CELL BIOLOGY: A Series of Monographs

EDITORS

D. E. BUETOW
Department of Physiology and Biophysics
University of Illinois
Urbana, Illinois

I. L. CAMERON
Department of Cellular and Structural Biology
The University of Texas
Health Science Center at San Antonio
San Antonio, Texas

G. M. PADILLA
Department of Physiology
Duke University Medical Center
Durham, North Carolina

A. M. ZIMMERMAN
Department of Zoology
University of Toronto
Toronto, Ontario, Canada

Recently published volumes

Ian R. Brown (editor). MOLECULAR APPROACHES TO NEUROBIOLOGY, 1982
Henry C. Aldrich and John W. Daniel (editors). CELL BIOLOGY OF *PHYSARUM* AND *DIDYMIUM*. Volume I: Organisms, Nucleus, and Cell Cycle, 1982; Volume II: Differentiation, Metabolism, and Methodology, 1982
John A. Heddle (editor). MUTAGENICITY: New Horizons in Genetic Toxicology, 1982
Potu N. Rao, Robert T. Johnson, and Karl Sperling (editors). PREMATURE CHROMOSOME CONDENSATION: Application in Basic, Clinical, and Mutation Research, 1982
George M. Padilla and Kenneth S. McCarty, Sr. (editors). GENETIC EXPRESSION IN THE CELL CYCLE, 1982
David S. McDevitt (editor). CELL BIOLOGY OF THE EYE, 1982
P. Michael Conn (editor). CELLULAR REGULATION OF SECRETION AND RELEASE, 1982
Govindjee (editor). PHOTOSYNTHESIS. Volume I: Energy Conversion by Plants and Bacteria, 1982; Volume II: Development, Carbon Metabolism, and Plant Productivity, 1982
John Morrow (editor). EUKARYOTIC CELL GENETICS, 1983
John F. Hartmann (editor). MECHANISM AND CONTROL OF ANIMAL FERTILIZATION, 1983
Gary S. Stein and Janet L. Stein (editors). RECOMBINANT DNA AND CELL PROLIFERATION, 1984
Prasad S. Sunkara (editor). NOVEL APPROACHES TO CANCER CHEMOTHERAPY, 1984
Burr G. Atkinson and David B. Walden (editors). CHANGES IN EUKARYOTIC GENE EXPRESSION IN RESPONSE TO ENVIRONMENTAL STRESS, 1985
Reginald M. Gorczynski (editor). RECEPTORS IN CELLULAR RECOGNITION AND DEVELOPMENTAL PROCESSES, 1986
Govindjee, Jan Amesz, and David Charles Fork (editors). LIGHT EMISSION BY PLANTS AND BACTERIA, 1986
Peter B. Moens (editor). MEIOSIS, 1987
Robert A. Schlegel, Margaret S. Halleck, and Potu N. Rao (editors). MOLECULAR REGULATION OF NUCLEAR EVENTS IN MITOSIS AND MEIOSIS, 1987
Monique C. Braude and Arthur M. Zimmerman (editors). GENETIC AND PERINATAL EFFECTS OF ABUSED SUBSTANCES, 1987
E. J. Rauckman and George M. Padilla (editors). THE ISOLATED HEPATOCYTE: USE IN TOXICOLOGY AND XENOBIOTIC BIOTRANSFORMATIONS, 1987
Heide Schatten and Gerald Schatten (editors). THE MOLECULAR BIOLOGY OF FERTILIZATION, 1989
Heide Schatten and Gerald Schatten (editors). THE CELL BIOLOGY OF FERTILIZATION, 1989
Anwar Nasim, Paul Young, and Byron F. Johnson (editors). MOLECULAR BIOLOGY OF THE FISSION YEAST, 1989
Mary Pat Moyer and George H. Poste (editors). COLON CANCER CELLS, 1990

Molecular Biology of the Fission Yeast

Edited by

Anwar Nasim
Division of Biological Sciences
National Research Council of Canada
Ottawa, Ontario, Canada

Paul Young
Department of Biology
Queen's University
Kingston, Ontario, Canada

Byron F. Johnson
Molecular Genetics Group
Division of Biological Sciences
National Research Council of Canada
Ottawa, Ontario, Canada

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Berkeley Boston
London Sydney Tokyo Toronto

Academic Press, Inc.
San Diego, California 92101

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Molecular biology of the fission yeast / edited by Anwar Nasim, Paul Young, Byron F. Johnson.
p. cm. -- (Cell biology)
Includes bibliographies and index.
ISBN 0-12-514085-1 (alk. paper)
1. Schizosaccharomyces pombe. 2. Fission (Biology) 3. Molecular microbiology. I. Nasim, A. (Anwar), Date. II. Young, Paul (Paul G.) III. Johnson, Byron F. IV. Series.
QK623.S23M63 1989
589.2'33--dc19 88-36980
CIP

Printed in the United States of America
89 90 91 92 9 8 7 6 5 4 3 2 1

To Urs Leupold, J. M. Mitchison, and C. F. Robinow

Preface

The fission yeast, *Schizosaccharomyces pombe*, although known to science for less than a century (Lindner, 1893) and actively studied for only about forty years, has become one of the best characterized organisms. Since the early studies of Leupold, Mitchison, and Robinow, *S. pombe* has been considered the best defined yeast after *Saccharomyces cerevisiae*. In some areas, it has been the paradigm. A not-so-subtle implication is that the fission yeast has been "... some special object particularly suited for the study of each of the more important problems" (A. Krogh, Nobel Laureate, 1920). This volume attempts to show how the fission yeast has been that "special object" in a variety of important areas of modern research.

The diversity of experimental approaches and the ease with which the novel techniques of gene manipulation and cloning have been applied to *S. pombe* have obviously generated an ever-increasing interest in using the fission yeast as a biological system. Some recent experiments, whereby a human homolog of a cell cycle mutant of *S. pombe* has been found, have led to new ways of examining the functional similarities between simple unicellular eukaryotes and the highly differentiated complex systems.

This volume is the first attempt to assemble the lore of the fission yeast. It recognizes that a large body of literature has been accumulated and attempts to provide an overview of most of it, although inevitably some areas will be judged to have been treated too lightly.

The dominant themes for many years emphasized cell biology and genetics. Currently, a much broader interest in the molecular biology of *S. pombe* is developing. Findings regarding the conservation of some cell cycle genes, attributes of the RNA processing system, and the structure of the centromeres and chromosomes stimulate broad interest.

Among others, this book is addressed to the many new investigators and laboratories adopting this system. We hope it leads to the development of new molecular tools for investigating problems in *S. pombe* as well as to the definition of areas of metabolism and biology beyond the major themes of the past.

We are extremely thankful to all those who have contributed to this volume. It has been a great joy and feeling of personal satisfaction to have worked with all these colleagues. Along with all the other efforts being undertaken to focus on *S. pombe* as one of the organisms particularly suitable for genetics and cell biology, we hope this volume will help to focus on *S. pombe* as one of the organisms particularly suitable for modern research.

Several aspects of the molecular or cellular biology of the fission yeast cell have been reviewed recently, thus these aspects are not included, or, at least, are not emphasized in this volume. The references to those reviews are as follows:

Calleja, G. B. (1987). Cell aggregation. *In* "The Yeasts" (A. H. Rose and J. S. Harrison, editors), Volume 2 (2nd edition). Academic Press, London.

Phipps, J., Nasim, A., and Miller, D. R. (1985). Recovery, repair, and mutagenesis in *Schizosaccharomyces pombe*. *Advances in Genetics* **23,** 1–73.

Robinow, C. F., and Johnson, B. F. (1989). Yeast cytology. *In* "The Yeasts" (A. H. Rose and J. S. Harrison, editors), Volume 3 (2nd edition). Academic Press, London.

Anwar Nasim
Paul Young
Byron F. Johnson

1

Genetics Overview

P. MUNZ,* K. WOLF,† J. KOHLI,* AND U. LEUPOLD*

*Institut für Allgemeine Mikrobiologie
Universität Bern
CH-3012 Bern, Switzerland

†Institut für Mikrobiologie und Weinforschung
Johannes Gutenberg-Universität
D-6500 Mainz, Federal Republic of Germany

I. INTRODUCTION

A. History

Schizosaccharomyces pombe was first isolated from an East African millet beer, called *Pombe,* by Lindner (1893) who described it as a yeast dividing by cell fission and forming four-spored linear asci. Studies on

MOLECULAR BIOLOGY OF THE
FISSION YEAST

ascus formation in the related eight-spored species *Schizosaccharomyces octosporus* led Beijerinck (1894) to believe that it was "nowhere clearer than here that the ascus and the ascospores are formed without a sexual act." This was disputed by Schiønning (1895) who thought if the possibility of a sexual act in the lower Ascomycetes was conceivable it would be precisely in this species in which ascus formation was found to be preceded by the pairwise fusion of cells. The sexual nature of these events became clearly established when Hoffmeister (1900) showed that the fusion of cells in *S. octosporus* is accompanied by nuclear fusion. Guillermond (1901) confirmed the results of Schiønning and Hoffmeister in a more detailed analysis of spore formation in both *S. octosporus* and *S. pombe*.

In spite of the demonstration of these and other clear cases of sexuality in yeasts that pointed to a life cycle involving a regular alternation of nuclear phases (Guillermond, 1905; Kniep, 1928), references to the possibility of a parthenogenetic formation of asci not preceded by sexual fusion of cells are repeatedly found in the literature on yeast of the following decades. The concept of regular alternation of haploid and diploid nuclear phases in the life cycle of yeasts was firmly established only when Winge and co-workers showed that sexual fusion of ascospores or cells derived from them was a regular feature of the life cycle of several yeast species of the genus *Saccharomyces* (Winge, 1935) and that sporulation of a cell clone derived from a strain of bakers' yeast was accompanied by the genetic segregation of morphological characters in each of the four-spored asci formed (Winge and Laustsen, 1937). This demonstrated that sexual fusion took place between spores or cells of haploid constitution and that it gave rise to diploid cell clones which under suitable conditions underwent meiosis to produce haploid ascospores again.

It was also Winge who suggested *Schizosaccharomyces pombe* as a potentially useful organism for genetic studies when the senior author of this chapter (U.L.) visited the Physiological Department of the Carlsberg Laboratory in Copenhagen as a young student in 1946 and again in 1948/1949. Considering the results obtained in *Saccharomyces*, it was clear that the early observations of Schiønning, Hoffmeister, and Guillermond, which showed that ascospore formation in *Schizosaccharomyces* immediately follows conjugation, pointed to a haplontic life cycle in which the diploid phase is restricted to the zygote formed by the sexual fusion of haploid cells. As in *Saccharomyces* and in higher Ascomycetes, ascospore formation was likely to be preceded by meiosis and to give rise to haploid ascospores which on germination would yield haploid cells again (Fig. 1).

The early observations on the conjugation of sister cells in *S. octosporus* and *S. pombe* (Schiønning, 1895; Guillermond, 1901, 1903, 1931)

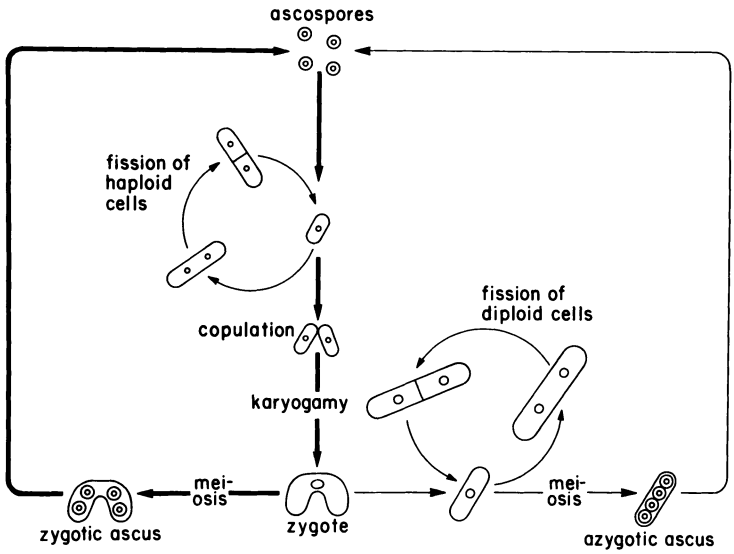


Fig. 1. Life cycle of *S. pombe* (homothallic strain). The left part of the figure (bold lines) shows the normal haplontic life cycle. The right part (thin lines) demonstrates the events that take place when zygotes develop into diploid cells. [Reproduced from "Handbook of Genetics" (R. C. King, ed.), Vol. 1, p. 396, Plenum Press, New York and London, 1974, by copyright permission of Plenum Publishing Corporation, New York.]

and on the ability of cell clones derived from single cells or spores of these yeasts to sporulate abundantly in pure culture (Beijerinck, 1897, 1898) had already made it clear that a homothallic mating behavior is common in the genus *Schizosaccharomyces*. In *Saccharomyces*, the finding that many cell clones derived from single ascospores were capable of early pairwise fusion of cells (Winge, 1935; Winge and Laustsen, 1937) pointed in the same direction. That heterothallic strains belonging to two self-sterile but cross-fertile mating types (called *a* and α) could also be found in this genus was only discovered several years later by Lindegren and Lindegren (1943), and it again took a few years before Winge and Roberts (1949) were able to show that a single pair of alleles *D/d* (now called *HO/ho*) is responsible for the early diploidization observed in homothallic clones and its lack in heterothallic clones derived from single spores.

B. Mating Types and Life Cycle

The first genetic analysis confirming the haplontic nature of the normal life cycle of fission yeast was carried out by Leupold (1950). The strain of *S. pombe* studied was obtained from the yeast collection of the Cen-

traalbureau voor Schimmelcultures in Delft. It had originally been isolated from grape juice by Osterwalder (1924) who first described it as a new species, *Schizosaccharomyces liquefaciens* Osterwalder. Since it differed from *Schizosaccharomyces pombe* Lindner only in its marked ability to liquefy gelatin, it was later renamed *Schizosaccharomyces pombe* Lindner, strain *liquefaciens* (Osterwalder), by Stelling-Dekker (1931).

From the Delft culture of this strain of *S. pombe,* Leupold (1950) isolated two types of homothallic clones differing in their fertility, of which only one (designated h^{90} because it formed about 90% spores in pure culture) has survived and needs to concern us here. In addition, heterothallic clones (designated h^+ and h^-) belonging to two opposite mating types, called (+) and (−), and some sterile clones were isolated from the same culture. In crosses between heterothallic strains of opposite mating type, the two parental types segregated 2:2 in each of the spore tetrads analyzed, and so did the two parental types in spore tetrads from the crosses of homothallic with heterothallic strains of either mating type. It was concluded that homothallism as well as heterothallism of the two mating types (+) and (−) were determined by a series of three alleles, h^{90}, h^+, and h^-. Rare spontaneous genetic events, interpreted to result from mutations among the three allelic states, were found to interconvert the three types of mating behavior, each of the three types giving rise to the other two types. However, h^- strains appeared to be able to do so only when derived from h^+ strains by secondary mutations. No mating-type mutants were observed in the original h^- isolates derived from the Delft strain or in the h^- progeny of their crosses with h^+ or h^{90} strains.

The two types of h^- strains, one stable and one unstable, were later recognized as two separate types, and additional secondary variants of homothallic and heterothallic strains (described in this volume, Chapter 2 by Egel, Mating-Type Genes, Meiosis, and Sporulation) were discovered. Their isolation was greatly facilitated when it was found that iodine, which stains sporulating colonies black and nonsporulating colonies or colony sectors yellow (Beijerinck, 1898), can be applied in the form of iodine vapor. A brief exposure will not kill the cells in the interior of the treated colonies and will therefore permit their isolation (Leupold, 1955).

The further elucidation of the genetic and physical basis of the inheritance of mating type in *S. pombe* has been one of the most fascinating topics of research in this yeast since the first analysis carried out by Leupold (1950). The development of the field may be traced by consulting the relevant sections in the reviews of Gutz *et al.* (1974) and Egel *et al.* (1980) on the genetics of *S. pombe* and in the review of Crandall *et al.* (1977) on the physiology of mating in yeasts. The present state of knowledge is summarized elsewhere in this volume (Chapter 2 by Egel, Mating-

Type Genes, Meiosis, and Sporulation). Suffice it to point out here that mobile genes assigned to three closely linked loci *mat1*, *mat2*, and *mat3* have turned out to exert the primary control of mating type in *S. pombe*. In homothallic h^{90} strains, they cooperate in a cassette mechanism very similar to that which has been found to underlie mating-type switching in homothallic strains of *Saccharomyces cerevisiae*. At the expression locus *mat1*, *P* (plus) information copied from the silent cassette *mat2-P* and *M* (minus) information copied from the silent cassette *mat3-M* are exchanged every few cell generations. In heterothallic h^+ and h^- strains, however, *P* or *M* information is stabilized at *mat1* as a result of aberrant recombination events in the mating-type region.

Judging from its mating-type constitution, h^{90} clearly represents the true wild type of *S. pombe*. Normal h^+ strains and stable h^- strains of the type originally isolated from the Delft culture of *S. pombe* strain *liquefaciens* (later called h^{+N} and h^{-S} to distinguish them from secondary heterothalic strains of the same mating type but differing in their mating-type interconversions) arise directly by rare but recurrent events from the homothallic h^{90} type. Although the precise genetic constitution of the homothallic wild-type and of several heterothallic variants including h^{+N} and h^{-S} with respect to the *mat* genes is known today from physical analysis, the symbols h^{90}, h^+, and h^- (with or without additional superscripts to indicate the various heterothallic subtypes) are still widely used as a short notation of the mating-type constitution of strains.

Although the haplontic nature of the normal life cycle of *S. pombe* was confirmed by the results obtained by Leupold (1950) in his first genetic analysis, subsequent studies have shown that it is nevertheless possible to propagate the organism vegetatively in the diplophase. This opened new possibilities for genetic analysis. It turned out that haploid strains regularly contain rare diploid cells arising presumably by endomitosis (Leupold, 1955). On solid media containing Magdala red (phloxin B), diploid cells develop into colonies that stain darker than haploid colonies, owing to a higher percentage of dead cells which are stained by the dye (Gutz *et al.*, 1974; Kohli *et al.*, 1977).

Diploid cell clones of constitution h^{90}/h^{90} isolated from haploid strains of the homothallic constitution h^{90} are capable of undergoing meiosis and spore formation directly, without preceding conjugation. The so-called azygotic asci thus formed retain the shape of the diploid cells from which they have arisen (Leupold, 1955). They are thus clearly distinguishable from the dumbell-shaped zygotic asci resulting from the conjugation of haploid (or diploid) homothallic or heterothallic cells. Diploid cell clones of constitution h^+/h^+ or h^-/h^- isolated from haploid strains of the heterothallic constitution h^+ or h^- are incapable of sporulation but retain the

heterothallic mating behavior of the haploid strains from which they have been derived. Matings between heterothallic cells of opposite mating type will lead to the various types of tetrad segregations expected of triploid or tetraploid zygotes if one or both partner cells are diploid, respectively (Leupold, 1956; Ditlevsen and Hartelius, 1956; Niwa and Yanagida, 1985). Depending on the physiological conditions, tetraploid zygotes may also fail to undergo karyogamy and may produce eight haploid ascospores resulting from a twin meiosis in each of the two separate diploid nuclei, instead of four diploid spores resulting from a single meiosis in a tetraploid nucleus (Gutz, 1967a,b).

Diploid cells arising by endomitosis are expected to be homozygous for all genes of the chromosomal genome. Diploids of heterozygous constitution may be obtained from young mass matings of complementing auxotrophic mutant strains of opposite heterothallic mating type. This is achieved by plating samples of the cell mixture on minimal medium after it has been pregrown on a sporulation medium until zygote formation has started. This selects for rare zygotes that fail to undergo meiosis and propagate vegetatively instead, thus giving rise to prototrophic diploid cell clones of heterozygous constitution for any allele difference distinguishing the two haploid parents. Owing to their heterozygous mating-type constitution h^+/h^-, these diploids are unstable. Under conditions inducing sporulation, they will undergo meiosis followed by the formation of azygotic asci (Fig. 1). Stable diploids of homozygous constitution h^+/h^+ and h^-/h^- may be derived from them, however, by replating vegetative cell material from young diploid colonies on sporulation medium and selecting for nonsporulating colonies. Stable diploids arise from mitotic crossing-over between the mating-type locus and the centromere and become homozygous for markers located distal to the crossover event (Leupold, 1970).

A more efficient method of producing stable diploids makes use of the properties of the mating-type allele *mat2-B102* (formerly called *mei1-B102*) which in heterozygous constitution h^-/*mat2-B102* blocks meiosis (Egel, 1973). It therefore allows direct selection for stable prototrophic diploids from mass matings of h^- and *mat2-B102* strains carrying complementing auxotrophic mutations (Kohli *et al.*, 1977).

The heterozygous diploids obtained by one or the other of these methods have been used in the analysis of such problems as complementation, mitotic recombination, and haploidization. It was found that even the relatively stable diploids of constitution h^+/h^+, h^-/h^-, and h^-/*mat2-B102* will eventually undergo spontaneous haploidization, which is due to a rare nondisjunction of chromosomes during mitosis followed by the successive loss of chromosomes. The first step, nondisjunction of chromo-

somes, is induced by *p*- and *m*-fluorophenylalanine in *S. pombe* as well as in other fungi. Since linked markers segregate as a unit in the process, the method can be used to assign heterozygous markers to linkage groups as a first step in chromosomal mapping (Gutz, 1966; Flores da Cunha, 1970; Adondi and Heslot, 1970; Kohli *et al.*, 1977).

C. Fission Yeast as an Organism for Genetic Research

A number of *S. pombe* strains of independent origin, referred to in various chapters of this volume, have been studied and are still studied for comparative purposes. But the vast majority of the mutant strains used in studies on the genetics and molecular biology of fission yeast are descendants of three strains, one homothallic (strain *968:* h^{90}) and two heterothallic (strains *972* and *975:* h^{-S} and h^{+N}, respectively), which have all been derived from the same Delft culture and therefore from the same homothallic isolate of *S. pombe* strain *liquefaciens* originally described by Osterwalder (1924). This monolithic origin provides for a high degree of homogeneity in the genetic background of the mutant strains of *S. pombe* studied. This is an advantage that is lacking in some of the other yeasts and molds used in genetic research where wild-type strains of widely different sources have contributed to the genetic makeup of the mutant strains used today.

The usefulness of an organism for research in the field of genetics and molecular biology depends to a large degree on the infrastructure provided by the number and linkage relationships of the chromosomal and organelle genes already known. A detailed list of 469 genes in *S. pombe* has been published by Kohli (1987). It gives information on the corresponding gene products or mutant phenotypes and cites representative publications that provide additional information on gene function, gene structure, or mapping data. It also proposes rules for the genetic nomenclature in *S. pombe* and gives both the three-letter symbols already in use and a number of newly proposed symbols for future use in the designation of new genes. A genetic stock center is currently being established in England. Information can be obtained from its curator Barbara Kirsop, National Collection of Yeast Cultures, Food Research Institute, Colney Lane, Norwich NR4 7UA, England.

Chromosomal mapping was started by Leupold (1958) and carried further by Flores da Cunha (1970). The first extensive chromosome map showing the location of 118 genes was published by Kohli *et al.* (1977). A revised version containing additional markers was presented by Gygax and Thuriaux (1984). The demonstration of three linkage groups by these

genetic analyses agrees with cytological observations, discussed in this volume by Robinow and Hyams in Chapter 8 on cytology, which confirm that the haploid set contains three chromosomes in *S. pombe*.

An updated genetic map showing the location of 162 chromosomal genes is given in Section II of this chapter. Seventy-seven additional genes which have already been assigned to individual chromosomes or even chromosome arms but which have not yet been mapped precisely may be looked up in the gene list published by Kohli (1987). Section II also presents an analysis of the underlying tetrad data that confirms the conclusion of Snow (1979a) that *S. pombe* shows little if any interference between crossover events in adjacent chromosome regions. Egel *et al.* (1980) have suggested that this could be due to the apparent lack of synaptonemal complexes in this yeast (Olson *et al.*, 1978), assuming that the latter prevent chiasmata as the cytological basis for interference (Egel, 1978). At present the total map length is about 2100 cM (centimorgans). This is known to be an underestimate arising from incomplete mapping of the chromosomes. The DNA content per haploid spore is approximately 1.5×10^{-14} g (Bostock, 1970; Nurse and Thuriaux, 1977), corresponding to 3–4 times the amount of DNA found in *Escherichia coli*. Thus, the genome comprises approximately 15,000 kilobases, a size comparable to that of *Sacch. cerevisiae*. The physical mapping of the *S. pombe* genome by pulsed-field gel electrophoresis is also in progress (Smith *et al.*, 1987). As in most fungi, the DNA of *S. pombe* is largely unmethylated (Antequera *et al.*, 1984).

D. Other Areas of Genetic Research

Fine-structure maps based on intragenic meiotic and mitotic recombination, as well as corresponding complementation maps based on interallelic complementation, have been constructed for a number of protein structural genes. In the case of several genes coding for multifunctional enzymes involved in purine and aromatic biosynthesis, these studies have yielded valuable information on the functional organization of the gene. Fine-structure maps have also been constructed for several genes coding for serine and leucine tRNAs. The fine-structure studies carried out in both protein and tRNA structural genes have at the same time yielded information not only on the specificity of the mutagens used to induce the underlying mutations but also on various aspects of meiotic and mitotic recombination. Aspects of gene conversion that have received particular attention include the general marker effect of map expansion and specific marker effects owing either to a site-specific induction of gene conversion

in one case (the M26 site in *ade6*) or to a defective mismatch repair in other cases (several sites in the serine tRNA genes *sup3, sup9,* and *sup12*). The fine-structure studies mentioned and most of the studies relating to specific aspects of allelic recombination have been reviewed by Gutz *et al.* (1974), Egel *et al.* (1980), and Phipps *et al.* (1985) and will not be discussed further in this volume.

Research on one interesting aspect of recombination was only at its beginning when the last general review on the genetics of fission yeast was written (Egel *et al.*, 1980). It concerns the rare meiotic and mitotic information transfer between dispersed but homologous members of gene families that code for related gene products. In *S. pombe,* this process of intergenic conversion has been studied in some detail in a family of serine tRNA genes during the last few years, and the results are reviewed by Kohli *et al.* in Chapter 3 of this volume, Informational Suppression, Transfer RNA, and Intergenic Conversion.

An important topic related to recombination concerns recovery, repair, and mutagenesis in *S. pombe*. This field has recently been reviewed by Phipps *et al.* (1985), and relevant work on recombination also discussed. Another topic of importance which has recently been reviewed by Wolf (1987) concerns the mitochondrial genome of *S. pombe*. A map of the mitochondrial genome accompanied by a brief review of the state of research in the field is included in Section III of this chapter.

