 268

Sulfur dioxide
  differential sensitivity of plants, 277–278
  dry deposition, 261–264
  effect on stress tolerance, 277
  emissions and immissions, 258–261
  and hydration products
    oxidative detoxification, 271–272
    pH stat mechanisms, 274–275
    radical scavengers and antioxidants, 272
    reductive detoxification, 273–274
    sulfate sequestration, 272–273
    toxicity, 265–266
  interference with cation nutrition, 275–276
  radical formation, 266–267
  wet deposition, 265
Sulfuric acid, acidification by, 268–271
Sulfurous acid, acidification by, 268–271
Survival, *see* Cell survival

T

Tetrahymena
  chemosensory behavior
    effect of chemoattractants, 221
    intracellular messenger systems, 222–223
    overlap with other signals, 223–224
    receptors, 221–222
  factors produced by, 206–207
  signaling systems, 195
  survival and proliferation
    autocrine signals
      cell-to-cell interactions, 199–201
      complex media, 202–203
      nutrient media, 199
    role of signal molecules, 196
      receptors, 197–198
      signal compound homologies, 197
      signal imprinting and receptor induction, 198–199
Tetrahymena pyriformis
  cell survival and proliferation, 217
  β-endorphin-related receptor, 221–222
Tetrahymena setosa, cell survival and proliferation, 217–218
Tetrahymena thermophila
  autocrine signal molecules, 201–202
  biological activity of insulin fragments, 205–206
  biphasic response to insulin, 203–205
  cell death, mechanisms, 211–212
    death induction by staurosporine, 215–216
    in low-density cultures, 212–215
  intracellular messenger systems, 222–223
  mitogenic actions, 206
  role of intracellular messenger systems, 207–211
  role of protein tyrosine kinase, 210–211
Tetrahymena vorax, cell survival and proliferation, 218
Thioredoxins, sulfitolysis, 268
Toxicity, sulfur dioxide and hydration products, 265–266
Transcription
  chloroplast RNA polymerases, 136–138
  mutations, 141
  nucleus-encoded RNA polymerase, 138–141
Transforming growth factor α, in *Paramecium,* 190
Translation
  mRNA, coupling with metabolism, 149–151
  plastid, effect of nuclear mutations, 153–155
  RNA-binding proteins, 151–153
Trypanosoma, apoptosis in, 191–193
Tumor heterogeneity
  animal tumors, 9–10
  and cell fusion, 31–32
  experimental and clinical cancer research, 32–33
  human cancers, 10–12
  normal cell populations, 37–38
  origins, genetic instability, in tumor progression, 27–28
  in prognosis, 33–34
  in treatment, 34–36
Tumor progression
  cellular heterogeneity as basis
    animal tumors, 9–10
    clonal evolution of cancer, 12–14

human cancers, 10–12
Nowell hypothesis, 7–9
subpopulation interactions, 14–20
epigenetic mechanisms
adaptation, 29–30
alterations in DNA methylation, 28–29
cell fusion and tumor heterogeneity, 31–32
relationship to differentiation, 30–31
examples, 6–7
experimental and clinical research, 32–33
Foulds' definition, 4
Foulds' rules, 4–6
genetic stability and instability, 27–28
relationship to cellular societies and organizations, 38–39
variability in behavior
among patients, 2–3
over time, 3
Tumors, *see also* Cancer
environment, role in genetic instability, 25–27
subpopulation interactions, 14–20

W

Wet deposition, sulfur dioxide, 265

Z

Zona pellucida
binding to sperm, 60–61
glycoprotein, role in gamete interactions, 61–62
interaction with sperm
galactosyltransferase, 77–78
proteins, 74–76
encoding genes, 63–65
sequence analysis, 65–70
recombinant proteins, 73–74
role of PH-20, 82–83
role of proacrosin, 83
role of rabbit sperm autoantigens, 84–85
role of spermadhesins, 81
role of zona receptor kinase, 79–81
sp56 protein, 78–79
structural considerations, 62–63
ZP1, 70, 72–73
ZP2, 70, 72
ZP3, 70–72
as part of egg, 59
Zona receptor kinase, interaction with zona pellucida, 79–81

This Page Intentionally Left Blank