A number of research topics not covered elsewhere in this volume are briefly mentioned, and references are given to facilitate screening of the literature for more detailed information. Several genes coding for glycolytic enzymes have been isolated and sequenced (Vassarotti and Friesen, 1985; Russell, 1985). Enzymes involved in glycerol metabolism and mutations in the corresponding genes have been characterized (Vasiliadis *et al.*, 1987; Gancedo *et al.*, 1986). Genes coding for malic enzyme and the malate transport system have been identified by mutation (Osothsilp and Subden, 1986). Acid and alkaline phosphatases and their secretion or intracellular localization in connection with glycosylation have been studied. The corresponding genes are defined by mutation and cloning, and the structural gene coding for secreted acid phosphatase has been sequenced (Schweingruber *et al.*, 1986a,b; Elliott *et al.*, 1986; Dhamija *et al.*, 1987). Another group is studying phospholipid metabolism by analysis of enzymes and mutants and by isolation of the corresponding genes (Fernandez *et al.*, 1986). Fluri and Kinghorn (1985) have published work on the genes and enzymes and on the regulation of purine catabolism. Several publications exist on cadmium-binding peptides (Murasugi *et al.*, 1984). Ribosomal proteins of fission yeast have been compared with the corresponding ones of other organisms (Otaka *et al.*, 1986), and two genes

coding for ribosomal proteins have been sequenced and their expression analyzed (Nischt *et al.*, 1987).

The tandem repeats coding for ribosomal RNAs have been located on chromosome III (Toda *et al.*, 1984); the restriction pattern is known and so are the nucleotide sequences of the 5.8 S RNA gene, part of the 17 S RNA gene, and the external transcribed spacer (Schaack *et al.*, 1982; Balzi *et al.*, 1985). Ribosomal 5 S RNA genes are dispersed over the whole genome, and the primary structure for several of these genes has been published (Tabata, 1981; Mao *et al.*, 1982). Currently several groups are studying the splicing mechanism of polymerase II transcripts, and initial data have appeared recently (Käufer *et al.*, 1985; Ares, 1986; Mertins and Gallwitz, 1987).

Other important areas of research on fission yeast to which genetic analysis has contributed are reviewed in detail in this volume and therefore need no special comments in this brief introduction. The main aim here is to familiarize the reader with those aspects of the life cycle and mating behavior of *S. pombe* that are of relevance for routine genetic analysis. For a more detailed description of the basic methods used in the genetics of fission yeast, the reader is referred to the reviews of Leupold (1970) and Gutz *et al.* (1974).

II. THE GENETIC CHROMOSOME MAP

A. Introductory Remarks

The first extensive genetic map of *S. pombe* was presented by Kohli *et al.* (1977). Subsequently a revised version containing additional markers was published by Gygax and Thuriaux (1984). These maps are based on the analysis of two-factor crosses. The observed frequencies of the three tetrad types, parental ditype (PD), nonparental ditype (NPD), and tetratype (T), were used to obtain estimates of the map distances in centimorgans with the equation derived by Perkins (1949):

$$x_p = 50(T + 6NPD)/(PD + NPD + T) \quad (1)$$

Let us briefly recall principles of mapping. Recombination events (crossover events) involving two chromatids at a time occur during the meiotic four-strand stage. They are distributed along the synapsed chromosomes. More crossover events will occur in long chromosome segments than in short ones. Every crossover event at the four-strand stage gives exactly two recombination sites (crossover sites) among the four

single chromatids. Again, long intervals are expected to have more crossover sites than short ones. It is thus natural to set the genetic distance proportional to the average number of crossover sites per chromatid in the interval studied.

If for an interval we analyze M tetrads and observe a total of U crossover events, then the average number of crossover events per tetrad is $u = U/M$. Likewise, a total of V crossover sites observed among N single chromatids gives an average of $v = V/N$ crossover sites per chromatid. We imagine for the moment that we may count (with a magic eye) unambiguously crossover events in tetrads and crossover sites in chromatids for any interval. This allows us to calculate the averages u and v. Following Haldane (1919) we choose 1 as the proportionality constant and call the unit of genetic length morgan (M). Thus, assuming 118 crossover sites have been counted among 100 chromatids, $v = 118/100 = 1.18$, and the genetic distance, x, is

$$x = 1v = 1.18 \text{ M} = 118 \text{ cM} \tag{2}$$

Since for every two crossover sites generated one crossover event takes place, 118 crossover sites per 100 chromatids is equivalent to 59 crossover events per 25 tetrads. Thus $u = 59/25 = 2.36$, and, in order not to get differing results from random spore analyses, this is divided by 2 to give

$$x = (1/2)u = 1.18 \text{ M} \tag{3}$$

To calculate the map distance of an interval we must know the average number of crossover sites per chromatid or the average number of crossover events per tetrad. But experimentally we observe parental spores and recombinant spores (recombinants) at the random spore level or the three tetrad types, PD, NPD, and T, at the tetrad level. The mapping problem arises because the observed entities are not always faithful indicators of recombination sites or events. Thus, in a cross

$$\frac{a\ +}{+\ b}$$

parental spores could have arisen by an even number of crossover sites (0, 2, 4, ...), whereas recombinants by an odd number (1, 3, 5, ...). Likewise, PDs could have originated by 0, 2, 3, ... crossover events, Ts by 1, 2, 3, ... events, and NPDs by 2, 3, ... events. On the other hand, if in tetrad analysis only PDs and Ts are found this means that tetrads with 2 or more crossover events are rare or did not occur, for otherwise some NPDs should be observed (assuming lack of chromatid interference). This in turn means that PDs are tetrads with exactly 0 events and Ts tetrads with exactly 1 event. In this case the observed tetrads correctly indicate

the number of crossover events having occurred in the interval, the average u can be determined, and hence the map distance calculated.

Perkins' formula treats a situation in which chromatid interference is absent and only 0, 1, or 2 crossover events are occurring in every tetrad. Tetrads with 3 or more events will again produce PDs, Ts, and NPDs. If the sample contains such tetrads a certain number of recombination events will not be taken into account when it should. This leads to an underestimation of the map distance. If an underestimation of less than or equal to 3% is accepted, Perkins' values can be used up to $x_p = 35$ cM in *Sacch. cerevisiae* (Mortimer and Schild, 1985) and up to $x_p = 20$ cM in *S. pombe*. This difference is due to different degrees of interference as discussed below.

B. Snow's Procedure

To deal with the problem of long genetic distances, Snow (1979a,b) has presented sets of equations in which tetrads of crossover rank 3 and higher are also taken into account. In system I (interference not estimated) the number of tetrads with 0, 1, 2, ..., r crossover events is assumed to follow a Poisson distribution with mean $u = 2x$ [see Eq. (3)]. Thus, the proportion of tetrads with r crossover events in the interval is

$$p(r) = e^{-2x}[(2x)^r/r!] \tag{4}$$

The expected relative frequencies of the three tetrad types is given by

$$P(\text{PD}) = m_1 = \tfrac{1}{6} + \tfrac{1}{2}e^{-2x} + \tfrac{1}{3}e^{-3x} \tag{5}$$

$$P(\text{NPD}) = m_2 = \tfrac{1}{6} - \tfrac{1}{2}e^{-2x} + \tfrac{1}{3}e^{-3x} \tag{6}$$

$$P(\text{T}) = m_3 = \tfrac{2}{3} - \tfrac{2}{3}e^{-3x} \tag{7}$$

In system II (interference estimated) the effect of interference is treated in addition. The model used (Barratt *et al.*, 1954) introduces an interference factor k. The $p(0)$ term is kept unchanged throughout, $p(1)$ is multiplied by 1, $p(2)$ by k, $p(3)$ by k^2, and so on. Then all terms except $p(0)$ have to be multiplied by a factor S, itself dependent on x and k, such that the sum of all probabilities again adds up to 1. The relative frequencies of the three tetrad types is then given by

$$P(\text{PD}) = \text{m}_4 = e^{-2x} + \tfrac{1}{6}[(1 - e^{-2x})(e^{2kx} + 2e^{-kx} - 3)/(e^{2kx} - 1)] \tag{8}$$

$$P(\text{NPD}) = \text{m}_5 = \tfrac{1}{6}[(1 - e^{-2x})(e^{2kx} + 2e^{-kx} - 3)/(e^{2kx} - 1)] \tag{9}$$

$$P(\text{T}) = \text{m}_6 = \tfrac{2}{3}[(1 - e^{-2x})(e^{-kx})(e^{3kx} - 1)/(e^{2kx} - 1)] \tag{10}$$

Positive interference is the case when $0 < k < 1$; for $k = 1$ interference is absent, the Poisson probabilities are not modified; and $k > 1$ means negative interference.

Let us agree that in Eqs. (8)–(10) x stands for x_i, i for "interference estimated," but omitting the subscript for typographical reasons. If we knew x in system I or x_i and k in system II we could calculate the proportions of the three tetrad types. But the situation is just reversed: we observe numbers of PD, NPD, and T and would like to know x or x_i and k, respectively. This is achieved with the maximum likelihood procedure. Assume we observe a_1 PD, a_2 NPD, and a_3 T tetrads among a total of M. The probability of obtaining such a result, given the expectations, m_1, m_2, and m_3 (system I), is

$$\text{prob}(a_1,a_2,a_3) = (M!/a_1!a_2!a_3!)(m_1)^{a_1}(m_2)^{a_2}(m_3)^{a_3} \tag{11}$$

Now, m_1, m_2, and m_3 are themselves functions of x, and inserting different values of x will produce different probabilities. The method can be thought of as varying x until the maximum value of $\text{prob}(a_1,a_2,a_3)$ is found. The corresponding x then represents the maximum likelihood estimate. In an analogous way, that x_i value and that k value are chosen in system II for which $\text{prob}(a_1,a_2,a_3)$ takes on the largest value.

In system I (interference not estimated) the obtained x directly represents the average number of crossover sites per chromatid, $2x$ the average number of crossover events per tetrad. The situation in system II (interference estimated) is different. Here $2x_i$ is the average of a hypothetical underlying Poisson distribution whose terms have been modified by k and S. It is obvious that the average number of crossover events per tetrad, $2x'$, will be smaller than $2x_i$ if $k < 1$ and larger than $2x_i$ if $k > 1$. Their mutual relation (Snow, 1979b) is

$$2x' = 2x_i[k(1 - e^{-2x_i})/(1 - e^{-2kx_i})] \tag{12}$$

Since $2x'$ is the average number of events per tetrad, x' is the average number of crossover sites per chromatid, thus the map distance in morgans. Multiplying x' by 100 gives the distance in centimorgans.

Using the computer program kindly provided by Dr. R. Snow, we have evaluated data presented in the two *S. pombe* mapping papers (Kohli *et al.*, 1977; Gygax and Thuriaux, 1984) as well as more recent published and unpublished tetrad data. An example of some computer calculations is given in Fig. 2. All values are in centimorgans. $x(P) = x_p$, calculated according to Perkins' formula. x represents the maximum likelihood estimate of the map distance from system I (interference not estimated, unmodified Poisson distribution assumed). From system II (interference estimated) one obtains simultaneously $x(i) = x_i$, and k. These two values

CHROMOSOME I CHROMOSOME I CHROMOSOME I CHROMOSOME I CHROMOSOME I CHROMOSOME I

Gen1	Gen2	Ref	PD	NPD	T	N	x(P)	x	s.e.	x(i)	s.e.	k	s.e.	x'	s.e.
ade2	ade4	1	62	36	192	290	70.3	122.1	19.5	120.6	18.8	0.87	0.24	108.8	27.5
ade2	aro3	1	75	0	5	80	3.1	3.2	1.5	3.2	1.4	0.25	2.47	3.2	1.4
ade2	leu2	1	244	9	194	447	27.7	32.3	2.5	32.1	2.4	0.60	0.21	28.6	2.7
ade2	lys7	2	24	0	8	32	12.5	14.5	5.3	14.4	5.1	0.03	0.35	12.6	3.9
ade2	mei2	11	35	1	9	45	16.7	13.9	4.4	14.0	4.7	3.55	4.28	19.3	13.0
ade2	min3	1	30	0	1	31	1.6	1.7	1.7	1.7	1.7	----	----	1.7	1.9
ade2	ste9	26	99	0	6	105	2.9	3.0	1.2	3.0	1.2	0.22	2.26	2.9	1.2
ade2	sup2	2	62	0	6	68	4.4	4.6	1.9	4.6	1.9	0.14	1.42	4.5	1.8
ade2	ura2	11	27	2	16	45	31.1	29.1	7.3	29.4	7.8	1.98	1.76	37.6	20.4
ade3	arg3	1	16	2	15	33	40.9	42.5	11.7	42.9	12.4	1.46	1.32	50.5	29.2
ade3	cdc15	3	55	0	7	62	5.6	6.0	2.3	6.0	2.3	0.09	0.92	5.7	2.1
ade3	eth2	3	11	0	20	31	32.3	54.3	15.9	51.9	12.1	0.00	0.04	32.3	4.4
ade3	eth3	3	14	1	16	31	35.5	43.9	12.6	43.5	11.9	0.61	0.64	37.4	13.5
ade3	lys2	1	120	14	174	308	41.9	54.0	5.0	53.3	4.7	0.65	0.19	45.5	5.5
ade3	lys2	3	36	9	66	111	54.1	71.0	11.6	70.7	11.5	0.92	0.38	67.7	19.0
ade3	lys2	T	156	23	240	419	45.1	58.0	4.6	57.4	4.4	0.74	0.17	50.6	5.8
ade3	lys3	1	186	111	486	783	73.6	114.6	10.3	117.3	11.3	1.43	0.47	156.7	53.3
ade3	lys3	3	67	24	143	234	61.3	84.6	10.4	84.7	10.6	1.03	0.31	86.3	20.9
ade3	lys3	T	253	135	629	1017	70.7	105.9	7.6	107.7	8.2	1.29	0.27	131.1	27.4
ade3	lys5	1	47	11	69	127	53.1	62.4	9.1	63.0	9.6	1.28	0.55	72.3	23.5
ade3	mes1	11	34	1	31	66	28.0	35.1	7.0	34.7	6.5	0.38	0.39	28.4	5.8
ade3	met2	2	80	1	37	118	18.2	20.1	3.4	20.1	3.4	0.55	0.56	18.4	3.5
ade3	met5	2	8	1	8	17	41.2	44.1	17.0	44.4	17.7	1.30	1.61	49.6	37.4
ade3	pro1	1	43	6	51	100	43.5	49.6	7.9	49.7	8.0	1.09	0.53	51.5	14.7
ade3	pro2	1	151	66	372	589	65.2	96.9	8.4	96.8	8.4	0.99	0.19	95.8	15.7
ade3	pro2	3	76	35	185	296	66.7	98.3	12.1	98.8	12.5	1.08	0.31	104.1	27.4
ade3	pro2	T	227	101	557	885	65.7	97.4	6.9	97.5	7.0	1.01	0.16	98.4	13.7
ade3	rad9	3	10	2	11	23	50.0	51.8	17.1	52.8	19.0	1.88	2.26	75.0	74.9
ade3	tps18	2	81	0	16	97	8.2	9.0	2.3	9.0	2.3	0.03	0.28	8.3	1.9
ade3	ura1	1	69	37	200	306	69.0	113.8	16.1	112.9	15.8	0.91	0.24	105.3	25.4
ade3	ura3	1	193	0	41	234	8.8	9.7	1.6	9.6	1.5	0.01	0.10	8.8	1.2
ade3	ura3	3	189	0	30	219	6.8	7.4	1.4	7.4	1.3	0.02	0.18	6.9	1.2
ade3	ura3	11	61	0	7	68	5.1	5.4	2.1	5.4	2.1	0.10	1.02	5.2	1.9
ade3	ura3	T	443	0	78	521	7.5	8.1	0.9	8.1	0.9	0.01	0.06	7.5	0.8
ade3	vir1	1	17	4	27	48	53.1	65.0	15.6	65.3	16.1	1.12	0.74	69.2	33.1
ade4	cdc4	2	62	0	3	65	2.3	2.4	1.4	2.4	1.4	0.50	5.31	2.4	1.4
ade4	leu2	1	77	40	218	335	68.4	110.9	14.6	110.2	14.4	0.92	0.23	103.5	23.5
ade4	min3	3	69	39	152	260	74.2	103.4	14.2	108.0	0.0	----	----	-----	-----
ade4	rad1	3	48	14	95	157	57.0	76.7	10.9	76.5	10.9	0.95	0.33	74.1	18.7
ade4	rad2	2	135	0	12	147	4.1	4.3	1.2	4.3	1.2	0.07	0.76	4.1	1.1
ade4	tps19	2	75	9	97	181	41.7	50.7	6.0	50.4	5.9	0.81	0.30	46.5	8.2
ade4	ura2	1	184	141	585	910	78.6	149.3	19.0	152.6	20.9	1.41	0.78	207.8	124.8
arg3	arg11	7	25	0	0	25	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.0	0.0
arg3	cdc3	3	49	12	83	144	53.8	67.8	9.6	67.9	9.7	1.04	0.39	69.3	18.4
arg3	his6	1	50	12	71	133	53.8	61.8	8.8	62.6	9.4	1.41	0.62	76.0	26.5
arg3	his6	3	43	14	84	141	59.6	78.7	12.0	79.1	12.3	1.09	0.43	83.4	26.1
arg3	his6	T	93	26	155	274	56.8	69.7	7.2	70.4	7.6	1.24	0.36	79.8	18.6

Fig. 2. Sample of results obtained by computer calculation from the observed numbers of the three tetrad types. For further discussion, see the text.

TABLE I

Interference Value *k*

Chromosome	Data set[a]	Sample size	Sample mean	Sample standard error
I	A	37	0.98	0.24
	B	71	1.17	0.67
II	A	35	1.07	0.31
	B	65	1.05	0.43
III	A	5	0.99	0.42
	B	26	0.87	0.48
I, II, and III	A	77	1.02	0.29
	B	162	1.07	0.56

[a]A, k values for which standard error of k smaller than $k/2$.
B, k values for which standard error of k smaller than k.

inserted into Eq. (12) lead to the map distance x' where interference has been taken into account. The standard errors (SE) of x, x_i, k, and x' are also given. For $k = 1$, $x = x_i = x'$.

Table I summarizes data on the k values. One immediately sees that k in *S. pombe* is near 1 for all three chromosomes. This has been noted before by Snow (1979a). We felt justified in restricting the sample of k values to those for which the SE was not exceedingly large. For k values from all three chromosomes whose SE does not exceed $k/2$ the average is $k = 1.02$. Figure 3 gives an indication of the spread of k. Although k values of 0.2 and greater than 2 are observed, the peaks are at $0.9 < k < 1.1$. We also have plotted k against x'. For x' near 40 cM $k \approx 0.9$, x' near 70 cM $k \approx 1.0$, x' near 100 cM $k \approx 1.1$. In *Sacch. cerevisiae* the average interference for several intervals is 0.36 (Mortimer and Schild, 1980). Thus the two organisms are rather different with respect to interference.

In Fig. 4A x' plotted against x_p; in Fig. 4B x is plotted against x_p. The lines going through the points are not statistical best-fit curves. Instead they are the theoretical curves obtained by inserting the expectations of system I [interference not estimated, $k = 1$, Eqs. (5)–(7)] into the Perkins formula

$$x_p = (1/2)(m_3 + 6m_2) \tag{13}$$

m_2 and m_3 are functions of x; x and x_p are initially in morgans, subsequently converted to centimorgans. Plotted then is the inverse function $x = f(x_p)$.

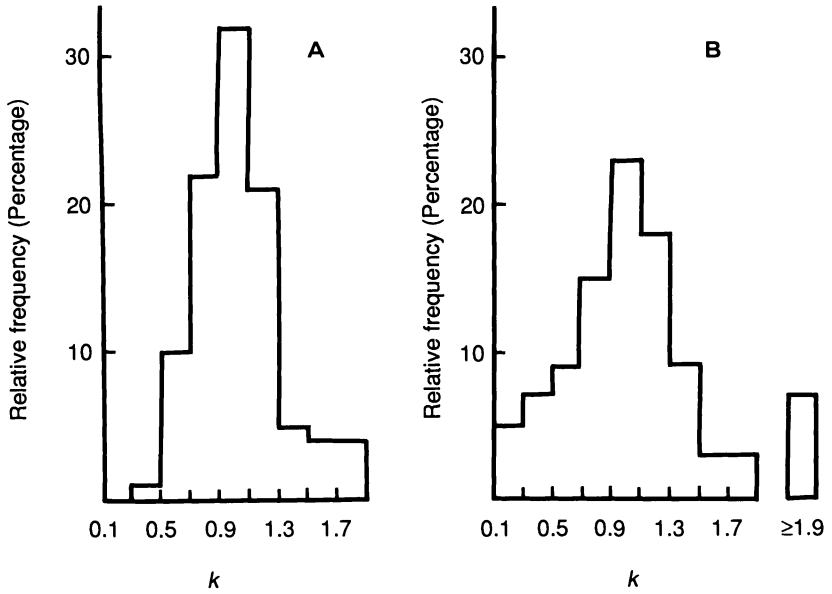


Fig. 3. Frequency distribution of interference values k. Data of the three chromosomes are pooled. A is based on 77 k values whose standard error (SE) obtained in the maximum likelihood procedure does not exceed $k/2$. B is based on 162 entries whose SE does not exceed k. The A values are contained within the B set.

Since in *S. pombe* k is rather close to 1 we felt that at least for the time being and for most practical purposes this theoretical curve could represent the situation basically as well as a statistical best-fit curve. Indeed, because of $k = 1$ the plots in Fig. 4A and 4B are rather similar. In addition, the lines drawn seem to represent the points well at least to a first approximation. In *Sacch. cerevisiae* with $k = 0.36$ the above argumentation would not hold, and Ma and Mortimer (1983) have derived an empirical equation that relates x' to x_p

$$x' = (80.7x_p - 0.883x_p^2)/(83.3 - x_p) \quad (14)$$

In *S. pombe*, Table II may be used to obtain x from x_p. Table II is constructed in exactly the same way as the lines in Fig. 4A and 4B. The suggestion is to choose the x_p value in Table II nearest to that of x_p calculated from the experiment and look up the corresponding x value. Especially in the upper range of Table II this procedure leads to only rough estimates of the map distance. Nevertheless, taken together, x is expected to be a better estimate of the map distance than x_p.

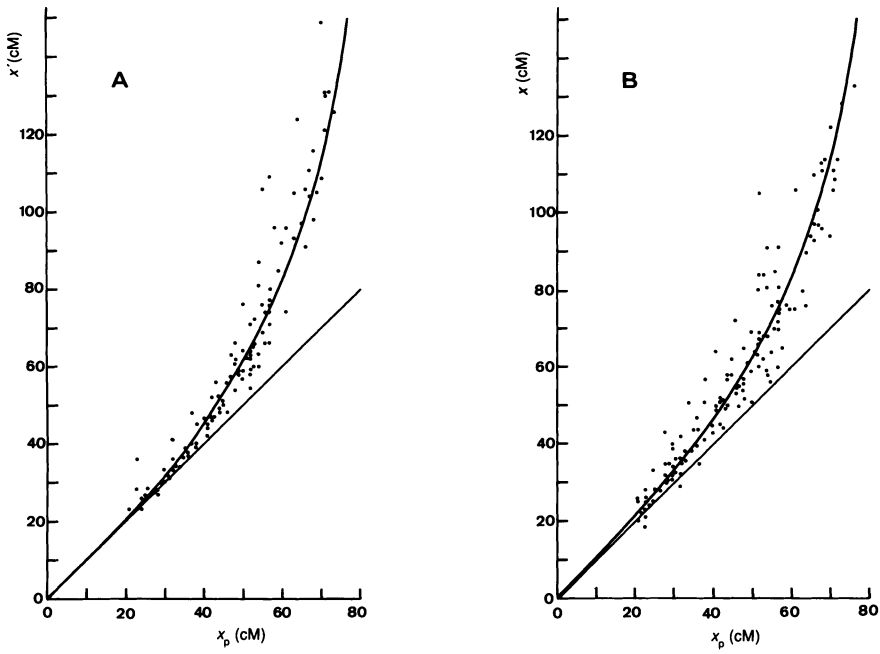


Fig. 4. (A) Plot of map distances x' (interference estimated) against x (Perkins), x_p. (B) Plot of x (interference not estimated) against x_p. x' and x were calculated by the method of Snow (1979a,b). Data of the three chromosomes are pooled. Only crosses with $M \geq 60$ tetrads have been taken into account. The numerous points for which $x_p < 20$ cM have been omitted. These points are all close to the bisector. The curved lines shown in A and B are the same; as discussed in the text, they are not statistical best-fit lines.

C. The Genetic Map

Figure 5 shows the map of *Schizosaccharomyces pombe*. It is based on tetrad data presented by Kohli *et al.* (1977) and Gygax and Thuriaux (1984), as well as on more recent published and unpublished data. x' values were used to construct the map, but, since in *S. pombe* interference is practically absent, basically the picture would not have been much different if x values had been used instead.

The mapping procedure developed by Snow (1979a,b) has its greatest merit when analyzing long distances where no intercalating additional markers have yet been found. In a hypothetical situation where markers are spaced at 10-cM intervals throughout the genome it could be dispensed with. In the present case the computer calculations proved very valuable since they allowed a thorough study of interference in *S. pombe*.

TABLE II

Corresponding Values of x and x_p (in Centimorgans) for $k = 1$ (No Interference)

x	x_p	x	x_p	x	x_p	x	x_p	x	x_p
21	20.3	41	36.8	61	49.7	81	59.5	105	67.8
22	21.2	42	37.5	62	50.3	82	59.9	110	69.2
23	22.1	43	38.2	63	50.9	83	60.3	115	70.4
24	23.0	44	38.9	64	51.4	84	60.7	120	71.5
25	23.8	45	39.6	65	51.9	85	61.1	125	72.6
26	24.7	46	40.3	66	52.5	86	61.5	130	73.5
27	25.6	47	41.0	67	53.0	87	61.9	135	74.4
28	26.4	48	41.7	68	53.5	88	62.3	140	75.2
29	27.3	49	42.4	69	54.0	89	62.7	145	75.9
30	28.1	50	43.0	70	54.5	90	63.0	150	76.6
31	28.9	51	43.7	71	55.0	91	63.4	155	77.2
32	29.8	52	44.3	72	55.5	92	63.7	160	77.8
33	30.6	53	45.0	73	56.0	93	64.1	165	78.3
34	31.4	54	45.6	74	56.4	94	64.4	170	78.7
35	32.2	55	46.2	75	56.9	95	64.8	175	79.2
36	33.0	56	46.8	76	57.3	96	65.1	180	79.5
37	33.7	57	47.4	77	57.8	97	65.4	185	79.9
38	34.5	58	48.0	78	58.2	98	65.7	190	80.2
39	35.3	59	48.6	79	58.7	99	66.0	195	80.5
40	36.0	60	49.2	80	59.1	100	66.4	200	80.8

A large part of the left arm of chromosome I (*cdc12* to *his6*) has the reverse orientation relative to the one given by Gygax and Thuriaux (1984). This finding is based on an analysis involving mitotic recombination (Lehmann and Munz, 1987). Therefore *his6* is a proximal marker of this segment. Crosses between *his6* and *aro5* give 79 PD, 60 NPD, and 253 T. Although here PDs are not significantly in excess over NPDs it is expected that significance would be attained in sufficiently large samples. In any case, from these numbers $x_p = 78.2$ cM and $x' = 190$ cM ($k = 1.3$) have been calculated. On the other hand, *aro5* is clearly linked to *lys1*. The location of *lys1* on the right arm of chromsome I is at present tentative. The other compatible orientation would be obtained by inverting the segment *cen1–lys1–cyh1–pma1* with *lys1* as a center of rotation.

According to Gygax and Thuriaux (1984) *ade10* and *ura4* (chromosome III) are linked (PD/NPD/T is 42/5/54). For the same cross a tetrad ratio of 57/48/172 is found by one of us (P. Munz) and a ratio of 22/23/89 by H. Schmidt (personal communication). The latter data do not support direct genetic linkage. According to Schmidt recombination is reduced at least fivefold on a homozygous *swi5* background. On this background *ade10* × *ura4* gives a tetrad ratio of 113/10/80, corresponding to $x_p = 34.5$ cM and

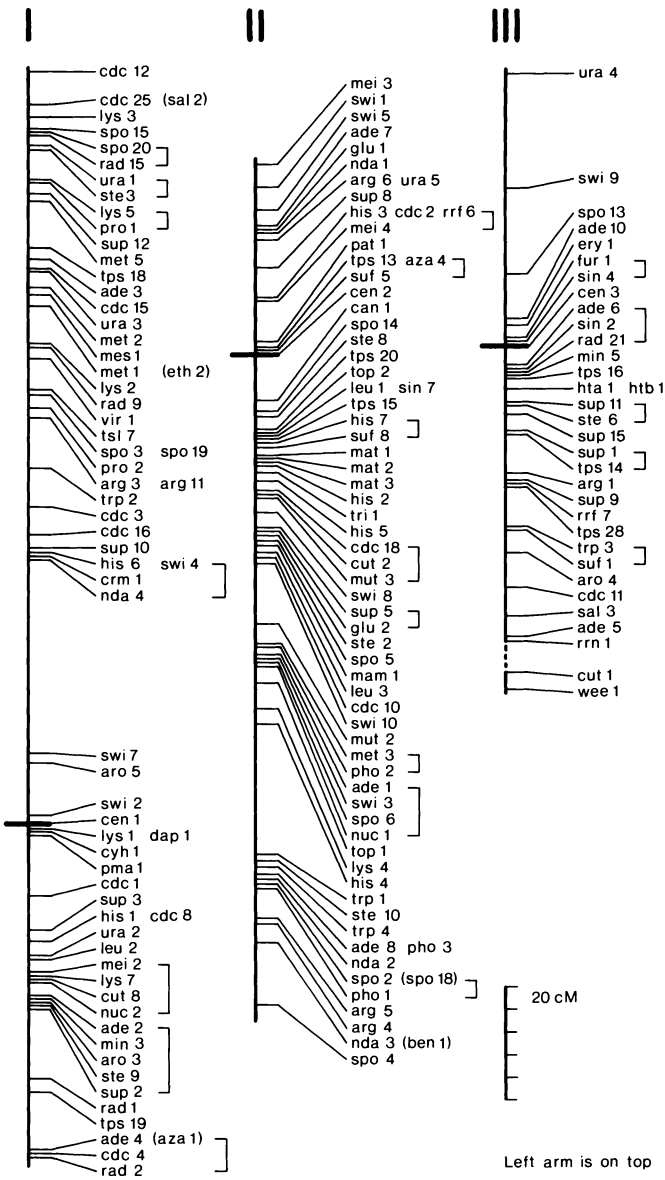


Fig. 5. Genetic chromosome map of *S. pombe*. Alleles are drawn on the same line, one of them in parentheses. If in tetrad analysis no recombinant-containing tetrads (T and NPD) have yet been observed between two nonallelic genes they are shown on one line. The order of genes enclosed by a square bracket to their right is arbitrary. Centromeres (*cen*) are indicated by bold lines. A survey of additional markers allocated to chromosomes or chromosome arms may be found in the review by Kohli (1987). *sup15* (chromosome III) is an omnipotent nonsense suppressor (B. Mathez and P. Munz, unpublished). *crm1* (chromosome I) is involved in the maintenance of chromosome structure (M. Yanagida, personal communication).

$x' = 38$ cM. This implies that under normal circumstances *ade10* and *ura4* are at least 190 cM apart. In addition *swi9* is weakly linked to both *ade10* and *ura4* and is located between these two genes (H. Schmidt, personal communication). *ura4* shows no signs of linkage to *arg1* and *ade5* (P. Munz, unpublished) and is thus placed at the far end of the left arm of chromosome III. Neither Toda *et al.* (1984) nor Hirano *et al.* (1986) could confirm linkage between *ade5* and *wee1* on chromosome III as reported by Kohli *et al.* (1977). The position of *wee1* thus remains to be reestablished.

Undoubtedly more changes will occur and inconsistencies will have to be resolved. Map distances will always wobble to some extent, necessarily so since they are derived from the study of samples. The order of markers, on the other hand, should be rather invariant if experiments have been planned and performed carefully. Mapping may be more demanding today, partly because the new markers isolated tend to have more subtle phenotypes than the auxotrophs of the golden ages.

III. THE MITOCHONDRIAL GENOME

A. Mitochondria of Fission Yeast

Mitochondrial genetics has been developed primarily in *Saccharomyces cerevisiae* which is able to survive with grossly altered (rho^-) or without mitochondrial DNA (rho^0). Yeasts sharing this property are called petite positive. *Schizosaccharomyces pombe* and the majority of yeasts are unable to produce viable rho^- or rho^0 mutants and are classified as petite negative (Bulder, 1964). Petite negativity in *S. pombe* does not imply that it is unable to survive without a functional respiratory chain, since mitochondrially inherited respiratory-deficient point and deletion mutants (mit^-) have been isolated, as well as various nuclear mutants defective in all parts of the respiratory chain and in oxidative phosphorylation (for a review, see Wolf, 1987). This inability to obtain mutants devoid of mitochondrial protein synthesis (syn^-, rho^-, rho^0) points to the necessity of mitochondrial protein synthesis even under fermentative conditions. The mitochondrial DNA content, expressed as percentage of total DNA, has been determined to be 6% in logarithmic cells and up to 14% in stationary cells (Bostock, 1969). The length of the circular mitochondrial genome has been determined as 6 μm by electron microscopy and restriction enzyme analysis (Del Giudice, 1981; Manna *et al.*, 1981; Anziano *et al.*, 1983). DNA sequencing of the entire genome of *S. pombe*

strain *ade7-50* (strain *50* for short) from the Leupold collection revealed a length of 19,430 base pairs (bp) and a G + C content of 30.1% (B. F. Lang, personal communication).

Using heterologous hybridization with gene probes from *Sacch. cerevisiae*, gene maps could be established for the two *S. pombe* strains *50* (Lang and Wolf, 1984) and *EF1* (Zimmer *et al.*, 1984). The compiled data and supplementary information from DNA sequencing (B. F. Lang, unpublished results) have led to the gene map depicted in Fig. 6. The genome contains genes for large and small ribosomal RNA (*rnl* and *rns*), which are separated by three tRNA genes. The three subunits of ATPase map in the genes *atp6, atp8,* and *atp9*. The latter two genes are separated by one of the two main tRNA gene clusters. Two of the three genes encoding subunits of cytochrome *c* oxidase, *cox2* and *cox3*, are continuous. The gene encoding cytochrome *b* (*cob*) is continuous in strain *EF1* but mosaic in strain *50*. Analysis of 28 *S. pombe* strains (Zimmer *et al.*, 1987) has shown that only 6 strains possess a mosaic *cob* gene, while the

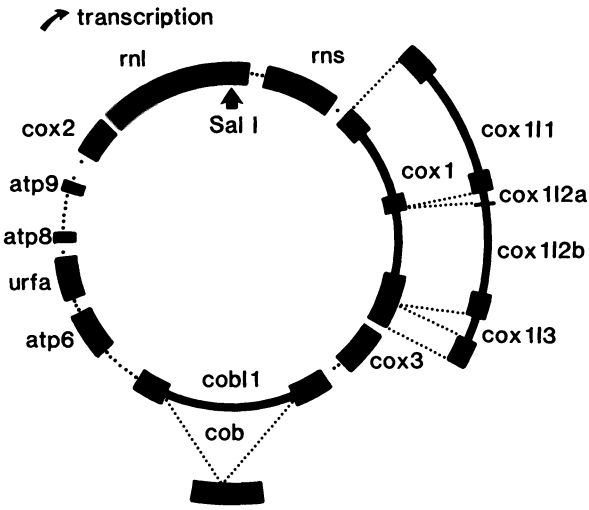


Fig. 6. Map of the *S. pombe* mitochondrial genome. The inner circle represents the genome of strain *50*, the outer segment the genome of strain *EF1*. Genes (exons) are drawn as thick bars, introns as thin bars. Dots indicate tRNA genes. *cobI1*, cob intron; *cox1I1*, *cox1I2a*, *cox1I2b*, *cox1I3*, introns in the *cox1* gene; *Sal*I, conserved restriction site in the 3′ part of *rnl*. The arrow indicates the direction of transcription and the location of the main promoter between *cox2* and *rnl*. The gene order is *rnl–tmk/tms/tmn–rns–tmm2–cox1–cox3–tmr1/tmt–cob–tmm1/tmf/tmd/tmg/tml1/tmw/tml2–atp6–tmh/tmp/tmq–urfa–tmi–atp8–tmc/tmv/tmy/tmr2/tmm3/tms–atp9–tma/tme–cox2*. (For genetic nomenclature, see Kohli, 1987.)

others harbor continuous genes. The *cox1* gene also shows a strain-dependent variation in the number of introns (2–4). The analyses of Zimmer *et al.* (1987) have shown that in natural isolates two *cox1* introns (*cox1I1* and *cox1I2a*) are found in all strains, whereas the other two introns (*cox1I2b* and *cox1I3*) are optional. A single intergenic unassigned reading frame (*urfa*) is located between *atp6* and *atp8*. It potentially encodes a polypeptide of 227 amino acids (Kornrumpf, 1984). The 25 tRNA genes are organized in two main clusters (between *atp8* and *atp9*, and *cob* and *atp6*, respectively; see legend to Fig. 6). With the exception of the regions between *cox1* and *cox3*, and *cox2* and *rnl*, respectively, tRNA genes are found to separate the different genes.

B. Signals for Transcription Initiation and RNA Processing

Transcription very likely starts from a promoter in front of *rnl* (sequence motif 5′-ATATATGTA-3′, where the last A is the first transcribed nucleotide) and possibly from a second one (5′-ATATGTGA-3′) in front of *cox3* (B. F. Lang, personal communication). All genes are transcribed from one strand (Lang *et al.*, 1983). As in mammalian mitochondrial genomes, transcripts are punctuated by tRNAs (Lang *et al.*, 1983). All genes are followed by (T + C)-rich regions, which may serve as recognition sites for 3′-processing.

C. Mitochondrial Genes

1. Gene for Large Ribosomal RNA

The DNA sequence of the *rnl* gene has been determined by Lang *et al.* (1987). In the direction of transcription, the gene is located between *cox2* and a cluster of three tRNA genes (*tmk*, *tms*, and *tmn*). Both the 5′ and the 3′ ends of the rRNA have been mapped precisely: whereas the 5′ end can be assigned unambiguously to a single nucleotide position, multiple 3′ ends occur within a run of 8 U residues. Thus, the rRNA is between 2818 and 2826 nucleotides long. Unlike its counterparts in *Sacch. cerevisiae* and *Aspergillus nidulans*, the *S. pombe* gene does not contain an intron. Comparison of potential secondary structure among the three fungal mitochondrial rRNAs and the *E. coli* rRNA has defined a common secondary structure core, held together by long-range hydrogen bonding interactions. A 5.8 S-like structure is present within the 5′-terminal region; in contrast, no 4.5 S-like structure is evident. An evolutionary evaluation of highly conserved regions of a small set of rRNA sequences suggests that *S. pombe* mitochondria diverged from a mitochondrial protofungal branch

earlier than either *A. nidulans* or *Sacch. cerevisiae* mitochondria. This points to a more primitive, conserved character of the mitochondrial genome of *S. pombe*.

2. *Gene for Small Ribosomal RNA*

The *rns* gene has been sequenced by H. Trinkl (personal communication): it is flanked by the tRNA genes *tmn* and *tmm*. At the 3′ end there is a cluster of 9 C nucleotides, which may function in RNA processing (see *rnl*). Like the large rRNA, the small rRNA molecule can be folded into a secondary structure which fits well the proposed structure of the *E. coli* molecule.

3. *tRNA Genes and Fungal Mitochondrial Evolution*

Inspection of the DNA sequence of *S. pombe* strain *50* has revealed 25 typical tRNA cloverleafs (B. F. Lang, A.-P. Sibler, G. Dirheimer, and R. P. Martin, personal communication). The reading of TGA and ATA is of particular interest, since it sets *S. pombe* again apart from most other fungal mitochondrial systems. Whereas in the ubiquitous mitochondrial proteins tryptophan is specified by TGG only, some TGA codons are found in two intronic open reading frames (orfs) and in *urfa*. It is puzzling that the *tmw* (tryptophan tRNA) gene has the anticodon CCA rather than TCA as in all other mitochondrial systems. From comparisons of highly conserved regions of mitochondrial proteins it is evident that ATA specifies isoleucine in *S. pombe* rather than methionine (which is the case in *Sacch. cerevisiae* and mammalian mitochondria). The tRNA that clearly decodes isoleucine has the anticodon GAT, which would recognize ATC and ATT but not ATA. The ATA-specific $tRNA_{Ile}$ is most probably encoded by one of the three tRNA genes with the anticodon CAT. The C residue in its anticodon might then be modified in such a way as to prevent a C–G wobble, while allowing a C–A wobble. The availability of almost the entire complement of mitochondrial tRNAs from five fungi (*Neurospora crassa, A. nidulans, Sacch. cerevisiae, Torulopsis glabrata,* and also *S. pombe*) has provided the opportunity to relate the five mitochondria to a common ancestor (Cedergren and Lang, 1985). These authors could show that *A. nidulans* and *N. crassa* sequences cluster as do *Sacch. cerevisiae* and *T. glabrata; S. pombe* is more distant.

4. *Uninterrupted Protein Coding Genes*

Simple uninterrupted protein coding genes are the two genes *cox2* and *cox3;* the three genes for ATPase subunits, *atp6, atp8,* and *atp9;* and *urfa*.

5. Mosaic cob Gene

Two genes, *cob* and *cox1*, may contain a variable number of introns (Zimmer *et al.*, 1987). The intron in the *cob* gene of several *S. pombe* strains belongs to the small family of group II introns (according to the nomenclature of Michel and Dujon, 1983). The common characteristics of these introns are the presence of highly conserved sequences at their untranslated 3′ ends and the potential of folding long stretches of their RNA into a highly conserved structure. The orf in the *cob* intron is in phase with the upstream exon and has the potential of coding for a protein rich in basic and polar residues. Recently, Michel and Lang (1985) have detected significant sequence similarity between group II intron orfs and several RNA-dependent polymerases of viral and transposable element origins. Dujon *et al.* (1986) were able to express this orf in *E. coli*, but a direct proof for a reverse transcriptase-like function is still missing.

6. Group I Introns in the cox1 Gene

The *cox1* gene of *S. pombe* strain *50* contains two group I introns with orfs in phase with the upstream exons. This type of intron is the most frequently found one among mitochondrial introns. The general features of group I introns are short conserved sequence elements that are always in the same order. Base pairing between these sequence motifs is essential for RNA splicing (Waring and Davies, 1984). Intron *cox1I2b* is exactly inserted at the position where an intron (intron 3) is found in the *cox1* gene of *A. nidulans* (Lang, 1984; Waring *et al.*, 1984). The existence of highly homologous introns within otherwise less conserved genes and the fact that exon sequences adjacent to the intron are also highly conserved argue in favor of a horizontal gene transfer (for a review, see Wolf and Del Giudice, 1987). In *S. pombe* strain *EF1*, the *cox1* gene is interrupted by two additional group I introns without orfs, which are the shortest members of this intron family so far (Trinkl and Wolf, 1986). These two introns (*cox1I2a* and *cox1I3*) are located at identical positions as the introns 4 and 5β in the *Sacch. cerevisiae cox1* gene, respectively.

D. Intron DNA Splicing in the Mitochondrial Genome

It was first observed by Gargouri *et al.* (1983) that intron mutants can revert by clean excision of the intron DNA sequence exactly at the splice points. This intron DNA splicing has been observed in *Sacch. cerevisiae* for several group I and group II introns (for a review, see Wolf, 1987).

Clean excision of introns at the DNA level has been shown by Merlos-Lange *et al.* (1987) in *S. pombe* for the group I intron *cox1I2a* and the group II *cob* intron. Using the technique of intron DNA splicing, a strain with only one intron could be constructed (Merlos-Lange *et al.*, 1987). It has been hypothesized (Gargouri *et al.*, 1983) that the genes are processed by a recombination between a cDNA reverse transcribed from a (partially) spliced mRNA precursor and the intron-containing genome. It remains to be tested whether the reverse transcriptase potentially encoded by the *cob* intron plays a role in this process.

ACKNOWLEDGMENTS

We thank R. Snow for kindly sending the computer program used in mapping and S. Kunz for setting it up in Bern. The mapping publications of R. K. Mortimer and D. Schild have been stimulating reading. Thanks are also due to all those working with *S. pombe*, especially H. Schmidt, H. Gutz, C. Shimoda, and M. Yanagida, whose published and unpublished mapping data have been incorporated in the present map without explicit reference. The work carried out in the laboratories of the authors was supported by the Swiss National Science Foundation (P. Munz, J. Kohli, and U. Leupold) and the Deutsche Forschungsgemeinschaft (K. Wolf).

REFERENCES

Adondi, G., and Heslot, H. (1970). Etude des conversions mitotiques au niveau du gène *ad-9* de *Schizosaccharomyces pombe*. *Mutat. Res.* **9,** 41–58.

Antequera, F., Tamame, M., Villanueva, J. R., and Santos, T. (1984). DNA methylation in fungi. *J. Biol. Chem.* **259,** 8033–8036.

Anziano, P. Q., Perlman, P. S., Lang, B. F., and Wolf, K. (1983). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. I. Isolation and physical mapping of mitochondrial DNA. *Curr. Genet.* **7,** 273–284.

Ares, M. (1986). U2 RNA from yeast is unexpectedly large and contains homology to vertebrate U4, U5, and U6 small nuclear RNAs. *Cell* **47,** 49–59.

Balzi, E., Di Pietro, A., Goffeau, A., Van Heerikhuizen, H., and Klootwijk, J. (1985). The RNA polymerase I initiation site and the external transcribed spacer of the fission yeast *Schizosaccharomyces pombe* ribosomal RNA genes. *Gene* **39,** 165–172.

Barratt, R. W., Newmeyer, D., Perkins, D. D., and Garnjobst, L. (1954). Map construction in *Neurospora crassa*. *Adv. Genet.* **6,** 1–93.

Beijerinck, M. W. (1894). *Schizosaccharomyces octosporus*, eine achtsporige Alkoholhefe. *Centralbl. Bakteriol. Parasitenkd.* **16,** 49–58.

Beijerinck, M. W. (1897). Weitere Beobachtungen über die Octosporushefe. *Centralbl. Bakteriol. Parasitenkd. II. Abt.* **3,** 449–455, 518–525.

Beijerinck, M. W. (1898). Ueber Regeneration der Sporenbildung bei Alkoholhefen, wo diese Funktion im Verschwinden begriffen ist. *Centralbl. Bakteriol. Parasitenkd. II, Abt.* **4,** 657–663, 721–730.

Bostock, C. J. (1969). Mitochondrial DNA of the fission yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **195,** 579–581.

Bostock, C. J. (1970). DNA synthesis in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **60,** 16–26.

Bulder, C. J. E. A. (1964). Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts. *Antonie von Leeuwenhoek* **30,** 1–9.

Cedergren, R., and Lang, B. F. (1985). Probing fungal mitochondrial evolution with tRNA. *BioSystems* **18,** 263–268.

Crandall, M., Egel, R., and MacKay, V. L. (1977). Physiology of mating in three yeasts. *In* "Advances in Microbial Physiology" (A. H. Rose and D. W. Tempest, eds.), pp. 307–398. Academic Press, New York.

Del Giudice, L. (1981). Cloning of mitochondrial DNA from the petite negative yeast *Schizosaccharomyces pombe* in the bacterial plasmid pBR322. *Mol. Gen. Genet.* **184,** 465–470.

Dhamija, S. S., Fluri, R., and Schweingruber, M. E. (1987). Two genes control three alkaline phosphatases in *Schizosaccharomyces pombe*. *Curr. Genet.* **11,** 467–474.

Ditlevsen, E., and Hartelius, V. (1956). A "gigas" mutant in *Schizosaccharomyces pombe* induced by X rays. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **26,** 41–49.

Dujon, B., Colleaux, L., Jacquier, A., Michel, F., and Monteilhet, C. (1986). Mitochondrial introns as mobile genetic elements; the role of intron-encoded proteins. *In* "Extrachromosomal Elements in Lower Eukaryotes" (R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollaender, eds.), pp. 5–27. Plenum, New York.

Egel, R. (1973). Commitment to meiosis in fission yeast. *Mol. Gen. Genet.* **121,** 277–284.

Egel, R. (1978). Synaptonemal complex and crossing-over: Structural support or interference? *Heredity* **41,** 233–237.

Egel, R., Kohli, J., Thuriaux, P., and Wolf, K. (1980). Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu. Rev. Genet.* **14,** 77–108.

Elliott, S., Chang, C., Schweingruber, M. E., Schaller, J., Rickli, E. E., and Carbon, J. (1986). Isolation and characterization of the structural gene for secreted acid phosphatase from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **261,** 2936–2941.

Fernandez, S., Homann, M. J., Henry, S. A., and Carmon, G. M. (1986). Metabolism of the phospholipid precursor inositol and its relationship to growth and viability in the natural auxotroph *Schizosaccharomyces pombe*. *J. Bacteriol.* **166,** 779–786.

Flores da Cunha, M. (1970). Mitotic mapping of *Schizosaccharomyces pombe*. *Genet. Res.* **16,** 127–144.

Fluri, R., and Kinghorn, J. R. (1985). Induction control of purine catabolism in *Schizosaccharomyces pombe*. *Curr. Genet.* **9,** 573–578.

Gancedo, C., Llobell, A., Ribas, J.-C., and Luchi, F. (1986). Isolation and characterization of mutants from *Schizosaccharomyces pombe* defective in glycerol catabolism. *Eur. J. Biochem.* **159,** 171–174.

Gargouri, A., Lazowska, J., and Slonimski, P. P. (1983). DNA-splicing in introns in a gene: A general way of reverting intron mutations. *In* "Mitochondria 1983" (R. J. Schweyen, K. Wolf, and F. Kaudewitz, eds.), pp. 259–268. De Gruyter, Berlin.

Guillermond, A. (1901). Recherches histologiques sur la sporulation des Schizosaccharomycètes. *C. R. Hebd. Séances Acad. Sci.* **133,** 242–244.

Guillermond, A. (1903). Remarques sur la copulation du *Schizosaccharomyces Mellacei*. *Ann. Soc. Bot. Lyon,* 49–55.

Guillermond, A. (1905). Recherches sur la germination des spores et la conjugaison chez les levures. *Rev. Gen. Bot.* **17,** 337–376.

Guillermond, A. (1931). Recherches sur l'homothallisme chez les levures. *Rev. Gen. Bot.* **43,** 49–86.

Gutz, H. (1966). Induction of mitotic segregation with *p*-fluorophenylalanine in *Schizosaccharomyces pombe*. *J. Bacteriol.* **92,** 1567–1568.

Gutz, H. (1967a). "Twin meiosis" and other ambivalences in the life cycle of *Schizosaccharomyces pombe*. *Science* **158,** 796–798.

Gutz, H. (1967b). Zwillingsmeiose—eine neue Beobachtung bei einer Hefe. *Ber. Dtsch. Bot. Ges.* **80,** 555–558.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. *In* "Handbook of Genetics" (R. C. King, ed.), Vol. 1, pp. 395–446. Plenum, New York.

Gygax, A., and Thuriaux, P. (1984). A revised chromosome map of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 85–92.

Haldane, J. B. S. (1919). The combination of linkage values, and the calculation of distances between the loci of linked factors. *J. Genet.* **8,** 299–309.

Hirano, T., Funahashi, S., Uemura, T., and Yanagida, M. (1986). Isolation and characterization of *Schizosaccharomyces pombe cut* mutants that block nuclear division but not cytokinesis. *EMBO J.* **5,** 2973–2979.

Hoffmeister, C. (1900). Zum Nachweise des Zellkerns bei *Saccharomyces*. *Sitzungsber. Dtsch. Naturwiss. Med. Ver. Böhmen Lotos Prag* **48,** 250–262.

Käufer, N. F., Simanis, V., and Nurse, P. (1985). Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. *Nature (London)* **318,** 78–80.

Kniep, H. (1928). "Die Sexualität der niederen Pflanzen." Fischer, Jena.

Kohli, J. (1987). Genetic nomenclature and gene list of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **11,** 575–589.

Kohli, J., Hottinger, H., Munz, P., Strauss, A., and Thuriaux, P. (1977). Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidization. *Genetics* **87,** 471–489.

Kornrumpf, D. (1984). Charakterisierung des mitochondrialen urf a Gens in *Schizosaccharomyces pombe* durch Klonierung und Sequenzierung eines Mutatorstammes. Diploma thesis, University of Munich.

Lang, B. F. (1984). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe:* Highly homologous introns are inserted at the same position of the otherwise less conserved *cox1* genes in *Schizosaccharomyces pombe* and *Aspergillus nidulans*. *EMBO J.* **3,** 2129–2136.

Lang, B. F., and Wolf, K. (1984). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 2. Localization of genes by interspecific hybridization in strain *ade7-50h*$^-$ and cloning of the genome in small fragments. *Mol. Gen. Genet.* **196,** 465–472.

Lang, B. F., Ahne, F., Distler, S., Trinkl, H., Kaudewitz, F., and Wolf, K. (1983). Sequence of the mitochondrial DNA, arrangement of genes and processing of their transcripts in *Schizosaccharomyces pombe*. *In* "Mitochondria 1983" (R. J. Schweyen, K. Wolf, and F. Kaudewitz, eds.), pp. 313–329. de Gruyter, Berlin.

Lang, B. F., Cedergren, R., and Gray, M. W. (1987). The mitochondrial genome of the fission yeast, *Schizosaccharomyces pombe*. Sequence of the large subunit ribosomal RNA gene, comparison of potential secondary structure in fungal mitochondrial LSU rRNAs, and evolutionary considerations. *Eur. J. Biochem.* **169,** 527–537.

Lehmann, E., and Munz, P. (1987). Concerning the map of chromosome I of *Schizosaccharomyces pombe*. *Curr. Genet.* **11,** 419–420.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterothallie bei *Schizosaccharomyces pombe*. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **24,** 381–480.

Leupold, U. (1955). Methodisches zur Genetik von *Schizosaccharomyces pombe*. *Schweiz. Z. Allg. Pathol. Bakteriol.* **18,** 1141–1146.

Leupold, U. (1956). Some data on polyploid inheritance in *Schizosaccharomyces pombe*. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **26,** 221–251.

Leupold, U. (1958). Studies on recombination in *Schizosaccharomyces pombe*. *Cold Spring Harbor Symp. Quant. Biol.* **23,** 161–170.

Leupold, U. (1970). Genetical methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* **4,** 169–177.

Lindegren, C. C., and Lindegren, G. (1943). A new method for hybridizing yeast. *Proc. Natl. Acad. Sci. U.S.A.* **29,** 306–308.

Lindner, P. (1893). *Schizosaccharomyces Pombe* n. sp., ein neuer Gärungserreger. *Wochenschr. Brau.* **10,** 1298–1300.

Ma, C., and Mortimer, R. K. (1983). Empirical equation that can be used to determine genetic map distances from tetrad data. *Mol. Cell. Biol.* **3,** 1886–1887.

Manna, F., Del Guidice, L., Schreil, W. H., and Wolf, K. (1981). Electron microscopy of native and cloned mitochondrial DNA. *Mol. Gen. Genet.* **184,** 469–470.

Mao, J., Appel, B., Schaack, J., Sharp, S., Yamada, H., and Söll, D. (1982). The 5S RNA genes of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **10,** 487–500.

Merlos-Lange, A.-M., Kanbay, F., Zimmer, M., and Wolf, K. (1987). DNA splicing of mitochondrial group I and group II introns in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **206,** 273–278.

Mertins, P., and Gallwitz, D. (1987). Nuclear pre-mRNA splicing in the fission yeast *Schizosaccharomyces pombe* strictly requires an intron-contained, conserved sequence element. *EMBO J.* **6,** 1757–1764.

Michel, F., and Dujon, B. (1983). Conservation of RNA secondary structures in two intron families including mitochondrial-, chloroplast- and nuclear-encoded members. *EMBO J.* **2,** 33–38.

Michel, F., and Lang, B. F. (1985). Mitochondrial class II introns encode proteins related to the reverse transcriptase of retroviruses. *Nature (London)* **316,** 641–643.

Mortimer, R. K., and Schild, D. (1980). Genetic map of *Saccharomyces cerevisiae*. *Microbiol. Rev.* **44,** 519–571.

Mortimer, R. K., and Schild, D. (1985). Genetic map of *Saccharomyces cerevisiae,* Edition 9. *Microbiol. Rev.* **49,** 181–212.

Murasugi, A., Nakagawa, C. W., and Hayashi, Y. (1984). Formation of cadmium-binding peptide allomorphs in fission yeast. *J. Biochem. (Tokyo)* **96,** 1375–1380.

Nischt, R., Gross, T., Gatermann, K., Swida, U., and Käufer, N. F. (1987). Sequence and regulatory responses of a ribosomal protein gene from the fission yeast *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **15,** 1477–1492.

Niwa, O., and Yanagida, M. (1985). Triploid meiosis and aneuploidy in *Schizosaccharomyces pombe:* An unstable aneuploid disomic for chromosome III. *Curr. Genet.* **9,** 463–470.

Nurse, P., and Thuriaux, P. (1977). Controls over the timing of DNA replication during the cell cycle of fission yeast. *Exp. Cell Res.* **107,** 365–375.

Olson, L. W., Edén, V., Egel-Mitani, M., and Egel, R. (1978). Asynaptic meiosis in fission yeast? *Hereditas* **89,** 189–199.

Osothsilp, C., and Subden, R. E. (1986). Malate transport in *Schizosaccharomyces pombe*. *J. Bacteriol.* **168,** 1439–1443.

Osterwalder, A. (1924). *Schizosaccharomyces liquefaciens* n. sp., eine gegen freie schweflige Säure widerstandsfähige Gärhefe. *Mitt. Geb. Lebensmittelunters. Hyg.* **15,** 5–28.

Otaka, E., Ooi, T., Itoh, T., and Kumazaki, T. (1986). Examination of protein sequence homologies III. Ribosomal protein YS25 from *Saccharomyces cerevisiae* and its counterparts from *Schizosaccharomyces pombe,* rat liver, and *Escherichia coli. J. Mol. Evol.* **23,** 337–342.

Perkins, D. D. (1949). Biochemical mutants in the smut fungus *Ustilago maydis. Genetics* **34,** 607–626.

Phipps, J., Nasim, A., and Miller, D. R. (1985). Recovery, repair and mutagenesis in *Schizosaccharomyces pombe. Adv. Genet.* **23,** 1–72.

Russell, P. R. (1985). Transcription of the triose-phosphate-isomerase gene of *Schizosaccharomyces pombe* initiates from a start point different from that in *Saccharomyces cerevisiae. Gene* **40,** 125–130.

Schaack, J., Mao, J., and Söll, D. (1982). The 5.8S RNA gene sequence and the ribosomal repeat of *Schizosaccharomyces pombe. Nucleic Acids Res.* **10,** 2851–2864.

Schiønning, H. (1895). En ny og ejendommelig Ascusdannelse hos en Gjaersvamp. *Medd. Carlsberg Lab.* **4,** 77–84.

Schweingruber, M. E., Fluri, R., Maundrell, K., Schweingruber, A.-M., and Dumermuth, E. (1986a). Identification and characterization of thiamin repressible acid phosphatase in yeast. *J. Biol. Chem.* **261,** 15877–15882.

Schweingruber, A.-M., Schönholzer, F., Keller, L., Schwaninger, R., Trachsel, H., and Schweingruber, M. E. (1986b). Glycosylation and secretion of acid phosphatase in *Schizosaccharomyces pombe. Eur. J. Biochem.* **158,** 133–140.

Smith, C. L., Matsumoto, T., Niwa, O., Kleo, S., Fan, J.-B., Yanagida, M., and Cantor, C. R. (1987). An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. *Nucleic Acids Res.* **15,** 4481–4490.

Snow, R. (1979a). Maximum likelihood estimation of linkage and interference from tetrad data. *Genetics* **92,** 231–245.

Snow, R. (1979b). Comment concerning maximum likelihood estimation of linkage and interference from tetrad data. *Genetics* **93,** 285.

Stelling-Dekker, N. M. (1931). Die Hefesammlung des "Centraalbureau voor Schimmelcultures." I. Teil: Die sporogenen Hefen. *Verh. K. Ned. Akad. Wet., Afd. Natuurkd., Reeks 2* **28,** 1–547.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in *Schizosaccharomyces pombe. Nucleic Acids Res.* **9,** 6429–6438.

Toda, T., Nakaseko, Y., Niwa, O., and Yanagida, M. (1984). Mapping of rRNA genes by integration of hybrid plasmids in *Schizosaccharomyces pombe. Curr. Genet.* **8,** 93–97.

Trinkl, H., and Wolf, K. (1986). The mosaic *cox1* gene in the mitochondrial genome of *Schizosaccharomyces pombe:* Minimal structural requirements and evolution of group I introns. *Gene* **45,** 289–297.

Vasiliadis, G. E., Sloan, J., Marshall, J. H., and May, J. W. (1987). Glycerol and dihydroxyacetone metabolizing enzymes in fission yeasts of the genus *Schizosaccharomyces. Arch. Microbiol.* **147,** 263–267.

Vassarotti, A., and Friesen, J. D. (1985). Isolation of the fructose-1,6-triphosphatase gene of the yeast *Schizosaccharomyces pombe.* Evidence for transcriptional regulation. *J. Biol. Chem.* **260,** 6348–6353.

Waring, R. B., and Davies, R. W. (1984). Assessment of a model for intron RNA secondary structure relevant to RNA self-splicing—A review. *Gene* **28,** 277–291.

Waring, R. B., Brown, T. A., Ray, J. A., Scazzocchio, C., and Davies, R. W. (1984). Three variant introns of the same general class in the mitochondrial gene for cytochrome oxidase subunit 1 in *Aspergillus nidulans*. *EMBO J.* **3,** 2121–2128.

Winge, Ø. (1935). On haplophase and diplophase in some Saccharomycetes. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **21,** 77–112.

Winge, Ø., and Laustsen, O. (1937). On two types of spore germination, and on genetic segregations in *Saccharomyces*, demonstrated through single-spore cultures. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **22,** 99–119.

Winge, Ø., and Roberts, C. (1949). A gene for diploidization in yeasts. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **24,** 341–346.

Wolf, K. (1987). Mitochondrial genes of the fission yeast *Schizosaccharomyces pombe*. *In* "Gene Structure in Eukaryotic Microbes" (J. R. Kinghorn, ed.), pp. 69–91. SGM Special Publication 22, IRL Press, Oxford.

Wolf, K., and Del Giudice, L. (1987). Horizontal gene transfer between mitochondrial genomes. *ISI Newslett.* **4,** 103–120.

Zimmer, M., Lückemann, G., Lang, B. F., and Wolf, K. (1984). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 3. Gene mapping in strain *EF1* (CBS 356) and analysis of hybrids between the strains *EF1* and *ade7-50h*$^-$. *Mol. Gen. Genet.* **196,** 473–481.

Zimmer, M., Welser, F., Oraler, G., and Wolf, K. (1987). Distribution of mitochondrial introns in the species *Schizosaccharomyces pombe* and the origin of the group II intron in the gene encoding apocytochrome *b*. *Curr. Genet.* **12,** 329–336.

2

Mating-Type Genes, Meiosis, and Sporulation

RICHARD EGEL

Institute of Genetics
University of Copenhagen
DK-1353 Copenhagen K, Denmark

I. INTRODUCTION

Homothallism is a widespread phenomenon in fission yeasts of the genus *Schizosaccharomyces*. It was first described for *Schizosaccharo-*

MOLECULAR BIOLOGY OF THE
FISSION YEAST

myces octosporus by Schiønning (1895) and analyzed genetically for *Schizosaccharomyces pombe* by Leupold (1950). Both authors observed that ascus formation was often preceded by conjugation within a newly divided pair of sister cells. A glimpse of Schiønning's amazement about his findings still emanates from the title of his paper: "A new and peculiar way of forming an ascus in a yeast."

When Leupold (1950) started to investigate the genetics of *S. pombe*, his culture of the Osterwalder strain contained homothallic as well as heterothallic subtypes. As a first approximation it appeared that both homothallism and heterothallism were controlled by a narrow region on chromosome II, with some recombination going on between two subloci (Leupold, 1958). For the general purpose of doing crosses with genetically defined parents, heterothallic strains are, of course, most practical to work with. On the other hand, many aspects of sporulation and related processes are more easily studied in the homothallic h^{90} strain, and most of this review, in fact, deals with the h^{90} configuration, which appears to be the wild type of the Osterwalder/Leupold strain.

Main emphasis is placed on recent developments, with special attention to the genetic and molecular aspects of mating-type switching and mating-type expression, as well as the induction of meiosis and sporulation. A more complete review of earlier literature on the subject is given in publications by Gutz *et al.* (1974), Crandall *et al.* (1977), Egel *et al.* (1980), Calleja *et al.* (1981), Phipps *et al.* (1985), and by Munz *et al.* in Chapter 1 of this volume.

II. LIFE CYCLE

A. Options

The major decisions during the life cycle of *S. pombe* are intimately controlled by the mating-type genes. It is therefore important to have a knowledge of the life cycle of fission yeast when considering mating-type genes and their expression, and a brief outline is given here. *Schizosaccharomyces pombe* is essentially a haploid organism. Although diploid zygotes are formed by fusion of two haploid cells, these usually undergo an immediate meiosis to form haploid spores, the diploid state being only a transitory phase. This normal life cycle (Fig. 1, bold arrows) is described first, followed by a summing up of the rarer diploid life cycles which can be induced in the laboratory (thin arrows in Fig. 1).

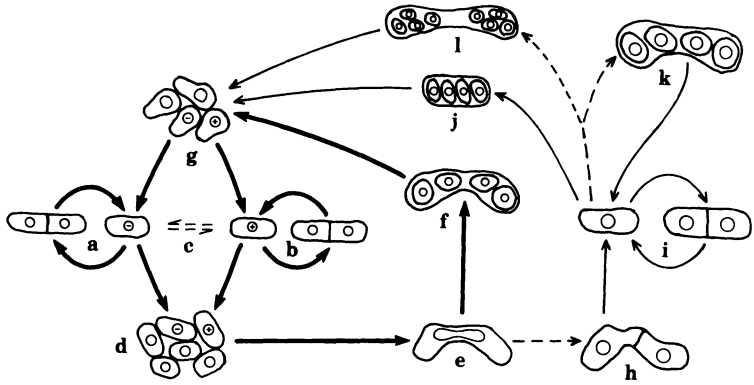


Fig. 1. Various options during the life cycle of *S. pombe*. The mainly haploid basic cycle is emphasized by bold arrows. Further explanations are given in the text.

1. Haploid Life Cycle

Under favorable conditions fission yeast cells reproduce asexually by means of the mitotic cell cycle. Cells grow by tip extension and divide by medial fission to form two equal sized daughter cells. This typically eukaryotic cell cycle is considered in detail in Chapter 5 by Fantes and Chapter 6 by Mitchison in this volume. Haploid cells can be of two mating types, "minus" or "plus". In heterothallic strains these mating types are perpetuated indefinitely, or nearly so (a and b in Fig. 1). Homothallic strains, on the other hand, undergo frequent switching of mating type in either direction with the result that sister cells often are of different mating types (c). Usually this has no physiological consequences until cells experience starvation. When this occurs, a mixed culture becomes sexually agglutinative (d), and diploid zygotes are formed by pairwise cell fusion (conjugation) and nuclear fusion (karyogamy) (e). Usually meiosis commences immediately, followed by sporulation to give four-spored asci (f). Eventually the ascus wall breaks down and the liberated haploid spores remain dormant until they encounter favorable growth conditions. Their germination (g) then restarts the cycle.

2. Diploid Life Cycles

Both conjugation and sporulation in *S. pombe* are favored by starvation. Hence, diploid zygotes usually have no other choice but to sporulate. Even if newly formed zygotes are transferred to growth medium,

most of them continue to sporulate rather than resume mitosis. A small percentage, however, restarts the vegetative cell cycle instead of meiosis under such circumstances (h in Fig. 1), thereby giving rise to a diploid colony (i). How a given diploid cell then reacts to starvation depends on its combination of mating-type genes. If it is still heterozygous (+/−) it can sporulate right away, giving rise to an azygotic ascus (j) which will liberate four haploid spores. If it has become homozygous for one or the other mating type, direct sporulation is not possible. Instead it can conjugate if a suitable partner cell is present. This leads to nuclear fusion and a tetraploid meiosis (or triploid with a haploid partner), and a four-spored ascus (k) is again formed. Yet another alternative can be observed if diploid cells are studied under conditions where conjugation is inefficient: nuclear fusion does not occur and instead both nuclei undergo meiosis on their own. This so-called twin meiosis (Gutz, 1967) leads to 6- to 8-spored asci (l).

The rare induction of diploid growth from early zygotes is not the only way of obtaining diploid colonies of *S. pombe*. Diploid subclones can occasionally arise in all vegetative cultures. This phenomenon is usually referred to as endomitosis. Such subclones have the great genetic advantage that they are homozygous for all the genetic markers of the original haploid mutant strain. In the reverse direction, haploidization can also occur, especially when nondisjunction is provoked by drugs such as *m*-fluorophenylalanine (Kohli *et al.*, 1977).

B. Responses to Starvation

All sexual activities of *S. pombe* are usually limited to a short period between vegetative growth and the stationary phase of a culture. A brief discussion of the physiological responses to starvation gives some necessary technical background on the mating-type genes, which direct sexual functions in this yeast.

Ascospores represent a dormant state selected for survival through periods of unfavorable conditions, which may include desiccation or consumption by a snail. Two spore characteristics in particular have been found most useful in the laboratory: resistance against snail gut enzymes (and other noxious agents) and accumulation of starch as a storage material. In genetic crosses, random spore analysis is facilitated by the selective digestion of vegetative cells by snail gut enzymes (Munz and Leupold, 1979) or their inactivation by 30% ethanol or acetone vapor (Gutz *et al.*, 1974; Egel, 1977a). The classic staining reaction of starch

with iodine has been adapted and modified by Leupold (1955) so as to identify spores in colonies on agar plates after treatment with iodine vapor. This technique is employed as an invaluable screening procedure at various steps in mating-type studies.

Even in purely heterothallic cultures, where mating partners are missing, the stationary cells acquire considerable resistance to moderate heat shock (47–48°C for 40 min or more). This dormant state is termed G_0 and can be attained from either G_1 or G_2 of the vegetative cell cycle (Costello *et al.*, 1986).

The initiation of mating-type related activities in batch cultures requires a critical G_1 window approximately one cell cycle before an asexual culture would become fully stationary (Egel and Egel-Mitani, 1974; Nurse and Bissett, 1981), and synchrony of conjugation and/or meiosis can be induced by various starvation protocols, conveniently by limiting or withdrawing a source of nitrogen. A different procedure is to shift a standing liquid culture, where the diffusion of oxygen is a limiting factor, to vigorous aeration by shaking (Calleja, 1973), which also indicates that active respiration and/or other mitochondrial functions are essential for the sexual cycle.

More generally, almost any sudden change in growth conditions induces a few cells to conjugate and/or sporulate. Even chemostat cultures can contain considerable numbers of zygotes and asci (McDonald *et al.*, 1982). Also, agar plates of most commonly used media support sporulation to some extent. As nutrients are never uniformly distributed throughout a colony of cells, there usually exists a transient zone where local conditions are more or less conducive to sporulation. Traditionally, malt extract or low-nitrogen media of defined composition are employed when sporulation is desired, as opposed to the yeast extract–glucose media used when sporulation should be suppressed (especially in liquid broth).

III. MATING-TYPE GENES

A. Homothallic Wild Type

When Leupold (1950) began genetic analyses, his stock culture of the Osterwalder strain already consisted of a mixture of homothallic and heterothallic subtypes. As the homothallic h^{90} strain appears complete and all the other configurations can be derived from it by rearrangement, the h^{90} genome is considered the wild type with respect to mating types.

1. *Expressed and Silent Cassettes*

Mating-type information is carried at three subloci, *mat1*, *mat2*, and *mat3*, situated on the right arm of chromosome II between the *his7* and *his2* markers. The actual mating type of a given cell is determined by whatever mating-type genes are inserted at *mat1*, the only expressible site (*mat1-M* for minus and *mat1-P* for plus). The other two loci, *mat2-P* and *mat3-M*, are silent storage sites, serving as a backup library. Their information is utilized during mating-type switching by copy transposition to *mat1*.

Originally the three subloci of the mating-type region were defined genetically by two meiosis-deficient mutants (*mei1*): *B102* with a missing *P* function and *B406* with a missing *M* function (Bresch *et al.*, 1968; Egel and Gutz, 1981; Egel, 1984a). The molecular organization of the h^{90} *mat* region has been worked out by Beach (1983), and the relevant DNA segments have been sequenced (Kelly *et al.*, 1988). While only the flanking sequences are unique for any one of these loci, the central cassettes are organized in a repetitive fashion (Fig. 2). In the chromosome they are positioned as direct repeats, connected by two spacer regions of roughly 15 kilobases (kb) each termed L and K (Beach and Klar, 1984).

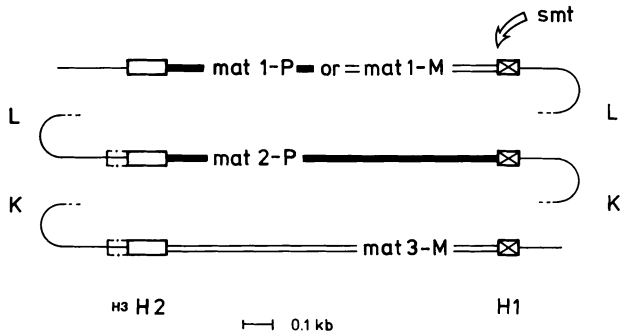


Fig. 2. Cassette structure at the three subloci of the mating-type region in the h^{90} strain of *S. pombe*. The cassettes have been aligned by their boxes of homology (*H1*, *H2*, *H3*), and the connecting spacers (L, K) are indicated in a loop-out configuration. This presentation is guided by the conviction of the author that the complex interactions during mating-type switching require folding of the entire region into a convoluted superstructure. The *smt* receptor signal for mating-type switching at the boundary between the internal cassette and the *H1* box can be activated only at the *mat1* locus.

2. *Cassette Structure*

The innermost segment of each cassette (1.1 kb) is specific for either mating type, and these differential segments are flanked by boxes of homology: *H1* of 59–64 base pairs (bp) is located to the right, and *H2* of 135 bp is located to the left at all three loci, whereas *H3* with 51 (of 55) identical base pairs is located to the left of *H2* at the two silent loci *mat2* and *mat3*. The unique *mat1* sequences on either side of the cassette itself display a strikingly skewed base composition: to the left of *H2* most of the G residues in G/C pairs are found in the upper strand, whereas to the right of *H1* most G residues are found in the lower strand. The significance of this asymmetry is not yet clear, but it might provide for some specialized chromatin structure surrounding the *mat1* cassette.

The *mat1 H1* box has particular significance for the homothallic switching mechanism. It is the site of a cis-active recognition signal for switching mating type (*smt*), which can mutate to reduced activity (Egel and Gutz, 1981; Beach, 1983). The *M* and *P* cassettes each contain two important open reading frames. These mating-type genes in a strict sense are transcribed from opposite strands, starting from centrally placed positions (Kelly *et al.*, 1988).

B. Mating-Type Switching

1. *Double-Strand Breaks at mat1*

Mating-type switching in an h^{90} cell is preceded by a double-stranded break in the chromosomal DNA close to the right-hand border of the *mat1* cassette (Beach, 1983). A small deletion (*C13-P11*) in that vicinity strongly reduces the percentage of cells containing the break. This deletion has been isolated as a "speckled" mutant with scattered streaks after iodine staining (Gutz and Fecke, 1979), indicative of a low rate of residual switching. In genetic crosses it maps just to the right of *mat1* (Egel and Gutz, 1981), defining the cis-active switching signal *smt*. A tight *smt* mutant with no residual mating-type switching has recently been described by Engelke *et al.* (1987). It is also caused by a deletion (O. Nielsen, unpublished data).

The *in vivo* generated cuts are present in some 20% of the cells in a vegetative h^{90} culture, and they are surprisingly persistent, lasting for about the length of an entire cell cycle (Beach, 1983). The cut molecules neither disappear nor accumulate significantly when different *cdc* mutants

are arrested at various points in the cell cycle. Corresponding data have been obtained by O. Nielsen (unpublished) by following a synchronized wild-type h^{90} culture through two successive cycles.

Even more surprising is the perfect viability of strains that show normal *in vivo* cutting activity at *smt* yet cannot switch mating-type cassettes. For example, Klar and Miglio (1986) constructed deletion strains that have lost both silent cassettes *mat2* and *mat3*. These strains do not show lethal sectoring even when cutting does occur at the remaining *mat1* cassette with the normal *smt* signal. Evidently *S. pombe* cells are able to self-heal the breaks in the absence of silent cassettes, when there is no additional information around for repair synthesis.

An enzyme activity *in vitro* able to generate the *smt*-specific cuts would, of course, have been helpful in the precise mapping of the broken ends, but such an activity could not be detected in cell-free extracts of *S. pombe* (A. Klar and R. Kostriken, personal communication). Therefore we resorted to a genomic sequencing procedure (Nielsen and Egel, 1989). As diagrammed in Fig. 3, the end points in the upper strand have been mapped for both mating types by this procedure, and mapping from either direction has given identical results. However, no signal has been obtained as yet for the corresponding end points in the lower strand. Proba-

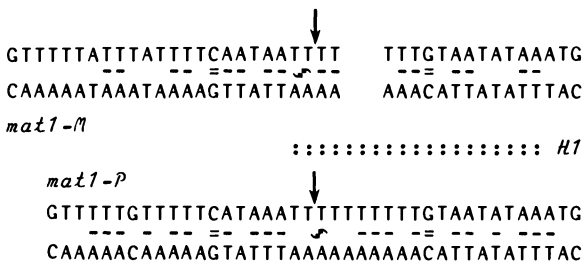


Fig. 3. DNA sequence of the *smt* site. The sequence of 40 base pairs around the boundary between the internal mating-type cassettes and the *H1* box is given for both mating types. Each sequence contains a so-called dirty palindrome (underlined). The dyad axis falls in a variable stretch of T/A pairs (7 and 10 bp for *M* and *P*, respectively), and the two sequences have been aligned so as to maximize DNA sequence similarity on either side of the T/A runs. The free ends of the double-stranded cuts at *mat1* have been mapped for the upper strands (arrows). In the lower strands the ends are protected *in vivo* and are still unmapped. The mapping was done by running chromosomal DNA containing the cuts alongside four lanes of Maxam/Gilbert reacted plasmid DNA (Nielsen and Egel, 1989). The common end point from which the fragment lengths were measured was generated by the same restriction endonuclease for all the lanes. After electrophoresis in a sequencing gel, the DNA was blotted to a membrane and hybridized to a single-stranded probe covering the expected site of cutting.

bly some (covalent?) modification is interfering with electrophoresis. Similarly, A. Klar (personal communication) has observed that three out of four end points for either mating type are protected against endonuclease degradation. The self-healing in *mat2/mat3* deletion strains can also be explained if the prospective enzyme producing the cut remains covalently bound to at least two of the ends on opposite sides, assuming that it leaves the DNA only after a switching event has been initiated or the original break has been resealed.

The breaks in the upper strand occur at the same position in both mating types, just to the right of the boundary between the internal cassette and the *H1* box, if a length polymorphism of 7 versus 10 T/A pairs in a row is disregarded. The target regions are partially self-symmetric, and the cutting sites fall into the central loops of the dirty palindromes. Although the corresponding end points in the lower strand are not yet known, the following prediction can be stated. Each time a *mat1-P* cassette is replaced by a new *mat1-M* cassette the mentioned polymorphic stretch of T/A pairs is shortened from 10 to 7. Therefore, the lower cuts in the *P* strand should not leave more than 7 A residues together, counting from the right-hand side, resulting in staggered cuts with 5′ overhangs. Alternatively, a few bases must be removed by proof editing before priming is possible at a sequence of perfect matching.

2. *Mutant swi Genes*

a. Standard Strains of Leupold's Stock. The above-mentioned speckled mutant is not the only type of mutant with a reduced rate of mating-type switching. There are others, and all are characterized by an uneven, radially striped appearance after the iodine reaction. A basic difference between them is that only a few (like the original speckled deletion) are linked to *mat1* and are cis active, whereas the majority carries fully recessive mutations not closely linked to the *mat* region (Gutz *et al.*, 1975; Egel *et al.*, 1984; Gutz and Schmidt, 1985). The phenotype of the latter class is termed "mottled," and the new switching genes are defined as *swi* genes. In most of the mutants (both *smt* and *swi*) the residual rates of mating-type switching are not equal in the two directions, and usually the frequency of *P* to *M* switches is higher than in the reverse direction, from *M* to *P*. The present number of *swi* genes is 10, and 7 of these have been mapped or at least allocated to one of the chromosome arms (Gutz and Schmidt, 1985).

The *swi* mutants can be grouped in several classes on the basis of their effects on the structure of *mat1* (Egel *et al.*, 1984; Gutz and Schmidt, 1985):

Class Ia (*swi1*, *swi3*, *swi7*): The frequency of double-stranded breaks at the *smt* signal is very much reduced, and this character is epistatic over mutants of the next class.

Class Ib (*swi2*, *swi5*, *swi6*): Cutting at *smt* occurs with wild-type frequency, but the breaks are not efficiently utilized for switching. This characteristic was the first evidence that self-healing of the double-stranded breaks might be a viable strategy in *S. pombe*, as verified by Klar and Miglio (1986).

Class II (*swi4*, *swi8*, *swi9*, *swi10*): Cutting at *smt* occurs normally, switching is also initiated, but it often fails to resolve properly. Viability is reduced, and the phenotype of older colonies is dominated by the fact that they tend to be overgrown by vigorous, heterothallic sectors of the h^{+N} type.

The main conclusions from investigation of *swi* mutants can be summarized as follows. The phenotypic classes define three major steps in the switching process: preconditioning (cutting at *smt* in the previous cell cycle), utilization (initiation of copy transposition), and resolution (regeneration of intact chromosomes). The residual switching activity observed in all the single mutants indicates that there exist alternative pathways (with low efficiency) that can lead to similar results as the wild-type swi^+ functions.

Screening for the mottled phenotype, of course, precludes the isolation of tight *swi* mutants which show no residual mating-type switching. Yet tight *swi* mutants have not been detected among other classes of completely self-sterile mutants. Perhaps they genuinely do not exist in *S. pombe*, although any mutant hunt can hardly claim to be exhaustive.

Is the leakiness of existing *swi* mutants due to residual activity of the affected gene product or to partial complementation by bypass activities from other genes? As far as *swi1* is concerned the answer is clearly in favor of the latter alternative. Of 16 *swi1* alleles tested, 5 are suppressed by an efficient opal nonsense suppressor (Schmidt *et al.*, 1987). It is not likely that all of them give leaky nonsense peptides with residual activity. Furthermore, when the chromosomal $swi1^+$ gene is disrupted by recombinant DNA techniques, the resulting $swi1::ura4^+$ transformants still express some mating-type switching at reduced frequency (Schmidt, 1987), similar to the originally isolated *swi1* mutants. Therefore the usual function of the $swi1^+$ protein can be carried out at low efficiency by another protein in the cell.

Various cumulative and/or pleiotropic effects have been reported by Gutz and Schmidt (1985) and Schmidt *et al.* (1987). In crosses with *swi7*

this marker is usually recovered in undersized colonies, and class I double mutants *swi1 swi7* and *swi3 swi7* are lethal. The reason for this lethality is still unexplained. In the only surviving class Ia double mutant, *swi1 swi3*, the residual rate of switching is not reduced below the level observed in the individual mutants. Similarly, no cumulative effects were observed in other double mutants within the same class (Ib/Ib or II/II, and tested in all the possible combinations). In double mutants from different classes, however, the iodine-positive sectoring is definitely reduced below the level characteristic for the respective single mutants, and triple mutants from all three classes are completely iodine negative. Of other effects tested *swi5*, *swi9*, and *swi10* mutants are sensitive to UV irradiation. Mutants of *swi5*, in particular, are also affected in general recombination, showing a 7- to 13-fold reduction in intragenic recombination at the *ade6* locus (Gutz and Schmidt, 1985).

The rearrangements accumulating in the class II mutants may arise as follows. Starting with a cut at *mat1-P* the distal 3′-ending strand (of the *H1* box) hybridizes to the *H1* box of *mat3-M* and serves as primer for DNA synthesis. A new strand is made throughout the internal part of the *M* cassette, and somewhere in *H2* the intermediate structure is resolved by switching the new strand back to *mat1*. Then synthesis in the reverse direction must complete the switching event. The original *P* cassette, previously residing at *mat1*, may find its way unreplicated into the unswitched sister cell.

If the *H2*-specific resolution step fails to be executed, synthesis of the growing strand primed by *H1* might continue beyond *H2*, all the way along the K region spacer, and into *mat2-P*. There again homology is found so as to switch the growing strand back to the recipient cassette *mat1-P*, either inside the cassette or in the *H2* box to which the resolution mechanism usually applies. As a result, instead of receiving a copy of just a *mat-M* cassette, the *mat1* locus now is split by a much longer insert of the entire silent domain from *mat2-P* to *mat3-M*. This exactly corresponds to the molecular structure of h^{+N}, described in a later section (cf. Table I,a), and the failure to resolve within the usual limits as followed by successful resolution at the second chance can formally explain the origin of this seemingly convoluted rearrangement.

Class II *swi* mutants also accumulate rearrangements involving the interval between *mat1* and *mat2*, either as a chromosomal tandem duplication or as a looped out, circular plasmid. These configurations may arise by aberrant switching from *mat1-M* to a copy of *mat2-P*, if resolution fails to occur at the *H2* box of *mat2* and copying of the donor cassette continues all the way into *mat1*. The *swi6* mutant, here grouped in class Ib, is

actually filling an intermediate position. The only rearrangement accumulating in this mutant corresponds to a tandem duplication of the entire *mat* region or the excision of a corresponding circle, which has not been detected (Egel *et al.*, 1984). This requires recombination between *mat1* and *mat3*, which usually interact during a switch from *P* to *M*.

How do the products of all these *swi* genes interact with the target sequences at *mat1* and the donor cassettes? It is not inconceivable that all the participating *H1* and *H2* boxes usually are held together in proximity by a major protein complex (a so-called transposome) from which the connecting lengths of DNA protrude as loops. The products of the 10 *swi* genes known already (and maybe more to come) are potential candidates to contribute to such a supporting complex structure. Circumstantial evidence for some physical interaction between *H1* and *H2* is indicated by the phenotype of a particular cis-acting mutant, *S-137* (R. Egel, unpublished observations). It is closely linked to *smt-C13-P11* (the first speckled mutant), and it mimics the resolution-defective phenotype of class II *swi* mutants, accumulating *mat1* insertions of the h^{+N} type.

b. Strains of Other Origin. Besides the standard genetic stocks based on the Leupold collection another strain of *S. pombe* (NCYC 132) has long been studied with regard to physiology of the cell growth and division cycle. Though not quite as easy to work with genetically, its mating-type system has been worked out to some extent (Calleja *et al.*, 1979, 1981). It is moderately homothallic with two distinguishable mating types, termed P with many switches and d with few. These are equivalent to (+) and (−), respectively, of Leupold's tester strains.

Gutz and Doe (1975) have studied the mating types of three other *S. pombe* strains of different geographical origins. Two of these isolates are moderately homothallic of the mottled colony type, whereas the third strain is heterothallic with very rare switches to the opposite mating type. Whether the differences compared to the Leupold strains are primarily determined at the level of a target signal (*smt*) or by additional switching genes (*swi*) has not been worked out; neither has this issue been resolved for the NCYC 132 strain mentioned before.

Sipiczki *et al.* (1985) have studied mating types in a strain of *S. pombe* var. *malidevorans*. The mating-type region was found equivalent to h^{90}. The colony phenotype after iodine staining was more "spotty," however. In addition "gray" colonies with reduced efficiency of sporulation were described as well as heterothallic colonies of two opposite mating types, found to be compatible with the Leupold testers. In this material the gray type and also the heterothallic colony types were attributed to additional genes outside of the mating-type region.

3. *Pedigree Patterns*

In the previous sections mating-type switching has been described at the level of a single cell. Discussion now focuses on the influence of a given switching event on the fate of closely related cells in a mitotic pedigree.

a. Miyata's Rule. The first evidence that switching and nonswitching cells are not randomly distributed in a mitotic lineage of wild-type h^{90} cells has been reported by Miyata and Miyata (1981). In minipedigrees observed under approaching nitrogen starvation, the appearance of a sister cell zygote is evidence of a switching event in the preceding cell division. In two pairs of sisters derived from the same grandmother cell, single switches occur most frequently, but simultaneous switching in both pairs has never been observed. Therefore, the grandmother cell of such four aligned cells divides asymmetrically: only one daughter cell is enabled to switch mating type in the next division, whereas the other daughter remains incapable of switching. The only morphological asymmetry observable in growing *S. pombe* cells is determined by the different ranks of growing tips and previous septa (fission scars) at either end of a given cell, but this is completely unrelated to the asymmetry in switching potential observed in the minipedigrees.

b. Chromosome Imprinting. In pedigrees of diploid cells (homozygous for the h^{90} configuration but marked in the *P* cassettes on one of the chromosomes) it has been observed that the asymmetric segregation of switching potential on one chromosome occurs essentially independent of the likewise asymmetric segregation on the other chromosome (Egel, 1984b). In other words, Miyata's rule is obeyed by each chromosome individually, and two sister cells are perfectly able to switch mating type in the next generation, as long as these switches occur on the two different chromosomes. This is the first indication that some structural asymmetry imprinted on the chromosome is responsible for the nonrandomness in the switching pattern.

In an elegant study Klar (1987) has provided evidence that an asymmetrically segregating precondition for the double-stranded breaks at *smt* follows one of the single DNA strands in semiconservative replication. In order to investigate the molecular segregation pattern he constructed two genetically engineered duplications of *mat1-P*. A DNA segment containing this cassette with some 200 bp on either side and a selectable $LEU2^+$ marker is inserted into the chromosome about 2 kb to the left of the original *mat1-P* cassette. In one construct both *mat-P* cassettes are ori-

ented in the same direction; in the other the inserted cassette is inverted relative to the original cassette. In both constructs two *smt* signals should be available on the same chromosome. Simultaneous cutting at both *smt* signals is, in fact, observed in the transformants bearing the directly repeated construct, whereas only one site or the other (but never both) is cut in the inverted orientation. The entire experiment has been carried out in the absence of switching, since both the silent cassettes had been deleted (cf. Klar and Miglio, 1986).

The absence of simultaneous cutting in the inverted orientation can be interpreted as follows. The two DNA strands constituting the *smt* signal are intrinsically unequal (cf. the run of 10 T/A pairs in *P-H1* of Fig. 3), and by semiconservative replication and mitosis the "T" strand and the "A" strand are pulled apart into different cells. In the direct repeat the two "T" sequences have the same polarity and are pulled into the same daughter cell. In the inverted repeat, however, the two "T" sequences have opposite polarity and are now located in different DNA strands. Hence the two "T" sequences are pulled into different cells. Assuming now that only the *smt* signal receiving the old "T" strand can be cut, then both *smt* signals can only be cut simultaneously in direct orientation but not in the inverted construct.

c. Recurrent Switching. In another series of diploid pedigrees switching has been allowed on only one of the chromosomes, the other chromosome being fixed in a heterothallic configuration (Egel and Eie, 1987). For h^{90}/h^{-U}, where the data are most comprehensive, the conclusions are as follows (always assuming that mating-type switching cannot occur without a preceding double-stranded break at *smt*). A newly switched-in *mat1-M* cassette begins from a virgin state, not able to switch immediately again in the next division. Hence it is not cut at *smt*. Usually such a cell initiates a so-called stem cell lineage: at each successive division the virgin state is preserved in one of the daughters, whereas the other is made competent for switching by obtaining the double-stranded break at *smt*. Each cell obtaining the cut at *smt* initiates another stem cell lineage: one of the daughters is not switched but inherits the cut at *smt*, whereas the other is switched to *mat1-P*, again in the virgin state with regard to cutting and switching. In these stem cell lineages uninterrupted chains of recurrent switching have been observed for up to seven switches in a row, limited only by the length of the experiment.

These observations impose certain restrictions on the putative switching mechanism. If 3′ priming of copy synthesis is a valid assumption, the newly switched, unbroken cassette should segregate with the lower strand (the "A" strand in the previous discussion) and the inheri-

table cut should segregate with the upper strand ("T"). Correspondingly, new cuts may be made only in the "T" branch behind the replication fork.

In the previous example (Klar, 1987), where cutting has been analyzed in the absence of switching, the frequent inheritance of the cut from the mother cell directly by one of the daughters is not likely to be a viable hypothesis. Otherwise the frequency of cutting at each *smt* site should approach 50% of the molecules (observed 26–30%). Presumably the self-healing necessary for successful replication restores the uncut virgin state for both daughter molecules. In wild-type strains, too, self-healing without switching may occur with variable and unknown frequencies. Therefore, the integration of all the observations into a deterministic, kinetic model of homothallism in *S. pombe* certainly is premature (for two attempts, cf. Johnson *et al.*, 1984; Egel, 1984b).

C. Mating-Type Functions

1. Subfunctions of Individual Cassettes

From the early genetic studies it is clear that the genes for either mating type serve more than one function (Bresch *et al.*, 1968; Meade and Gutz, 1976). Mating-type-specific subfunctions for conjugation and meiosis, in particular, can be differentially inactivated by mutation. The cloning of *mat* cassettes (Beach *et al.*, 1983; Beach, 1983) has opened the way for more direct analyses.

DNA sequencing of all the *mat* cassettes in *S. pombe* has suggested two open reading frames (*orf*), both for *mat1-P* and for *mat1-M* (Kelly *et al.*, 1988). In both cases the two reading frames are encoded by opposite strands in a divergent orientation. Our results (R. Egel, M. Egel-Mitani, and O. Nielsen, unpublished data) indicate that the *matP-B102* mutant allele differentially affecting meiosis is complemented only by the right-hand *orf* of *matP*. Kelly *et al.* (1988) have analyzed the different *orf* functions more systematically (Fig. 4), both by Northern blotting of the corresponding transcripts and by *in vitro* mutagenesis. One of these transcripts in either mating type is to some extent produced constitutively. These transcripts (*Pc* and *Mc*) are present even under growth conditions, although a further boost of transcription occurs on starvation. The other transcripts (*Pm* and *Mm*) are inducible in that they first appear after nitrogen starvation. If the constitutive transcripts are inactivated by nonsense mutations, the transformant cells cannot conjugate, whereas meiosis is blocked specifically if mutations are introduced into the inducible transcripts. In addition, the constitutive transcripts are also needed for

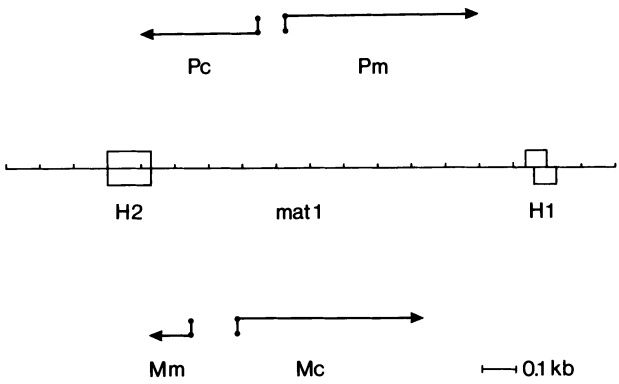


Fig. 4. Expression units of the *mat1* cassettes. The four functional open reading frames (*orf*) are indicated by arrows, pointing in the direction of transcription.

meiosis. Strictly speaking, therefore, each *mat* cassette is composed of two genes.

In the nomenclature adopted here precedence is given to the established symbols *Pm* and *Mm* for the meiotic function affected by the mutants *B102* and *B406* (Egel and Gutz, 1981; Egel, 1984a). Correspondingly *Pc* and *Mc* may read conjugation, which is affected in other mutants such as *JM56* (Meade and Gutz, 1976). Kelly *et al.* (1988), on the other hand, have proposed the postscripts *i* for inducible and *c* for constitutive, although strictly speaking both sets of transcripts respond to nitrogen starvation.

2. *Expression Signals*

Both *M* and *P* cassettes are active when inserted at *mat1*. Each one expresses two divergent transcripts initiated from within the cassette (Kelly *et al.*, 1988). Identical sequences are present in the respective silent cassettes as well. Why are they utilized at *mat1* but not at *mat2* or *mat3*? This question has not yet been studied systematically, and mutants allowing expression of the silent loci have not been described. Attempts to screen for such mutants have thus far proved unsuccessful (O. Nielsen, unpublished data). Hence it appears unlikely that negative transcriptional control is exerted on the silent *mat* cassettes by a simple repressor system.

A comparison of the various fusion cassettes produced by rearrangement (Beach and Klar, 1984) gives the only clue at present as to which additional sequences may be important for expression (cf. Table I, part c). Discussion of this point is continued in Section III,D,4.

D. Heterothallic Derivatives

1. Standard Strains h^{+N} and h^{-S}

The two heterothallic strains originally isolated together with h^{90} (Leupold, 1950, 1958) have turned out to be heterothallic for completely different reasons. The directly repeated arrangement of the three sets of homology boxes and the presence of double-stranded cuts at *smt* cause a destabilization of the entire mating-type region mitotically and meiotically. Recombination out of place can lead to many different rearrangements, and the standard heterothallic strains are just two examples, and incidentally the most readily isolated single-step events from h^{90}. The molecular details worked out by Beach and Klar (1984) explain in a rational way a wide range of previous genetic observations (e.g., Leupold, 1958; Gutz and Doe, 1973; Egel, 1976a,b).

The h^{-S} configuration can be described most easily. It arises as a deletion by loop-out recombination between *mat2-P* and *mat3-M*, retaining a single *M* cassette in the silent region in addition to the expressed *mat1-M* cassette. This strain can no longer switch because it has no silent *P* cassette, although the *smt* switching signal at *mat1* is normal. The h^{+N} configuration arises at least 10 times more frequently than h^{-S}, even though its origin is more complex. Its occurrence as a single-step event can best be comprehended as a faulty event of mating-type switching. It presumably results from delayed resolution of a switching intermediate during copy transposition (as discussed in Section III,B,2,a for class II *swi* mutants). In structural terms h^{+N} contains a duplication at *mat1-P*, composed of the entire spacer between the two silent cassettes together with an extra cassette. The expressed, most leftward cassette remains *P* for many cell divisions, resulting in an almost stable heterothallic (+) strain.

2. Combinatorial Nomenclature for Fusion Cassettes

The two rearrangements mentioned before (and others described below) all contain one or more mating-type cassettes in recombined, so-called fused surroundings. The three original subloci *mat1*, *mat2*, and *mat3* constitute the initial configuration. At each one of these a cassette is joined to a leftward (proximal) and a rightward (distal) extension. Apart from the three original arrangements there are six possible new combinations of the extending sequences, and the fusion cassette itself may be either *M* or *P*. Moreover, the two connecting spacer regions of the origi-

nal h^{90} configuration participate as well, in that they are excised or duplicated by the rearrangements.

In order to represent this multitude of possibilities systematically, the various rearrangements have been named in a combinatorial fashion according to the following conventions (Beach and Klar, 1984). The spacer between the silent cassettes *mat2* and *mat3* is termed the K region. The other spacer, between *mat1* and *mat2*, is termed the L region. The individual *mat* loci are assigned two numbers, separated by a colon. The first and the second number refer to the origin of the proximal and the distal extension, respectively. If the encoded information has to be indicated, a *P* or an *M* is added with a hyphen. The mating-type regions of the three standard strains h^{90}, h^{-S} and h^{+N} are described according to these rules in Table I,a.

TABLE I

Arrangement of *mat* Cassettes

a. Three standard strains	
h^{90}	*mat1-M,P* – –L– – *mat2-P* ==K== *mat3-M*
h^{-S}	*mat1:1-M* – –L– – *mat2:3-M*
h^{+N}	*mat1:2-P* ==K== *mat3:1-M,P* – –L– – *mat2:2-P* ==K== *mat3:3-M*
b. Other arrangements	
h^{-U}	*mat1:2-M* ==K== *mat3:1-M,P* – –L– – *mat2:2-P* ==K== *mat3:3-M*
h^{+R}	*mat1:2-P* ==K== *mat3:1-M,P* – –L– – *mat2:3-M*
h^{+S}	*mat1:1-P* – –L– – *mat2:3-P*
h^{+L}	*mat1:2-P* ==K== *mat3:3-M*
h^{-L}	*mat1:3-M*
circle	L– – *mat2:1^{0}-M,P* – –L
$h^{-S'}$	*mat1:1-M* – –L– – *mat2:1-M* – –L– – *mat2:3-M*
$h^{+N'}$	*mat1:2-P* =K= *mat3:2-P* =K= *mat3:1-M,P* –L– *mat2-P* =K= *mat3-M*
c. Occurrence of various subloci	
mat1-M	h^{90}, h^{-S}, $h^{-S'}$
mat1-P	h^{90}, h^{+S}
mat2-P	h^{90}, h^{+N}, $h^{+N'}$, $h^{-U'}$
mat3-M	h^{90}, h^{+N}, $h^{+N'}$, h^{-U}, h^{+R}, h^{+L}
mat1:2-M	h^{-U}
mat1:2-P	h^{+N}, $h^{+N'}$, h^{+R}, h^{+L}
mat1:3-M	h^{-L}
mat2:3-M	h^{-S}, $h^{-S'}$, h^{+R}
mat2:3-P	h^{+S}
mat2:1-M	circle, $h^{-S'}$
mat2:1-P	circle
mat3:1-M	h^{+N}, $h^{+N'}$, h^{+R}, h^{-U}
mat3:1-P	h^{+N}, $h^{+N'}$, h^{+R}, h^{-U}
mat3:2-P	$h^{+N'}$

3. *Additional Configurations*

The number of possible rearrangements in the *mat* region is very large, and many different mating-type configurations have been reported over the years. These are summarized, using the above nomenclature, in Table I,b. As before, essentially all the molecular interpretations are taken from Beach and Klar (1984).

a. h^{-U}. The rare switching product in the leftmost cassette from h^{+N} is known as h^{-U} (Gutz and Doe, 1973). In formal genetics it is not used as frequently as h^{-S}, but it has its merits when one quickly wants to isolate an isogenic strain with the opposite mating type from a multiply marked h^{+N} strain or vice versa.

b. h^{+R}. Crossing-over in the L region in a standard cross $h^{-S} \times h^{+N}$ can give rise to h^{90} progeny. The reciprocal recombinant is known as h^{+R} (Leupold, 1958).

c. h^{+S}. A stable h^{+} strain has been described by Gutz *et al.* (1986). In all its genetic properties it is analogous to h^{-S}. Therefore, the tentative configuration assigned to it in Table I,b appears most likely. An authentic strain for this constitution has been constructed by recombinant DNA techniques (L. Heim, personal communication).

d. h^{+L} **and** h^{-L}. In diploid strains quite frequently (10^{-3} to 10^{-2}) one of the *mat*-bearing chromosomes becomes abortive, resulting in a 2 : 2 segregation of viable and nonviable spores in entire subclones of the starting strain (Gutz and Angehrn, 1968). This can now be explained by loop-out recombination of the L region (including one cassette) from the h^{90} or the h^{-S} chromosome, leading to h^{+L} and h^{-L} respectively. Hence the L region must contain at least one essential gene with unknown function, in contrast to the K region, which can be lost without affecting viability.

e. Circular Plasmids. The loop-out product consisting of one cassette and the L region can be isolated in circular form from undigested h^{-S} DNA (Beach and Klar, 1984). It is able to replicate autonomously as a plasmid, in that it can rescue h^{-L} and h^{+L} segregants from haploid spores as well as complement *meil* mutations (H. Gutz and R. Egel, unpublished observations; L. Heim, personal communication). The frequency of loss is fairly high, however, so that reintegration into the chromosome is readily detectable by the appearance of vigorous sectors from the originally undersized colonies. In most of these the standard arrangement of *mat*

cassettes is regenerated, while others may result from integration at different sites (Engelke *et al.*, 1987).

The loss of the L-bearing plasmid does not stop cell division immediately but allows a variable number of residual divisions, depending on the nutritional state at the time of loss. This indicates that the product of the essential gene is usually present in greater than limiting amounts and is diluted out rather slowly.

f. $h^{-S'}$ and $h^{+N'}$. By reintegration of the above-mentioned circular plasmids or by displaced sister strand exchange various forms of concatenation can occur, without necessarily changing the expressed mating type. The two examples given by Beach and Klar (1984), $h^{-S'}$ and $h^{+N'}$, are included in Table I,b. Other combinations of this category probably exist but have not been described yet. Subclones of h^{90}, in particular, have occasionally been encountered carrying additional *mat*-hybridizing bands of unexplained origin (O. Nielsen, unpublished observations). Such hidden rearrangements can be quite annoying if they are not detected at the beginning of a new experimental series.

4. Expression of Fusion Cassettes

The expression or nonexpression of the various fusion cassettes of Table I,c can give certain hints as to which external sequences are important for transcriptional initiation inside the cassettes. It appears that all the combinations where the centromere-proximal (leftward) extension comes form *mat1* are expressed. Hence the distal extensions of either *mat2* or *mat3* alone are not sufficient to suppress activity at the silent loci. As *mat2 : 1-P* is expressed, at least on plasmids, the proximal *mat2* extension alone is not sufficient to suppress expression. On the other hand, *mat3 : 1* is not expressed. Therefore, the suppressive influence by the proximal *mat3* extension is so strong as to overcome a possible activating effect from the right by the distal *mat1* extension. Finally, the proximal *mat2* sequence together with the distal *mat3* extension in h^{+R} are strong enough to inactivate the intervening cassette.

E. Recombination in *mat* Region

The issue of intrastrand or sister strand recombination between the partially repetitive *mat* cassettes has already been addressed in the preceding section. This section discusses two anomalies concerning recombination between the *mat* regions of two homologous chromosomes.

1. Hot Spot at smt Signal

As discussed in Section III,B, the normal *smt* signal next to *mat1* is the site of double-stranded breaks *in vivo*. The cutting activity is highest for *mat1 : 1* cassettes, still considerable for *mat3 : 1*, but insignificant for most of the other configurations (Beach and Klar, 1984). In diploid strains homozygous for the *mat1 : 1 smt-n* configuration this site stands out as a hot spot of mitotic crossing-over (Angehrn and Gutz, 1968; Egel, 1981), leading to homozygosis for distal markers on the long arm of chromosome II. Correspondingly, in meiotic crosses homozygosis for *smt-n* is associated with considerable map expansion in the *his7/his2* interval (Meade and Gutz, 1978; Klar and Miglio, 1986).

2. Cold Zone in the K Spacer Region

Very much in contrast to the L region and the hot spot of *smt* associated recombination, harsh permafrost prevails throughout the K region spacer between the two silent cassettes. In fact, it was found impossible to genetically map the order of *mat2* and *mat3* relative to *mat1*, simply because meiotic crossing-over did not occur in that interval at a resolution of 0.001 cM (centimorgan) (Egel, 1984a). The unique ordering of the three subloci had to await the advent of gene cloning and restriction blot hybridization (Beach and Klar, 1984). By these molecular methods the physical distance between *mat2* and *mat3* has been estimated to be about 15 kb. It is not, therefore, altogether unreasonable to assume that the entire K spacer region is hidden away in some unusual chromatin structure, whereby it is rendered inaccessible to the meiotic recombination machinery.

What other evidence might point at specialized protein interactions within the K spacer chromatin? Recently (R. Egel, unpublished observations) the tight blockage of K recombination has been lifted in a peculiar pleiotropic mutant (*rik1* for recombination in the K spacer). This mutant was found as a second mutation in the *end1-458* strain of Uemura and Yanagida (1984). It is not linked to *end1* and is unrelated to the endonuclease activity determined by that gene. Actually it is linked to *ade6* on chromosome III (about 15 cM). The *rik1* marker was detected by its gray phenotype in combination with h^{90}, referring to the relatively weak iodine staining caused by reduced sporulation efficiency. In the combination h^{+N} *rik1* the K insertion at *mat1* (cf. Table I,a) is destabilized and frequently excised, resulting in abundant sectoring of gray h^{90} *rik1* subclones.

When crossing-over was analyzed for the *his7/his2* interval in the cross

his7 h^{90} *mat2-B102 rik1* × h^{90} *mat3-B406 his2 rik1*

up to 35% of the *his*$^+$ selected progeny from the *rik1* homozygous cross were gray *h*^{90} *rik1* colonies, indicating that the selected crossover in fact had occurred in the K region between *mat2-P* and *mat3-M*. A small percentage of gray colonies were even observed in *rik1* heterozygous crosses, but none in the wild-type control. On these grounds it is suggested that the unusual failure to recombine is not an intrinsic property of the K spacer DNA but is imposed on this sequence by the wild-type *rik1*$^+$ protein, which probably brings about the tight compaction of a sizable chromatin domain in that region.

IV. CONJUGATION

A. Sterility Genes

Sterile mutants in *S. pombe* by definition do not mate with *h*$^+$ or with *h*$^-$ tester strains. At the *h*^{90} *mat* region only deletions of the entire *mat1* cassette give such a phenotype (O. Nielsen, unpublished observation; A. Klar, personal communication). Most sterile mutants, however, map at a number of additional genes. Since normal crosses are impossible the genetic analysis of such mutants requires special methods. Using protoplast fusion, eight different *ste* genes have been identified (Thuriaux *et al.*, 1980; Girgsdies, 1982; Michael and Gutz, 1987). Mutants *ste5* and *ste6* allow azygotic meiosis, whereas the others are also nonsporulating as diploid cells. U. Leupold (personal communication) has characterized two additional *ste* genes from a new lot of nonsense-suppressible *ste* mutants. These were isolated from a homothallic, nonsense-suppressible *ade* mutant and subsequently selected for ade$^+$ papillae. If any of the papillae are able to sporulate the respective *ste* mutant is also nonsense-suppressible and can be crossed to tester strains in the presence of the suppressor.

Most of the *ste* mutants may be impaired in the general responses to starvation conditions. The *ste5* gene is allelic to *ras1* (Lund *et al.*, 1987), an oncogene homolog characterized by Fukui *et al.* (1986a; cf. Nadin-Davis *et al.*, Chapter 4, this volume). The sterility of *ras1* mutants can be overcome by transformation with a human *ras* sequence (Nadin-Davis *et al.*, 1986), indicating that some function has been conserved through evolution, although the specific cellular responses directed by the *ras* genes have diverged considerably.

Normal *ras1* function is needed both for conjugation and for meiosis, but not for vegetative growth in *S. pombe*. In permanently activated *ras1* mutants the vegetative cell cycle and meiosis appear normal. Conjugation, however, is very inefficient, although the growth of conjugation

tubes is initiated, especially in *mat1-P* cells, and even becomes exaggerated (Fukui *et al.*, 1986a; Nadin-Davis *et al.*, 1986). This indicates that a transient pulse of *ras1* activity relatively early after nitrogen starvation is most conducive to conjugation. Some later function during zygote formation may require a falling off of *ras1* activity again.

The requirement of *ras1* function for conjugation and meiosis can be bypassed by overexpression of another gene, *byr1*, which has been cloned and sequenced (Nadin-Davis and Nasim, 1988). Its sequence suggests that it probably encodes a protein kinase function. Disruption of the chromosomal *byr1* gene completely blocks both conjugation and meiosis. Allelism with other known *ste* loci has not yet been tested.

A key role in the release of sexual functions in response to starvation is played by the *pat1* gene (more fully described in Section V,A,1,c). Partial inactivation of the *pat1* protein derepresses conjugation (Beach *et al.*, 1985), still under control of the mating-type genes. Complete inactivation, however, precipitates meiosis and sporulation irrespective of mating type and ploidy (Iino and Yamamoto, 1985a; Nurse, 1985). The influence of the various *ste* gene mutations on this *pat1*-driven conjugation and/or meiosis was analyzed by Sipiczki (1988). None of the previously known mutants (*ste1–ste9*) interfered with *pat1*-driven sporulation, but untimely conjugation upon partial *pat1* inactivation was only possible in mutants *ste1*, *ste3*, and *ste8*. In addition, a new complementation group (*aff1*) of *ste* mutants was defined by completely sterile suppressors of the temperature-sensitive growth arrest of *pat1* strains.

B. Prezygotic Interactions

1. Sterility Genes Specific for One Mating Type

In several nonsporulating mutants only one of the mating types is blocked. When isolated from a homothallic starting strain their phenotype is heterothallic, although the *mat* region is still in the h^{90} configuration (Egel, 1973b). These mutants fall in two classes, *map* and *mam*, affecting *P* and *M* related subfunctions, respectively. Two genes are known for each class at present. Since h^{90} *map2* and h^{90} *mam2* strains still allow sexual agglutination whereas *map1* and *mam1* strains do not, the latter genes must direct earlier steps in the pathway.

The *map* and *mam* genes are primarily concerned with conjugation. Three of them are not essential for meiosis, since azygotic asci can be observed among homozygous diploid cells. Only *map1* also blocks meiosis in a homozygous diploid strain. Strangely enough, this deficiency can be complemented from cell to cell, without fusion, when wild-type h^+ cells are added to diploid *map1*/*map1* cells. This effect indicates that the

map1 function can be rescued by signal transmission at the cytoplasmic membrane.

2. *Diffusible Pheromones*

The exchange of transmitter substances (pheromones) between cells of opposite mating type has long been assumed also for *S. pombe*, but clear-cut "action at a distance" has only recently been demonstrated. Leupold (1950) obtained suggestive evidence for a morphological difference between mating cells of opposite mating type. DNA synthesis is retarded after nitrogen starvation in a mixed culture of h^+ and h^- cells compared to separately cultured strains (Egel and Egel-Mitani, 1974). In medium exchange experiments between h^+ and h^- cultures around the time of nitrogen starvation, certain sporulation-specific changes in protein patterns have been observed (Friedmann and Egel, 1978), though neither agglutinability nor morphological changes are induced by such treatment.

During their studies on *ras1* mutants Fukui *et al.* (1986a,b) observed that wild-type h^+ cells respond by elongation to the presence of h^- *ras1* mutants, even at a distance. Single h^+ cells also elongated in the vicinity of many h^- wild-type cells. No changes were observed in the reverse direction, however. Similar observations have been made by U. Leupold working with partially sterile *ste10* mutants (personal communication). He has extended these studies to various wild-type combinations by using a specially adapted oil chamber technique (Leupold, 1987). Diploid target cells of either mating type respond very well in this assay (Fig. 5), whereas haploid h^- cells show no response. The level of secretion, on the other hand, is independent of ploidy. This bioassay shows that cells of both mating types secrete active substances that induce the elongation of cells of opposite mating type. The elongation is clearly directional, resulting in bending toward the source of pheromone.

The physiological role of such factors is to synchronize the partner cells at the responsive G_1 window (the start concept in cell cycle studies; cf. Fantes, Chapter 5, this volume) and to coordinate the initiation of cell fusion to the apposed tips where the local pheromone concentration is highest. It is suggested that the pheromones secreted by *P* and *M* cells be referred to as *P*-factor and *M*-factor, respectively. These terms would in several respects be more practical than the originally proposed h^--factor (Fukui *et al.*, 1986b).

3. *Sexual Agglutination*

The first indication of sexual activity in liquid cultures of *S. pombe* is the aggregation in flocs of up to 10^5 cells (Calleja and Johnson, 1971; Egel,

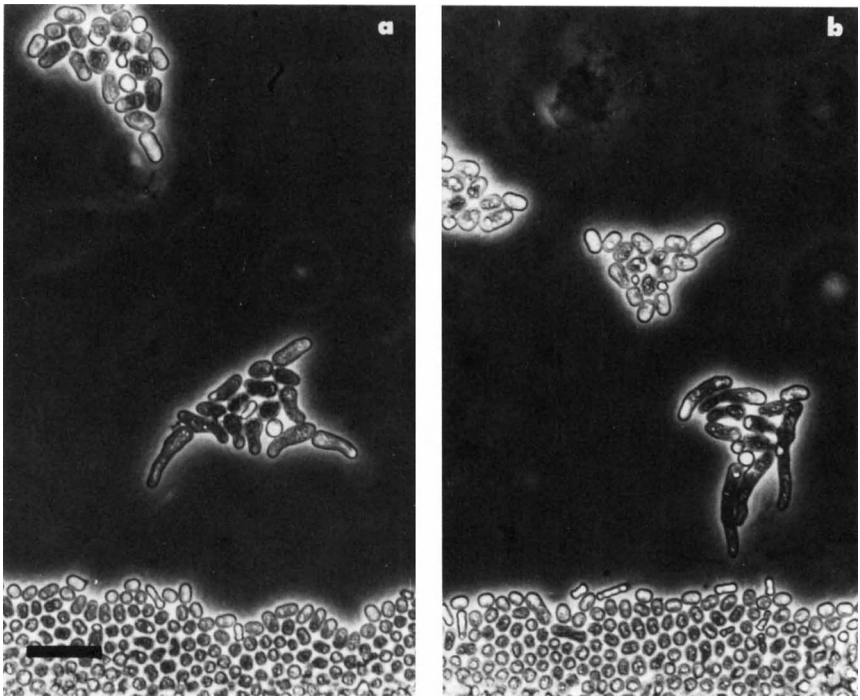


Fig. 5. Pheromone response by both mating types. Cells in the microcolonies are responding to pheromone released by the heavy inoculate at the bottom of each micrograph. The two-dimensional microcolonies are contained between a chip of sporulation agar and a coverslip. The photographs were taken after 42 hr. (a) Microcolonies, diploid h^-/h^-; main colony, haploid h^+. (b) Microcolonies, diploid h^+/h^+; main colony, haploid h^-. Bar, 20 μm. (Reproduced by courtesy of U. Leupold.)

1971). This does not happen in purely heterothallic cultures, nor do cells of separately cultured heterothallic strains flocculate immediately after mixing. Therefore, mutual induction is necessary, and this type of flocculation is rightly termed sexual agglutination. A certain degree of self-agglutination has been reported by Johnson *et al.* (1987).

A number of papers, reviewed by Calleja *et al.* (1981), describe the study of agglutination in the NCYC 132 strain of *S. pombe*. The connecting forces between cells are mainly hydrogen bonds between proteinaceous agglutinins. These proteins are probably localized on the fimbrialike sex hairs that appear on the surface of sexually induced cells (Calleja *et al.*, 1977). The most reasonable assumption is that different and complementary agglutinins are produced by the two mating types, but experimental methods to test this hypothesis directly have not yet been devel-

oped. A major hindrance is the need for mutual induction in a mixed culture, from which it is difficult to separate the individual components.

Wild-type activity of the ancillary mating-type genes *maml* and *mapl* is required for sexual agglutination. Their gene products may be involved in the induction mechanism or in agglutinin formation.

C. Cellular and Nuclear Fusion

1. Cell Wall Breakdown

a. Balancing Cross-Connections. Once sexual agglutination has occurred the flocculated cells associate two by two to form zygotes. This potentially hazardous endeavor requires considerable coordination at the macromolecular level. Ultimately, two cell wall regions must be dissolved to allow cytoplasmic fusion, but this process needs to be balanced by the formation of new cross-connections, otherwise the prezygotes might perish by osmotic lysis. Leupold's strains are very efficient in coping with this hazard, but the NCYC 132 strain is rather unbalanced in this respect (cf. Calleja *et al.*, 1981).

Conjugation usually occurs near the cell poles (Streiblova and Wolf, 1975). The proper localization of morphogenetic stimuli appears to be facilitated by the accumulation of cytoplasmic vesicles in the region of the developing conjugation tube (Hirata and Tanaka, 1982). The two processes of local breakdown of cell wall components and introduction of covalent cross-connections are affected differently by the addition of 2-deoxyglucose. This inhibitor of glucan synthesis gives rise to shear-sensitive zygotes and prezygotes that swell and sometimes rupture at their conjugation tubes (Kröning and Egel, 1974). Calleja *et al.* (1981) use different terms for the two stages: copulation for the formation of covalently linked cell pairs, which have become resistant to deflocculating agents such as detergents or proteases, and conjugation for cytoplasmic fusion after both cell walls have been breached.

b. Conjugation-Specific Glucanase. A major cell wall component of *S. pombe* is 1,3-β-glucan, and glucanases attacking this type of linkage can be liberated from cell wall preparations by autolysis (Reichelt and Fleet, 1981). While vegetative cells only contain one form (type I) of such an enzyme, a second species (type II) can be extracted from conjugating and sporulating cells. Glucanase II is about 30 times as active as glucanase I, using cell walls as a substrate. Activation by trypsin is possible but only during conjugation. This may indicate that only part of the enzyme is activated endogenously *in vivo*, presumably the fraction localized at the

fusing tips of the conjugation tube. In sporulating cells, on the other hand, all the enzyme appears to be activated by endogenous proteases, leading eventually to the complete breakdown of the ascus wall during spore liberation.

c. Mutant Affecting Cell Fusion. A mutant (*fus1*) blocked shortly before conjugation has been described (Bresch *et al.*, 1968; Egel, 1973a; Kröning and Egel, 1974). The separating cell walls are not dissolved if both prezygotic partner cells carry this mutation. Instead both cells elongate excessively and become highly vacuolated. This cell wall extension is confined to the conjugation tubes, and the original cell bodies are pushed further and further apart, often leading to horseshoe-shaped configurations.

Homothallic *fus1* mutants can be crossed successfully with both h^+ and h^- tester strains. Thus, the provision of wild-type *fus1* activity from one side only is sufficient for the breakdown of both separating walls, and the expression of the *fus1* gene is independent of the mating type. The respective gene product is likely to be a developmentally regulated glucanase or a glucanase-activating factor. Interestingly, prezygotic *fus1* cells can easily be detached from one another. Perhaps the formation of cross-connections is a second function of the glucanase itself. Alternatively, the newly broken ends of glucan chains may be the substrate for an independent connecting enzyme.

The prezygotic arrest in *fus1* mutants occurs at the G_1 phase level (Egel and Egel-Mitani, 1974), and it is readily reversible to the vegetative, mitotic cell cycle. Azygotic meiosis in diploid cells is not affected by *fus1* mutations.

2. *Karyogamy*

Immediately after successful cytoplasmic fusion the prezygotic nuclei fuse as well, usually close to the breach in the neck of the conjugation tube (cf. Conti and Naylor, 1960). This is initiated by the fusion of the two spindle pole bodies (Hirata and Tanaka, 1982). Mutants specifically blocked in karyogamy have not been described in *S. pombe*. The only example for a partial separation of nuclear from cellular fusion is the so-called twin meiosis (Gutz, 1967): when diploid cells conjugate under marginal conditions (certain batches of yeast extract have been found most conducive to this phenomenon), conjugation tubes may become exceedingly long and narrow, and nuclear fusion is often prevented long enough after cytoplasmic mixing to allow the induction of meiosis instead. The two nuclei then undergo meiosis and sporulation individually, and up to eight haploid spores are liberated.

V. SPORULATION

A. Meiosis

1. Meiotic Induction

When is the appropriate time to start meiosis? This question points at the most critical gamble during the life cycle of *S. pombe*, much as it does in many other organisms. A precocious decision will give other cells a chance to multiply further and outnumber the timid. If delayed too long, dwindling resources may not be sufficient for the bold to finish sporulation.

How this decision is normally made can be studied at various levels: Which mitotic functions are not required for meiosis? What other functions are essential for meiosis? What safeguards exist against precocious sporulation? How do all these functions interact?

a. Mitotic Start Mutants. Of the numerous temperature-sensitive cell division cycle (*cdc*) mutants only two allelic groups allow conjugation to be induced after their arrest at the restrictive temperature. These two are *cdc2* and *cdc10*, both of which block in G_1 at the start window (Nurse and Bissett, 1981). The control point defined by these mutants is further discussed by Fantes in Chapter 5 of this volume. In addition $cdc2^+$ function is also required before mitosis, but the *cdc2* cells arrested in G_2 cannot conjugate. These data complement earlier findings that conjugation of wild-type cells as well as prezygotic arrest of *fus1* mutants occur in G_1 (Egel and Egel-Mitani, 1974).

The arrest of *cdc2* mutants for 1–2 hr leads to a high incidence of diploidization by endomitosis (Egel, 1977b). This is probably due to the skipping of mitosis in the subpopulation arrested in G_2 and resumption of the cell cycle with DNA synthesis at the diploid level. If this endodiploidization takes place under conditions of nitrogen starvation it can be followed by the formation of two-spored azygotic asci at the restrictive temperature (Crandall *et al.*, 1977, p. 347). Beach *et al.* (1985) have also reported on the formation of two-spored asci with diploid spores produced by *cdc2-33* at 33°C, concluding that the initiation of meiosis as well as the performance of meiosis I are independent of *cdc2* function. Function of *cdc10*, on the other hand, is required for meiosis I (Beach *et al.*, 1985).

The product of the *cdc2* gene is a protein kinase that is phosphorylated under growth conditions but becomes dephosphorylated after nitrogen starvation (Simanis and Nurse, 1986). Conceivably it facilitates commitment to mitosis only in the active, phosphorylated state.

b. Meiotic Start Mutants. The functions missing in the above-mentioned mitotic start mutants are required for a cell to become committed to the vegetative cell cycle. Similarly a number of specialized functions are needed to commit a diploid cell to meiosis, provided that the physiological conditions are appropriate. Such meiotic start functions are missing in a class of mutants with the following phenotype (Bresch *et al.*, 1968; Egel, 1973a, 1984a; Shimoda *et al.*, 1985): mitosis and conjugation are not affected (normal growth and zygote formation), meiosis is blocked (no spores, no iodine reaction), and commitment to meiosis is not accomplished (diploid growth can readily be induced from the affected zygotes). All these mutants are blocked in G_1 before premeiotic DNA synthesis (Egel and Egel-Mitani, 1974), and all of them are subject to excessive elongation and vacuolization so long as their zygotes are arrested by starvation.

The genes affected in these mutants are *mat-P* and *mat-M* (formerly termed *mei1*), or rather the inducible meiotic genes of the *mat* cassettes *Pm* and *Mm* (see Fig. 4), as well as *mei2* and *mei3*, not linked to the *mat* region. In addition, the functions of *mat1-Pc*, *mat1-Mc*, *map1*, and various *ste* genes also participate in the induction of meiosis (cf. Sections III,C,1; IV,A; and IV,B,1).

c. Uncoupling Mutants. Untimely sporulation may be considered a nuisance even for diploid cells, but for haploid cells the precocious release of meiotic functions would usually be suicidal. The incorporation of reliable safeguards is therefore essential. Conditional mutants in which the normal meiotic control mechanisms are disengaged have been obtained by two approaches. Nurse (1985) isolated *ran1* (randy) mutants in which haploid sporulation is induced in high-nitrogen medium by a shift to elevated temperature. This temperature is therefore nonpermissive for growth. Iino and Yamamoto (1985a) isolated *pat1* (parthenogenetic) mutants as conditional suppressors of the *matP-B102* mutation. The two kinds of mutants are allelic, and *pat1* is now the agreed gene symbol.

In *pat1* mutants at the nonpermissive temperature sporulation is, in fact, independent of all mating-type activities. Meiosislike divisions are induced in haploid cells without any conjugation having taken place, and without any preceding endodiploidization. This circumvention of sex results in an azygotic type of ascus containing a variable number of uneven and usually nonviable, subhaploid spores. In diploid cells homozygous for *pat1*, on the other hand, induced meiosis and sporulation appear normal by the criteria of spore viability and genetic recombination (Iino and Yamamoto, 1985b).

Of the other meiotic start mutants, *mei3* is suppressed by *pat1* at the restrictive temperature whereas *mei2* is not. Conversely, suppressors of *pat1* mutants can readily be isolated by selecting for growth at the restric-

tive temperature, and most of these are allelic to *mei2* (Iino and Yamamoto, 1985b). This places *mei2* function at a critical position between the mitotic start window and commitment to meiosis. All the later functions for normal meiosis are also needed for *pat1*-driven sporulation.

d. Regulatory Cascade. The above-mentioned genes have been cloned and sequenced, and transcriptional analyses have supplemented the genetic observations. It is now possible to describe a detailed series of regulatory interactions (Fig. 6) that lead to the initiation of meiosis. Well before conjugation the stage is set for meiosis, since both the partly constitutive and the inducible genes of *mat1-P* and *mat1-M* are expressed in response to starvation. So long as the respective products are physically

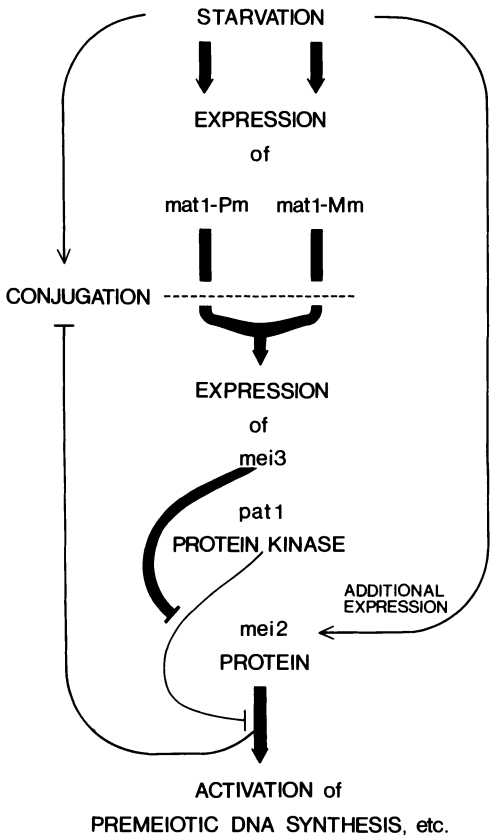


Fig. 6. Regulatory cascade of commitment to meiosis and sporulation (bold arrows). The basic expression of *pat1* is constitutive. Transcription of *mei2* varies inversely with cAMP concentration. Stimulation is indicated by arrows, inhibition by cross bars.

separated, however, no further steps are taken as far as meiosis is concerned. In haploid cells the inducible *mat* genes have no obvious function, but cytoplasmic mixing allows their products to interact immediately so as to discharge the following cascade. The mixing of two preformed compounds can explain why so few zygotes normally escape commitment to meiosis even when being transferred to growth conditions very early after conjugation. This binary interaction is the only indicator by which a cell can judge whether it is functionally diploid, a prerequisite for meiosis. As a result the *mei3* gene is transcribed if, and only if, the inducible products of the meiotic *mat1* genes *Pm* and *Mm* are present simultaneously (McLeod *et al.*, 1987), and the *mei3* function is to inactivate the *pat1* protein. A slightly different conclusion of Shimoda and Uehira (1985) was probably premature.

Comparative analysis of the *pat1* sequence suggests that the encoded product is a protein kinase (Beach *et al.*, 1985; McLeod and Beach, 1986). Its prime function appears to be inactivation of the *mei2* protein under vegetative conditions. Additional data indicate that the *pat1* protein indeed is a protein kinase *in vitro*, and that the *mei3* protein physically associates with it, inhibiting its kinase activity (McLeod and Beach, 1988). As with *pat1*, *mei2* is already expressed in growing cells (Shimoda *et al.*, 1987). The basal level is unaffected by various alleles of other *mei* genes. Some superimposed regulation does occur, however, since *mei2* transcripts are more abundant in sporulating cells. The *mei2* product, now released from inactivation, in turn activates premeiotic DNA synthesis and/or other sporulation functions. In addition it mediates the termination of earlier activities related to zygote formation such as conjugation tube extension.

e. Fine Tuning. The induction of meiosis is the ultimate goal of this regulatory cascade, when it is geared up to highest intensity. Before that, however, there is some fine tuning at intermediate levels. The two partly constitutive genes *pat1* and *mei2*, in particular, are pleiotropic and also affect conjugation to some extent. If the *pat1*-encoded protein kinase is only partially inactivated, this causes G_1 arrest at start and derepresses sexual conjugation, but it does not immediately provoke meiosis (Nurse, 1985; Beach *et al.*, 1985). In the absence of the *mei2* function, on the other hand, conjugation is slightly impaired. Conjugation tubes are longer and narrower than usual, and also the filamentous protrusions observed for *mei2*-blocked zygotes are much more slender than their counterparts in zygotes blocked by mutations at *mat1-Pm*, *mat1-Mm*, or *mei3* (Egel, 1973a).

The fine tuning of the cascade is influenced by the level of cyclic AMP (cAMP). Beach *et al.* (1985) observed that the requirement for the *pat1*-

encoded protein kinase in growing cells could be bypassed by the addition of cAMP. This effect was potentiated by caffeine, a known inhibitor of cAMP phosphodiesterase. A cAMP-dependent protein kinase competing with the *pat1* enzyme for a common substrate may account for these data, but more recent results suggest a different explanation. Maximal transcription of the *mei2* gene is induced by nitrogen starvation in cells of any mating type (Shimoda *et al.*, 1987). On the other hand, *mei2* transcription is completely abolished by added cAMP in the presence of caffeine (Watanabe *et al.*, 1988). A similar effect was observed for the inducible *mat1* transcripts *Pm* and *Mm*. This indicates that cAMP generally counteracts nitrogen starvation in the induction of life-cycle control genes. The *in vivo* concentration of cAMP during sporulation is indeed lower than in vegetative cells by 35% (Fukui *et al.*, 1986a).

A number of other gene functions are also involved in the transcriptional activation in response to starvation. The induction of *mat1-Pm* transcription requires functional products of both *map1* and *mat1-Pc* (O. Nielsen, unpublished data). Similarly, starvation-induced transcription of *mei2* depends on another gene, tentatively termed *steX* (Watanabe *et al.*, 1988), which can mutate to suppression of *pat1* arrest. The *steX* mutant itself is completely sterile and sporulation deficient, resembling *aff1* of Sipiczki (1988). Antagonistic action is exerted by the wild-type *pac1* gene, which at high copy number can suppress *pat1* arrest (Watanabe *et al.*, 1988). Evidently, the coupling of the sporulation cascade to starvation is a multichannel process and only partly understood.

2. *Synapsis and Recombination*

a. Linear Elements. The pairing of homologous chromosomes and accompanying chiasma formation are universal properties of meiosis. Homologous pairing is usually mediated by a characteristic structure, the synaptonemal complex, which is observable by electron microscopy. Such structures have never been detected in *S. pombe*. Instead, dense filaments termed linear elements appear during prophase at a stage when the nucleus is elongated with the fused spindle pole body at one end and a bundle of extranuclear microtubules running alongside (Olson *et al.*, 1978; Hirata and Tanaka, 1982). The number and extent of these filaments is quite variable and in no way related to the number of chromosomes (which is three) (Kohli *et al.*, 1977; Robinow, 1977; Umesono *et al.*, 1983). This stage with filaments accumulates in *mei4* mutants (Olson *et al.*, 1978; Shimoda *et al.*, 1985). Premeiotic DNA synthesis has occurred by then, but whether recombination has already been initiated is difficult to analyze, since the affected nuclei are blocked after commitment to meiosis and cannot be revived to allow colony formation.

b. Meiotic Recombination. *Schizosaccharomyces pombe* is very proficient indeed in terms of meiotic crossing-over. Its total map length spans at least 2,250 cM (Munz *et al.*, Chapter 1, this volume) with about 6 kb per map unit on average. This corresponds to an average of 45 crossovers per meiosis, distributed 19 : 15 : 11 between chromosomes I, II, and III, respectively. The average number of crossover events is unusually high, especially for the longest chromosome, and for individual nuclei it may be higher still, since by and large there is no crossover interference in *S. pombe* (Snow, 1979). Perhaps the absence of synaptonemal complexes and the absence of crossover interference are causally related (Olson *et al.*, 1978; Egel-Mitani *et al.*, 1982). Without interference the siting of crossover events is essentially random, although in certain regions recombination is absent, for example, in the K spacer between *mat2* and *mat3* (Egel, 1984a) or close to the centromere (Nakaseko *et al.*, 1986).

Gene conversion has also been studied in *S. pombe* (for a review, see Egel *et al.*, 1980). The basic conversion frequency per gene is rather low with a narrow peak around 0.6%, approximating a Poisson distribution (Gygax and Thuriaux, 1984). In most genes postmeiotic segregation occurs very rarely, indicating that efficient mismatch repair systems are present during meiosis. In recombination studies with *S. pombe* most emphasis has been put on various marker effects. In the opal mutant *ade6-M26* conversion frequencies are elevated about 10 times on either side of the mutant site (Gutz, 1971). A G/C to T/A transversion in this mutant gene brings the target sequence closer to the consensus of topoisomerase II (P. Szankasi, personal communication) and may provide new double-stranded breaks which can initiate recombination inside the gene. Certain G/C to C/G transversions at the anticodon of tRNA genes have a strong marker effect on intragenic recombination. This is related to inefficient repair since 25% of the aberrant asci show postmeiotic segregation (Munz *et al.*, 1983; cf. Egel *et al.*, 1980).

Intergenic (or allotopic) gene conversion occurs between dispersed isoacceptor tRNA genes (Munz *et al.*, 1982; Junker *et al.*, 1987). This intergenic conversion system has been used in a homothallic background to isolate mutants that affect recombination by either increasing or decreasing the meiotic instability of the reiterated tRNA/suppressor genes (Thuriaux, 1985).

3. Meiosis II

In contrast to meiosis I, the second meiotic division requires a functional *cdc2* product (Beach *et al.*, 1985), thereby stressing the general similarity between this division and mitosis. There are several mutants affecting meiosis II selectively: *tws1* leads to two-spored asci by the omis-

sion of meiosis II (Nakaseko *et al.*, 1984). Arrest by a conditional tubulin mutant *nda3* can also result in two-spored asci as can *cdc2* arrest (see Section V,A,1,a). In fact, *tws1* has been shown recently to be allelic with *cdc2* (Niwa and Yanagida, 1988). Evidently the completion of meiosis II is not always essential for spore formation. However, in two other mutants (*mes1* and *mes2*) binucleate zygotes incapable of sporulation accumulate after meiosis I (Bresch *et al.*, 1968; C. Shimoda, personal communication).

In wild-type sporulation the spindle pole body differentiates into a multiplaque structure (Tanaka and Hirata, 1982). Normally this modification has already commenced at the binucleate stage. In *mes1*, however, it is not initiated (Shimoda *et al.*, 1985).

B. Spore Formation

In the wild-type cell the first morphological indication of incipient sporulation is the above-mentioned modification of the spindle pole body between meiosis I and meiosis II. This is followed in meiosis II by the deposition of forespore membranes from coalescing vesicles, starting at the modified spindle pole bodies (Yoo *et al.*, 1973; Tanaka and Hirata, 1982). Eventually the four nuclei formed after meiosis II become entirely enclosed by both leaflets of the forespore membrane, and spore wall material is deposited between the two layers. A major constituent of the growing spore wall is a prominent sporulation-specific protein (Shimoda, 1983). Amylose is synthesized in the spores, as detected by the iodine reaction.

None of the above changes occurs in mutants of the 18–20 *spo* genes (Bresch *et al.*, 1968; Kishida and Shimoda, 1986), which have been screened among iodine-negative colonies. Conversely, not all iodine-positive derivatives of nonsporulating strains regain the ability to sporulate: in *vir1* mutants the synthesis of amylose becomes uncoupled from sporulation (Meade and Gutz, 1975). Since this mutant is recessive, the corresponding wild-type function is probably a negative regulator of starch formation, which is normally inactivated inside the developing spores.

C. Spore Germination

The dormant state of the spores should be given up only when the chances of sustained growth and division are reasonably high. Study of spore germination requires homogeneous starting material. Spores can be freed from vegetative cells by gradient centrifugation (Padilla *et al.*, 1975;

Nishi *et al.*, 1978) or by digestion of the vegetative cells (Munz and Leupold, 1979).

The kinetics of germination are strongly influenced by spore size. The pregermination interval varies from 5 to 10 hr, being shortest for large spores, whereas the interval between germ tube formation and first division (~4.5 hr) is independent of spore size (Padilla *et al.*, 1975). Ungerminated spores have a high refractive index, and the loss of this property is the earliest morphological change related to germination, followed by swelling and germ tube formation (Nishi *et al.*, 1978).

The loss of refractivity is fully induced by simple sugar solutions, glucose giving the fastest response, but swelling and germ tube formation require the presence of other nutrients as well (Shimoda, 1980). The ability to utilize fructose and maltose is very low in dormant spores and is attained only by germination on other sugars such as glucose. Internal carbohydrates, trehalose in particular, are also mobilized during germination (Inoue and Shimoda, 1981), although glycogen or amylose levels remain essentially constant for at least 8 hr. Since active trehalase is already present in the cell wall fraction of dormant spores, the rapid degradation of trehalose is probably mediated by export through the cytoplasmic membrane.

The first round of DNA replication takes place concurrently with germ tube formation. When DNA replication is inhibited by the presence of hydroxyurea or by *cdc10* arrest, the formation of the first germ tube is not inhibited, and a second germ tube even appears some 2.5 hr later (Shimoda and Nishi, 1982). The isolation of temperature-sensitive germination-deficient mutants has been reported (Shimoda, 1979), but most of these are no different from ordinary *cdc* mutants. The cycle is now complete and the germinated spores resume vegetative growth and division.

VI. SYNOPSIS

This chapter describes the various steps of sexual differentiation in *S. pombe*. The numerous mutants affecting this pathway at different levels are diagrammed in Fig. 7. Major highlights from recent work are the elucidation of the structure of the mating-type cassettes together with the rules of mating-type switching, and the cascade of interactions leading to meiosis.

The precursor state for mating-type switching is a double-stranded break at the expressed cassette. This break is long lasting and inheritable from cell to cell, being retained in the nonswitching stem cell from which

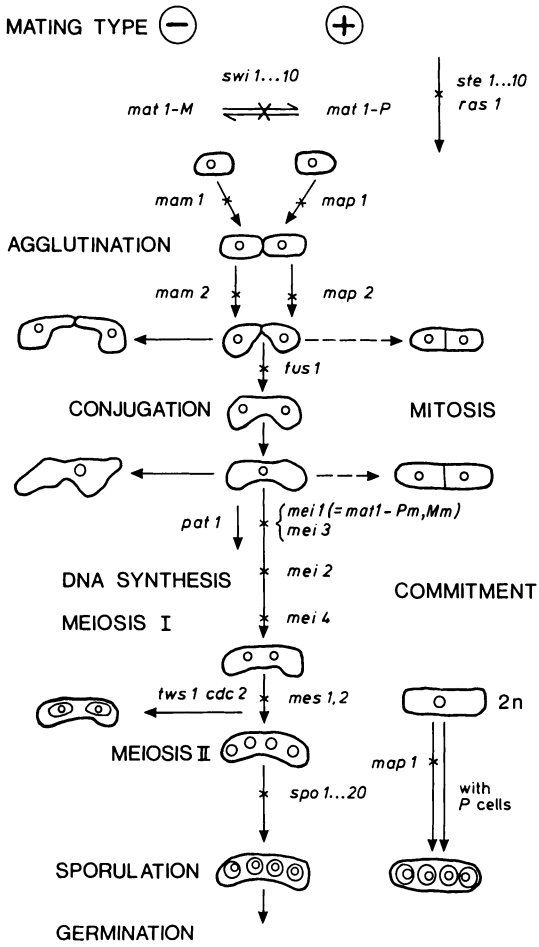


Fig. 7. Genes involved in conjugation, meiosis, and sporulation. Mutant phenotypes are as follows: *mat1-M,P*, expressed mating-type genes needed for conjugation and/or meiosis; *swi1 ... 10*, lowered frequency of mating-type switching; *ste1 ... 10* (*ras1* = *ste5*), no conjugation with either mating type; *mam1*, neither agglutination nor conjugation of *M* cells; *mam2*, no conjugation of agglutinating *M* cells; *map1*, neither agglutination nor conjugation of *P* cells; *map2*, no conjugation of agglutinating *P* cells; *fus1*, no cell fusion between *fus1* cells, aberrant conjugation tube extension, haploid growth recoverable; *mei1* (= *mat1-Pm,Mm*), *mei3*, *mei2*, no commitment to meiosis, no premeiotic DNA synthesis, aberrant zygote extension, diploid growth recoverable; *pat1*, unscheduled induction of meiosis bypassing *mat1* and *mei3*; *mei4*, blocked at a pachytenelike stage, no further divisions; *tws1*, *cdc2*, no meiosis II, sporulation possible with two-spored asci; *mes1,2*, no meiosis II, no sporulation; *spo1 ... 20*, no sporulation after meiosis II; diploid *map1/map1*, no meiosis, meiosis inducible by *P* cells without fusion.

switched daughters segregate recurrently. These switched daughter cells obtain a new cassette by copy transposition from the silent cassette of opposite mating type. New breaks are not formed at random: in some subtle way their appearance is linked to replication and limited to one of the branches behind the replication fork. These double-stranded breaks are protected *in vivo*. They can be self-sealed and are not lethal when no silent cassettes are available. Covalently bound proteins may be responsible for this protection.

The gene products encoded by the expressed mating-type cassettes are partly inducible by starvation. All of them are present before conjugation, but the complementary proteins of either mating type are physically separated until cytoplasmic fusion allows them to interact. When this occurs their combined action releases a cascade of positive and negative controlling devices committing the zygote to meiosis and sporulation. This mechanism depends on three proteins, only two of which are present before conjugation. The missing one is specifically induced by the zygotic interaction of the mating-type products. It functions as a trigger of meiosis in a double-negative fashion by inactivation of an antimeiotic protein kinase. So long as the kinase is active its target protein, a direct initiator of meiosis, is blocked. As soon as the kinase is inhibited the target protein can do its job.

In addition a better understanding of conjugation is being obtained. It is critical for the induction of conjugation that the meiotic cascade be set to an intermediate level in response to a lowered concentration of cyclic AMP. The exchange of diffusible pheromones has been shown for both mating types. The subsequent conveyance and processing of mating-type signals is severely impaired in oncogene homolog *ras1* mutants. This is the first in a series of sterility genes for which a biochemical function is being ascertained, and more work along these lines can be expected.

ACKNOWLEDGMENTS

I wish to express my gratitude to the numerous colleagues who have communicated their most recent findings to keep this review up to date. Unpublished work from this laboratory, contributions by Olaf Nielsen in particular, has been supported by the Danish Center for Microbiology. The proficient technical assistance of Tove Friis is gratefully appreciated. Lis Lefeldt and Poul Eriksen helped with the artwork, and Lis Svarth coped with the manuscript. The many constructive suggestions from careful and critical reading by Steve Aves and Olaf Nielsen are especially acknowledged. Finally I deeply regret that I could not possibly have cited the countless researchers who have made corresponding observations or arrived at similar conclusions from work on organisms other than fission yeasts.

REFERENCES

Angehrn, P., and Gutz, H. (1968). Influence of the mating-type on mitotic crossing over in *Schizosaccharomyces pombe*. *Genetics* **60,** 158.

Beach, D. H. (1983). Cell type switching by DNA transposition in fission yeast. *Nature (London)* **305,** 682–688.

Beach, D. H., and Klar, A. J. S. (1984). Rearrangements of the transposable mating-type cassettes of fission yeast. *EMBO J.* **3,** 603–610.

Beach, D. H., Nurse, P., and Egel, R. (1982). Molecular rearrangement of mating-type genes in fission yeast. *Nature (London)* **296,** 682–683.

Beach, D., Rodgers, L., and Gould, J. (1985). *ran1*$^+$ controls the transition from mitotic division to meiosis in fission yeast. *Curr. Genet.* **10,** 297–311.

Bresch, C., Müller, G., and Egel, R. (1968). Genes involved in meiosis and sporulation of a yeast. *Mol. Gen. Genet.* **102,** 301–306.

Calleja, G. B. (1973). Role of mitochondria in the sex-directed flocculation of a fission yeast. *Arch. Biochem. Biophys.* **154,** 382–386.

Calleja, G. B., and Johnson, B. (1971). Flocculation in a fission yeast: An initial step in the conjugation process. *Can. J. Microbiol.* **17,** 1175–1177.

Calleja, G. B., Yoo, B. Y., and Johnson, B. F. (1977). Fusion and erosion of cell walls during conjugation in the fission yeast (*Schizosaccharomyces pombe*). *J. Cell Sci.* **25,** 139–155.

Calleja, G. B., Johnson, B. F., Zuker, M., and James, A. P. (1979). The mating system of a homothallic fission yeast. *Mol. Gen. Genet.* **172,** 1–6.

Calleja, G. B., Johnson, B. F., and Yoo, B. Y. (1981). The cell wall as sex organelle in fission yeast. *In* "Sexual Interactions in Eukaroytic Microbes" (D. H. O'Day and P. A. Horgen, eds.), pp. 225–259. Academic Press, New York.

Conti, S. F., and Naylor, H. B. (1960). Electron microscopy of ultrathin sections of *Schizosaccharomyces octosporus*. II. Morphological and cytological changes preceding ascospore formation. *J. Bacteriol.* **79,** 331–340.

Costello, G., Rodgers, L., and Beach, D. (1986). Fission yeast enters the stationary phase G_0 state from either mitotic G_1 or G_2. *Curr. Genet.* **11,** 119–125.

Crandall, M., Egel, R., and MacKay, V. L. (1977). Physiology of mating in three yeasts. *Adv. Microb. Physiol.* **15,** 307–398.

Egel, R. (1971). Physiological aspects of conjugation in fission yeast. *Planta* **98,** 89–96.

Egel, R. (1973a). Commitment to meiosis in fission yeast. *Mol. Gen. Genet.* **121,** 277–284.

Egel, R. (1973b). Genes involved in mating-type expression of fission yeast. *Mol. Gen. Genet.* **122,** 339–343.

Egel, R. (1976a). The genetic instabilities of the mating type locus in fission yeast. *Mol. Gen. Genet.* **145,** 281–286.

Egel, R. (1976b). Rearrangements at the mating type locus in fission yeast. *Mol. Gen. Genet.* **148,** 149–158.

Egel, R. (1977a). Selective spore survival during replica-plating of fission yeast. *Arch. Microbiol.* **112,** 109–110.

Egel, R. (1977b). Frequency of mating-type switching in homothallic fission yeast. *Nature (London)* **266,** 172–174.

Egel, R. (1981). Mating-type switching and mitotic crossing-over at the mating-type locus in fission yeast. *Cold Spring Harbor Symp. Quant. Biol.* **45,** 1003–1007.

Egel, R. (1984a). Two tightly linked silent cassettes in the mating-type region of *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 199–203.

Egel, R. (1984b). The pedigree pattern of mating-type switching in *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 205–210.

Egel, R., and Egel-Mitani, M. (1974). Pre-meiotic DNA synthesis in the fission yeast. *Exp. Cell Res.* **88,** 127–134.

Egel, R., and Eie, B. (1987). Cell lineage asymmetry in *Schizosaccharomyces pombe:* Unilateral transmission of a high-frequency state for mating-type switching in diploid pedigrees. *Curr. Genet.* **12,** 429–433.

Egel, R., and Gutz, H. (1981). Gene activation by copy transposition in mating-type switching of a homothallic fission yeast. *Curr. Genet.* **3,** 5–12.

Egel, R., Kohli, J., Thuriaux, P., and Wolf, K. (1980). Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu. Rev. Genet.* **14,** 77–108.

Egel, R., Beach, D. H., and Klar, A. J. S. (1984). Genes required for initiation and resolution steps of mating-type switching in fission yeast. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3481–3485.

Egel-Mitani, M., Olson, L. W., and Egel, R. (1982). Meiosis in *Aspergillus nidulans:* Another example for lacking synaptonemal complexes in the absence of crossover interference. *Hereditas* **97,** 179–187.

Engelke, U., Koster, L., Gutz, H., Heim, L., and Schmidt, H. (1987). Molecular characterization of h^- mutants of *Schizosaccharomyces pombe*. *Curr. Genet.* **12,** 535–542.

Friedmann, K. L., and Egel, R. (1978). Protein patterns during sporulation in fission yeast. *Z. Naturforsch.* **33c,** 84–91.

Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986a). Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. *Cell* **44,** 329–336.

Fukui, Y., Kaziro, Y., and Yamamoto, M. (1986b). Mating pheromone-like diffusible factor released by *Schizosaccharomyces pombe*. *EMBO J.* **5,** 1991–1993.

Girgsdies, O. (1982). Sterile mutants of *Schizosaccharomyces pombe:* Analysis by somatic hybridization. *Curr. Genet.* **6,** 223–227.

Gutz, H. (1967). "Twin meiosis" and other ambivalences in the life cycle of *Schizosaccharomyces pombe*. *Science* **158,** 796–798.

Gutz, H. (1971). Site-specific induction of gene conversion in *Schizosaccharomyces pombe*. *Genetics* **69,** 317–337.

Gutz, H., and Angehrn, P. (1968). Lethal mutations in the mating-type region of *Schizosaccharomyces pombe*. *Genetics* **60,** 186.

Gutz, H., and Doe, F. J. (1973). Two different h^- mating types in *Schizosaccharomyces pombe*. *Genetics* **74,** 563–565.

Gutz, H., and Doe, F. J. (1975). On homo- and heterothallism in *Schizosaccharomyces pombe*. *Mycologia* **67,** 748–759.

Gutz, H., and Fecke, H. C. (1979). Probable promoter mutations in the mating-type region of *Schizosaccharomyces pombe*. *Hoppe-Seyler's Z. Physiol. Chem.* **360,** 274.

Gutz, H., and Schmidt, H. (1985). Switching genes in *Schizosaccharomyces pombe*. *Curr. Genet.* **9,** 325–331.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. *In* "Handbook of Genetics" (R. C. King, ed.), Vol. 1, pp. 395–446. Plenum, New York.

Gutz, H., Meade, J. H., and Walker, S. (1975). Mating type modifier genes in *Schizosaccharomyces pombe*. *Genetics* **80,** s38.

Gutz, H., Kapitza, P., and Köster, L. (1986). Stable h^+ strains of *Schizosaccharomyces pombe*. *Naturwissenschaften* **73,** 209.

Gygax, A., and Thuriaux, P. (1984). A revised chromosome map of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 85–92.

Hirata, A., and Tanaka, K. (1982). Nuclear behavior during conjugation and meiosis in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Appl. Microbiol.* **28,** 263–274.

Iino, Y., and Yamamoto, M. (1985a). Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. *Mol. Gen. Genet.* **198,** 416–421.

Iino, Y., and Yamamoto, M. (1985b). Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2447–2451.

Inoue, H., and Shimoda, C. (1981). Changes in trehalose content and trehalase activity during spore germination in fission yeast, *Schizosaccharomyces pombe*. *Arch. Microbiol.* **129,** 19–22.

Johnson, B. F., Calleja, G. B., and Zuker, M. (1984). Mating-type gene switching in a homothallic fission yeast. *J. Theor. Biol.* **110,** 299–312.

Johnson, B. F., Walker, T., Miyata, M., Miyata, H., and Calleja, G. B. (1987). Sexual coflocculation and asexual self-flocculation of heterothallic fission-yeast cells (*Schizosaccharomyces pombe*). *Can. J. Microbiol.* **33,** 684–688.

Junker, A., Lehmann, E., and Munz, P. (1987). Genetic analysis of particular aspects of intergenic conversion in *Schizosaccharomyces pombe*. *Curr. Genet.* **12,** 119–125.

Kelly, M., Burke, J., Smith, M., Klar, A., and Beach, D. (1988). Four mating-type genes control sexual differentiation in the fission yeast. *EMBO J.* **7,** 1537–1547.

Kishida, M., and Shimoda, C. (1986). Genetic mapping of eleven *spo* genes essential for ascospore formation in the fission yeast. *Schizosaccharomyces pombe*. *Curr. Genet.* **10,** 443–447.

Klar, A. J. S. (1987). Differential parental DNA strands confer developmental asymmetry on daughter cells in fission yeast. *Nature* (*London*) **326,** 466–470.

Klar, A. J. S., and Miglio, L. M. (1986). Initiation of meiotic recombination by double-stranded DNA breaks in *Schizosaccharomyces pombe*. *Cell* **46,** 725–731.

Kohli, J., Hottinger, H., Munz, P., Strauss, A., and Thuriaux, P. (1977). Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidization. *Genetics* **87,** 471–489.

Kröning, A., and Egel, R. (1974). Autolytic activities associated with conjugation and sporulation in fission yeast. *Arch. Microbiol.* **11,** 575–589.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterothallie bei *Schizosaccharomyces pombe*. *C. R. Trav. Lab. Carlsberg, Ser. Physiol.* **24,** 381–480.

Leupold, U. (1955). Metodisches zur Genetik von *Schizosaccharomyces pombe*. *Schweiz. Z. Allg. Pathol. Bakteriol.* **18,** 1141–1146.

Leupold, U. (1958). Studies on recombination in *Schizosaccharomyces pombe*. *Cold Spring Harbor Symp. Quant. Biol.* **23,** 161–170.

Leupold, U. (1987). Sex appeal in fission yeast. *Curr. Genet.* **12,** 543–545.

Lund, P. M., Hasegawa, Y., Kitamura, K., Shimoda, C., Fukui, Y., and Yamamoto, M. (1987). Mapping of the *ras1* gene of *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **209,** 627–629.

McDonald, I. J., Calleja, G. B., and Johnson, B. F. (1982). Conjugation in chemostat cultures of *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **128,** 1981–1987.

McLeod, M., and Beach, D. (1986). Homology between the *ran1*$^+$ gene of fission yeast and protein kinases. *EMBO J.* **5,** 3665–3671.

McLeod, M., and Beach, D. (1988). A specific inhibitor of the *ran1*$^+$ protein kinase regulates entry into meiosis in *Schizosaccharomyces pombe*. *Nature* (*London*) **332,** 509–514.

McLeod, M., Stein, M., and Beach, D. (1987). The product of the *mei3*$^+$ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J.* **6,** 729–736.

Meade, J. H., and Gutz, H. (1975). A new type of mutation in *Schizosaccharomyces pombe:* Vegetative iodine reaction. *Genetics* **80,** 711–714.

Mead, J. H., and Gutz, H. (1976). Mating-type mutations in *Schizosaccharomyces pombe.* Isolation of mutants and analysis of strains with an h^- or h^+ phenotype. *Genetics* **83,** 259–273.

Meade, J. H., and Gutz, H. (1978). Influence of the *mat1-M* allele on meiotic recombination in the mating type region of *Schizosaccharomyces pombe. Genetics* **88,** 235–238.

Michael, H., and Gutz, H. (1987). Sterility (*ste*) genes of *Schizosaccharomyces pombe. Yeast* **3,** 5–9.

Miyata, H., and Miyata, M. (1981). Mode of conjugation in homothallic cells of *Schizosaccharomyces pombe. J. Gen. Appl. Microbiol.* **27,** 365–371.

Munz, P., and Leupold, U. (1979). Gene conversion in nonsense suppressors of *Schizosaccharomyces pombe.* I. The influence of the genetic background and of three mutant genes (*rad2, mut1* and *mut2*) on the frequency of post-meiotic segregation. *Mol. Gen. Genet.* **170,** 145–148.

Munz, P., Amstutz, H., Kohli, J., and Leupold, U. (1982). Recombination between dispersed serine tRNA genes in *Schizosaccharomyces pombe. Nature (London)* **300,** 225–231.

Munz, P., Dorsch-Hasler, K., and Leupold, U. (1983). The genetic fine structure of nonsense suppressors in *Schizosaccharomyces pombe.* II. *sup8* and *sup10. Curr. Genet.* **7,** 101–108.

Nadin-Davis, S. A., and Nasim, A. (1988). A gene which encodes a predicted protein kinase can restore some functions of the *ras* gene in fission yeast. *EMBO J.* **7,** 985–993.

Nadin-Davis, S. A., Nasim, A., and Beach, D. (1986). Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. *EMBO J.* **5,** 2963–2971.

Nakaseko, Y., Niwa, Y., and Yanagida, M. (1984). A meiotic mutant of the fission yeast *Schizosaccharomyces pombe* that produces mature asci containing two diploid spores. *J. Bacteriol.* **157,** 334–336.

Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O., and Yanagida, M. (1986). Chromosome walking shows a highly homologous repetitive sequence present in all the centromere regions of fission yeast. *EMBO J.* **5,** 1011–1021.

Nielsen, O., and Egel, R. (1989). Mapping the double-strand breaks at the mating-type locus in fission yeast by genomic sequencing. *EMBO J.* **8,** 269–276.

Nishi, K., Shimoda, C., and Hayashibe, M. (1978). Germination and outgrowth of *Schizosaccharomyces pombe* ascospores isolated by Urografin density gradient centrifugation. *Can. J. Microbiol.* **24,** 893–897.

Niwa, O., and Yanagida, M. (1988). Universal and essential role of MPF/$cdc2^+$. *Nature (London)* **336,** 430.

Nurse, P. (1985). Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. *Mol. Gen. Genet.* **198,** 497–502.

Nurse, P., and Bissett, Y. (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. *Nature (London)* **292,** 558–560.

Olson, L. W., Eden, U., Egel-Mitani, M., and Egel, R. (1978). Asynaptic meiosis in fission yeast? *Hereditas* **89,** 189–199.

Padilla, G. M., Carter, B. L. A., and Mitchison, J. M. (1975). Germination of *Schizosaccharomyces pombe* spores separated by zonal centrifugation. *Exp. Cell Res.* **93,** 325–330.

Phipps, J., Nasim, A., and Miller, D. R. (1985). Recovery, repair and mutagenesis in *Schizosaccharomyces pombe. Adv. Genet.* **23,** 1–72.

Reichelt, B. Y., and Fleet, G. H. (1981). Isolation, properties, function, and regulation of endo-(1-3)-β-glucanases in *Schizosaccharomyces pombe*. *J. Bacteriol.* **147,** 1085–1094.

Robinow, C. F. (1977). The number of chromosomes in *Schizosaccharomyces pombe:* Light microscopy of stained preparations. *Genetics* **87,** 491–497.

Schiønning, H. (1895). En ny og ejendommelig Ascusdannelse hos en Gjaersvamp. *Medd. Carlsberg Lab.* **4,** 77–84.

Schmidt, H. (1987). Strains of *Schizosaccharomyces pombe* with a disrupted *swi1* gene still show some mating-type switching. *Mol. Gen. Genet.* **210,** 485–489.

Schmidt, H., Kapitza, P., and Gutz, H. (1987). Switching genes in *Schizosaccharomyces pombe:* Their influence on cell viability and recombination. *Curr. Genet.* **11,** 303–308.

Shimoda, C. (1979). Genetic analysis of temperature-sensitive germination-deficient mutants in the fission yeast, *Schizosaccharomyces pombe*. *Jpn. J. Genet.* **54,** 465–466.

Shimoda, C. (1980). Differential effect of glucose and fructose on spore germination in the fission yeast, *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **26,** 741–745.

Shimoda, C. (1983). Existence of a major spore wall protein (23K) in the fission yeast *Schizosaccharomyces pombe:* A possible sporulation-specific protein. *Arch. Microbiol.* **136,** 26–27.

Shimoda, C., and Nishi, K. (1982). Germ-tube formation is independent of DNA synthesis during spore germination in the fission yeast, *Schizosaccharomyces pombe*. *Plant Cell Physiol.* **23,** 1133–1136.

Shimoda, C., and Uehira, M. (1985). Cloning of the *Schizosaccharomyces pombe mei3* gene essential for the initiation of meiosis. *Mol. Gen. Genet.* **201,** 353–356.

Shimoda, C., Hirata, A., Kishida, M., Hashida, T., and Tanaka, K. (1985). Characterization of meiosis-deficient mutants by electron microscopy and mapping of four essential genes in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **200,** 252–257.

Shimoda, C., Uehira, M., Kishida, M., Fujioka, H., Iino, Y., Watanabe, Y., and Yamamoto, M. (1987). Cloning and analysis of transcription of the *mei2* gene responsible for initiation of meiosis in the fission yeast *Schizosaccharomyces pombe*. *J. Bacteriol.* **169,** 93–96.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene $cdc2^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45,** 261–268.

Sipiczki, M. (1988) The role of sterility genes (*ste* and *aff*) in the initiation of sexual development in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **213,** 529–534.

Sipiczki, M., Kucsera, J., and Dobo, E. (1985). Homothallic and heterothallic sexual types in *Schizosaccharomyces pombe* var. *malidevorans*. *Curr. Genet.* **9,** 263–272.

Snow, R. (1979). Maximum likelihood estimation of linkage and interference from tetrad data. *Genetics* **92,** 231–246.

Streiblova, E., and Wolf, A. (1975). Role of cell wall topography in conjugation of *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **21,** 1399–1405.

Tanaka, K., and Hirata, A. (1982). Ascospore development in the fission yeasts *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* var. *japonicus*. *J. Cell Sci.* **56,** 263–280.

Thuriaux, P. (1985). Direct selection of mutants influencing gene conversion in the yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **199,** 365–371.

Thuriaux, P., Sipiczki, M., and Fantes, P. A. (1980). Genetical analysis of a sterile mutant by protoplast fusion in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **116,** 525–528.

Uemura, T., and Yanagida, M. (1984). Isolation of type I and II DNA topoisomerase mutants from fission yeast: Single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO J.* **3,** 1737–1744.

Umesono, K., Hiraoka, Y., Toda, T., and Yanagida, M. (1983). Visualization of chromosomes in mitotically arrested cells of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **7,** 123–128.

Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C., and Yamamoto, M. (1988). The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *EMBO J.* **7,** 761–767.

Yoo, B. Y., Calleja, G. B., and Johnson, B. F. (1973). Ultrastructural changes of the fission yeast (*Schizosaccharomyces pombe*) during ascospore formation. *Arch. Mikrobiol.* **91,** 1–10.

3

Informational Suppression, Transfer RNA, and Intergenic Conversion

J. KOHLI,* P. MUNZ,* AND D. SÖLL†

*Institut für Allgemeine Mikrobiologie
Universität Bern
CH-3012 Bern, Switzerland

†Department of Molecular Biochemistry and Biophysics
Yale University
New Haven, Connecticut 06511

I. INTRODUCTION

The early work on informational suppression in *Schizosaccharomyces pombe* has been reviewed by Hawthorne and Leupold (1974). The first

MOLECULAR BIOLOGY OF THE
FISSION YEAST

molecular data on *S. pombe* suppressors and tRNA were summarized by Egel *et al.* (1980). This chapter deals mainly with three aspects. First we present the current state of informational suppression analysis with emphasis on the use of suppressors as tools for genetic and molecular analysis. Then the description of genetic recombination between dispersed suppressor tRNA genes follows. Finally the recent analysis of tRNA gene expression is described.

II. INFORMATIONAL SUPPRESSION IN *SCHIZOSACCHAROMYCES POMBE*

Many extragenic, allele-specific suppressor mutations have been isolated on the background of auxotrophic mutations. A convenient system for suppressor analysis is provided by the mutations in *ade6* and *ade7* that are purine auxotrophs and produce a red colony pigment on media with limiting adenine concentration. Rare changes between the red and white phenotypes can be scored visually on plates with up to one thousand colonies, and sectors are identified easily. A growing number of suppressible mutations are known, and the most frequently used ones are given in Table I. The best-studied suppressors are those coding for serine and leucine tRNAs recognizing nonsense codons of the UGA (opal) and UAA (ochre) type.

Additional mutations have been characterized that alter the efficiency of the suppressors (Fig. 1). One type of additional mutation is located within the affected suppressor genes: second-site mutations, third-site mutations, and fourth-site mutations. They have been obtained by sequential inactivation and reactivation of the suppressor gene by mutation. These multiply mutated suppressor tRNA genes are important tools for the study of intergenic conversion and gene expression discussed below. The other types of mutations affecting suppressor gene activity are extragenic. Antisuppressor mutations reduce (rarely increase) the efficiency of suppressors. Allosuppressors reactivate suppressors that carry a second-site mutation. Allosuppressors themselves have no suppressor activity.

A. Nonsense Suppressors Coding for tRNA

The best studied and most useful suppressors are the UGA (opal) suppressors. In combination with most UGA mutations they provide for growth rates close to those of wild-type strains. These strong suppressors have often been named efficient suppressors (e.g., *sup3-e*) in order to

TABLE I

Informational Suppressors in *Schizosaccharomyces pombe*

Suppressor (standard allele)	Map location	Mutations suppressed/not suppressed	Properties	References
tRNA nonsense suppressors				
sup3-5 (*sup3-e*)	I R	UGA suppressed: *ade6-704, ade1-40, ade7-84, glu1-57, leu3-155, lys1-37* UGA not suppressed: *ade6-712*	Inserts serine, strong suppressor	Rafalski *et al.* (1979); Hottinger *et al.* (1982)
sup9-169 (*sup9-e*)	III R	Like *sup3-5*	Like *sup3-5*	Willis *et al.* (1984)
sup12-1 (*sup12-e*)	I L	Like *sup3-5*	Like *sup3-5*, but haplolethal	Amstutz *et al.* (1985)
sup8-4 (*sup8-e*)	II L	UGA suppressed: *ade6-704, ade1-40, ade7-84, glu1-57, leu3-155, lys1-37, ade6-712*	Inserts leucine, strong suppressor	Wetzel *et al.* (1979); Sumner-Smith *et al.* (1984)
sup10-152 (*sup10-e*)	I L	Like *sup8-4*	Like *sup8-4*, but stronger	Munz *et al.* (1983)
sup3-18 (*sup3-i*)	I R	UAA suppressed: *ade7-413, arg1-230, trp1-312, leu3-82*	Inserts serine, weak suppressor	Hottinger *et al.* (1984)
sup8-1 (*sup8-i*)	II L	Like *sup3-18*	Inserts leucine, weak suppressor	Egel *et al.* (1980)
sup13-1		UAG suppressed: *trp1-566*	Weak suppressor	Krupp *et al.* (1985)
sup14-7		Like *sup13-1*	Like *sup13-1*	Krupp *et al.* (1985)
Omnipotent suppressors				
sup1-35	III R	UGA, UAA, UAG suppressed	Weak suppressor	Hawthorne and Leupold (1974)
sup2-413	I R	Like *sup1-35*	Weak suppressor	Hawthorne and Leupold (1974)
sup11-1	III R	*ade7-61c8-1* (frameshift), UGA, UAA suppressed	Weak suppressor	Hottinger (1980)
sup15-1	III R	UGA, UAA, UAG suppressed	Weak suppressor	P. Munz (unpublished)
Frameshift suppressors				
suf1, suf11	III, II	*ade7-C8:* frameshifts group I	Weak suppressor, 2 genes	Hottinger and Leupold (1981)
suf2–suf8	I, II, III	*ade7-C3, ade6-C5:* frameshifts group II	Weak suppressor, 7 genes	Hottinger and Leupold (1981)
suf10-1		*ade7-49c3-2:* frameshifts group III	Weak suppressor, 1 gene	Hottinger (1980)
suf9-1		*ade7-49c4-4:* frameshifts group IV	Weak suppressor, 1 gene	Hottinger (1980)
Missense suppressors				
sup4-486		*ade7-519, ade7-680, ade7-541:* missense	Weak suppressor	Hawthorne and Leupold (1974)
sup5-541	II R	Like *sup4-486*	Like *sup4-486*	Hawthorne and Leupold (1974)
sup6-465		*ade7-465:* missense	Weak suppressor	Hawthorne and Leupold (1974)
sup7-465	II L	Like *sup6-465*	Like *sup6-465*	Hawthorne and Leupold (1974)

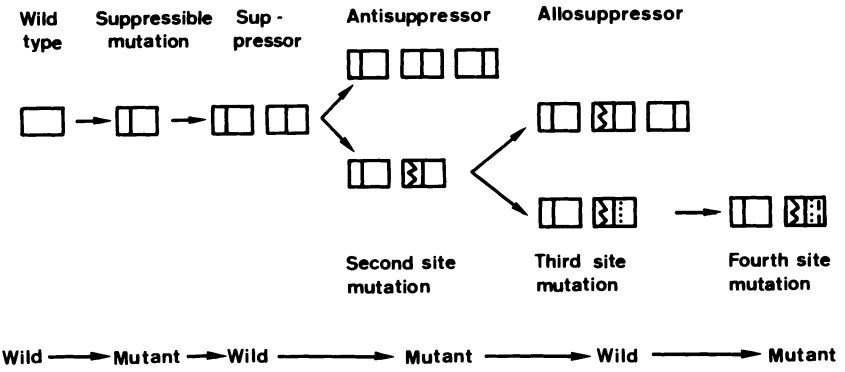


Fig. 1. Suppression genetics in *S. pombe*. Boxes indicate individual genes and arrows single mutation steps. The different vertical lines in the boxes symbolize the mutations of various types. At the bottom the phenotypes of the resulting strains are given. Often auxotrophy mutations in *ade6* or *ade7* are used. This allows a visual distinction between wild phenotype (white colonies) and mutant phenotype (red colonies).

distinguish them from the weak (inefficient, e.g., *sup3-i*) suppressors that read UAA (ochre) codons. There are three serine-inserting suppressor tRNA genes known (Table I) with almost identical sequences. Two of them (*sup3, sup9*) are derived from wild-type serine tRNA genes reading the codon UCA by substitution of the middle base of the anticodon. The third (*sup12*) is derived from the single gene that produces UCG reading tRNA by substitution of two bases in the anticodon (see below); it is not useful for routine genetic analysis owing to its haplolethal nature. The leucine-inserting suppressors *sup8* and *sup10* have a slightly different pattern of UGA suppression (Table I). Otherwise *sup8* has the same properties as the serine-inserting UGA suppressors. It was also created by substitution of the middle base of the anticodon and derives from a wild-type gene coding for UUA reading leucine tRNA. The *sup10* gene must differ considerably in its sequence from *sup8* (Sumner-Smith *et al.*, 1984) although its wild-type allele also codes for UUA reading tRNA (Munz *et al.*, 1981). The *sup10* suppressor is even stronger than the other UGA suppressors, and this leads to reduced colony size (oversuppression) and frequent accumulation of additional antisuppressor mutations that restore wild-type growth rate (Munz *et al.*, 1983).

There are two well-characterized UAA suppressors (*sup3* and *sup8*), and both are allelic to the respective UGA suppressors that insert either serine or leucine. The ochre suppressor anticodons are changed at the second position in comparison to wild-type and opal suppressor tRNAs. Both suppressors are weak (inefficient: *sup3-i, sup8-i*), and strains carry-

ing them grow slowly on selective medium. The red pigment of *ade7* UAA mutants is only partially reverted to a pink color, while the UGA suppressors form white colonies on the background of *ade7* UGA mutations. Unlike bacterial UAA suppressors there is no suppression of amber mutations by the *S. pombe* ochre suppressors (Krupp *et al.*, 1985). Also the recently isolated UAG (amber) suppressors are specific for their termination codon. Again they are weak suppressors. Thus, in contrast to *Escherichia coli* and *Saccharomyces cerevisiae* the nonsense suppressors most suitable for genetic studies in *S. pombe* are the opal suppressors.

B. Additional Mutations in Suppressor tRNA Genes

A large number of second-site mutations (see Fig. 1) abolishing suppressor activity have been obtained in the genes *sup3, sup9* (Hofer *et al.*, 1979), as well as *sup8* and *sup10* (Munz *et al.*, 1983) by spontaneous or induced mutation. With help of these second-site mutants fine-structure maps of the genes were constructed by meiotic and mitotic recombination analysis. Interesting properties of gene conversion have been described based on these data, among them marker effects arising from specific mutations (Thuriaux *et al.*, 1980; Kohli *et al.*, 1984). When the genes and many of the second-site mutations were sequenced, data were accumulated on mutation specificity (Willis *et al.*, 1984; Pearson *et al.*, 1985), and a new type of mutation hot spot was discovered (Heyer *et al.*, 1986). The second-site mutations have been useful for the determination of the *in vivo* decoding rules for serine and leucine tRNAs of *S. pombe* (Munz *et al.*, 1981).

Second-site mutants in *sup3* were subjected to mutagenesis, and strains showing temperature-sensitive suppression of UGA mutations were derived (Nurse and Thuriaux, 1984). As shown in Fig. 1 some of these derivatives carry three mutations in the *sup3* gene: The anticodon mutation, an inactivating second-site mutation, and the reactivating mutation for suppression at low temperature (25°C). Subsequently such a *sup3* allele was subjected to two more rounds of mutation. An allele carrying four mutations in *sup3* was inactive for suppression also at 25°C. Its reactivation yielded new types of fully active suppressors carrying three mutations. All these *sup3* alleles have been sequenced and the effect of the individual mutations assessed on inactivation and reactivation of tRNA gene expression (Willis *et al.*, 1986b). The results indicate that there is considerable flexibility for the sequence evolution of tRNA genes despite strong structural constraints for tRNA structure and function.

C. Antisuppressors and Allosuppressors

When active suppressor strains are mutagenized, one class of the resulting inactivating mutations are located in the antisuppressor genes that are distinct from the suppressor genes (Fig. 1). In this way 15 so-called *sin* genes (suppressor interacting) have been identified (Thuriaux *et al.*, 1975; Munz *et al.*, 1983). The products of these genes must be involved in the synthesis or function of the suppressor molecules. The antisuppressor gene *cyh1* was originally defined by the isolation of cycloheximide-resistant mutants which were then shown to reduce the efficiency of the omnipotent suppressor *sup1* (Thuriaux *et al.*, 1975). Afterward Coddington and Fluri (1977) showed that *cyh1* mutants have an altered protein of the large ribosomal subunit.

Subsequently the search for function of the antisuppressor genes concentrated on tRNA modification. Janner *et al.* (1980) determined that the *sin1* gene codes for the isopentenyltransferase. The *sin1* mutants lack isopentenyladenosine (i^6A) on the 3′ side of tRNA anticodons. In serine suppressor tRNAs this leads to strong reduction of suppressor efficiency. The leucine suppressor tRNAs are devoid of i^6A and are not affected in *sin1* mutants. Later it was found that the mutations in *sin3* and *sin4* lead to the loss of 5-(methoxycarbonylmethyl)-2-thiouridine (mcm^5s^2U) at the wobble position of the anticodons of *S. pombe* tRNAs (Heyer *et al.*, 1984; Grossenbacher *et al.*, 1986). The serine suppressor tRNAs are affected more strongly than the leucine suppressor tRNAs. Neither of the sequenced suppressor tRNAs carries mcm^5s^2U at the wobble position, but they carry related nucleoside modifications instead. Recently it was found that the *sin15* mutation leads to loss of 5-carbamoylmethyluridine, another nucleoside at wobble positions of certain tRNAs (A. Grossenbacher and C. Gehrke, unpublished). *sin15* reduces the efficiency of the overstrong *sup10* to an extent that growth retardation caused by oversuppression is abolished. A review on the genetics of tRNA modification in *S. pombe* and other organisms has been published by Kohli (1983).

The allosuppressors have been isolated as mutations that reactivate second-site alleles of *sup3* (Fig. 1; Nurse and Thuriaux, 1984). By themselves they show no suppressor activity; they are temperature-sensitive mutations (no allosuppression at 35°C) and map in five different genes (*sal1-sal5;* Kohli, 1987). Nothing is known about the products of the *sal* genes. The *sal3* mutants have elongated cells at 25°C. The same has been observed for the *sin3* antisuppressor (Grossenbacher *et al.*, 1986). Elongation of cells is a property typical for mutations in cell division cycle (*cdc*) genes. A clear-cut relation between allosuppression and cell cycle exists for the *sal2* mutations that are allelic to *cdc25*, a gene involved in

the control of mitosis (Nurse and Thuriaux, 1984). The *cdr1* and *cdr2* mutants fail to undergo quicker division after nutritional shift-down and accumulate cells larger than wild type in stationary phase. The *cdr* mutants also function as allosuppressors (Young and Fantes, 1984).

D. Omnipotent Suppressors

Two weak nonsense suppressors (*sup1* and *sup2*) act on UGA, UAA (Hawthorne and Leupold, 1974), and UAG mutations (Krupp *et al.*, 1985). It has also been observed that *sup1* and *sup2* enhance the efficiency of frameshift suppressors, although they do not suppress frameshift mutations themselves (Hottinger and Leupold, 1981). These data and the fact that mutations at different sites in the two genes lead to suppressor phenotypes with varying properties (Egel *et al.*, 1980) indicate that the gene products of *sup1* and *sup2* may be ribosomal proteins. The suppressor *sup11* is poorly characterized. It seems to suppress nonsense and frameshift mutations (Hottinger, 1980). Recently a new suppressor, *sup15-1*, was characterized (B. Mathez, unpublished). It is a weak suppressor of all three types of termination codons (Table I).

E. Frameshift and Missense Suppressors

Frameshift mutations have been characterized genetically by Munz and Leupold (1970) in the genes *ade6* and *ade7*. They served for the isolation of frameshift suppressors in 11 different genes that are classified into 4 groups (Table I). Best characterized are the group I suppressors (*suf1* and *suf11*) and the group II suppressors (7 genes, *suf2–suf8*). Group I suppressors resemble the *Sacch. cerevisiae* frameshift suppressors that code for proline tRNAs, and group II suppressors correspond to the *Sacch. cerevisiae* frameshift suppressors that insert glycine (Hottinger and Leupold, 1981). The group III and group IV suppressors (one gene known for each class) are less well characterized (Hottinger, 1980). All the frameshift suppressors are rather inefficient: the red pigment of the *ade6* and *ade7* mutations is not fully reverted to white. In addition frameshift suppressors lead to slow growth even on medium supplemented with adenine. This means that they have adverse effects on cell growth, and in consequence additional antisuppressor mutations are easily selected in *suf* strains (Hottinger and Leupold, 1981).

The four missense suppressors are weak and suppress only a few *ade7* mutations (Table I). They have no effect on nonsense mutations

(Hawthorne and Leupold, 1974) or frameshift mutations (Hottinger, 1980), and they have not been characterized in detail.

III. RECOMBINATION BETWEEN DISPERSED SUPPRESSOR tRNA GENES

Recombination between dispersed tRNA genes has been well documented in *S. pombe* (Munz *et al.*, 1982; Amstutz *et al.*, 1985; Heyer *et al.*, 1986; Kohli *et al.*, 1984), and the term "intergenic conversion" has been used for such recombination events. The primary observation which led to the characterization of the phenomenon came from fine-structure mapping studies in suppressor genes. In *sup3* and *sup9* (Section II), sets of second-site mutations are known (e.g., *sup3-UGA,rX*). Such alleles are suppressor inactive on the background of an appropriate nonsense mutation owing to the inactivating *rX* lesion. To map the second-site mutations not only heteroallelic crosses (*sup3-UGA,rX1* × *sup3-UGA,rX2*) were evaluated but for the purpose of control also selfings (*sup3-UGA,rX1* × *sup3-UGA,rX1*). It turned out that such selfings gave reversion frequencies of the order of 1×10^{-6}. This value is 50 times higher than that found in selfings of the nonsense mutation alone. The difference could not be readily explained by mutation. As we now know, the basis lies in a recombinational interaction between members of the corresponding tRNA gene family.

Most experiments have been done with the serine tRNA gene family: *sup3* on the right arm of chromosome I and *sup9* on chromosome III, both coding in their wild-type form for $tRNA^{Ser}_{UCA}$, and *sup12* on the left arm of chromosome I producing $tRNA^{Ser}_{UCG}$. These three genes are nearly identical, and from them active suppressor alleles have been obtained (e.g., *sup3-UGA*) as well as alleles with second-site mutations abolishing suppressor activity (e.g., *sup3-UGA,r36*). The symbols *sup3-UGA* and *sup3-e* are used interchangeably (Section II,A). On the background of the UGA nonsense mutation *ade6-704* active suppressors produce prototrophic and white colonies whereas inactive suppressors lead to auxotrophic colonies which are pink on media with limiting adenine concentrations such as yeast extract agar.

A. Meiotic Analysis

In a first experiment the number and the nature of revertants were compared in two crosses. In cross I, the control, an h^+/h^+ diploid was

crossed to an h^-/h^- diploid, both parents being homozygous for *ade6-704*. Here all suppressor genes are in the wild-type form. Cross II, the experimental, was the same except that both parents were in addition homozygous for *sup3-UGA,r36*. Diploid × diploid crosses produce tetraploid zygotes giving in turn diploid progeny spores. Prototrophic revertants could be selected among the spores in both cases. The control gave few revertants (5×10^{-8}), and they originated with approximately equal frequency from reversion at *ade6-704* as well as from forward mutation at *sup3* and *sup9*. In the experimental cross no change in *ade6* reversion was observed, but a marked increase in the frequency of events leading to the active suppressor state at *sup3* and *sup9* and a third locus, *sup12*, was seen. The following interpretation was given: *sup3-UGA* originated by incorporation of wild-type information from $sup9^+$ or $sup12^+$ into *sup3*, replacing the *r36* lesion. *sup9-UGA* and *sup12-UGA* originated by transfer of the suppressor-active anticodon information from *sup3-UGA,r36* into $sup9^+$ and $sup12^+$, respectively.

In the above experiment active suppressors were selected. This means that not all events between interacting tRNA genes are detected since suppressor-active anticodon information and secondary inactivating lesion have to be separated in the process. Thus it is expected that one of the end points of the event between matching partners has to be located between the relevant sites; otherwise coconversion would result, not leading to the active suppressor state. This drawback does not hold for a second set of experiments. Here active suppressor strains were selfed (e.g., *sup3-UGA* × *sup3-UGA*) and rare adenine dependent progeny clones isolated. Genetic tests revealed two classes: (1) strains with a functional wild-type allele (*sup3-UCA* and *sup3-UCG*) and (2) strains with alleles containing suppressor-active anticodon information combined with an inactivating lesion (*sup3-UGA,rX*). In the present context only the former are of interest. *sup3-UCA* alleles could have arisen by mutation, but the generation of *sup3-UCG* from *sup3-UGA* requires two base-pair changes. DNA sequencing has shown that the majority of both wild-type alleles originated by intergenic conversion.

Although the three genes of the serine tRNA gene family are sequence homologous some differences exist. Anticodon information may be such that the tRNA product recognizes the codons, UCA, UCG, or UGA; the introns differ (*sup3* and *sup9* have the same 15-bp intron which differs at six positions from the 16-bp intron of *sup12*), and a third molecular landmark occurs in the sequence corresponding to the tip of the extra arm where position 47 : 3 is occupied by T in *sup3* and by C in *sup9* and *sup12*. The DNA sequence of convertants from both types of experiment (selection of active suppressors on the one hand and isolation of suppressor-

negative clones on the other) has revealed that not only has anticodon information been transferred from donors to recipients but the intron sequence and in several cases the information corresponding to the tip as well. Thus, recombination between the dispersed members of the gene family explains the results far better than mutation.

B. Mitotic Analysis

Recombination between the dispersed members of the serine tRNA gene family has been most clearly demonstrated in meiosis. During mitosis the same events appear to occur, too, but they are 2–3 orders of magnitude less frequent. Two mitotic events have been analyzed at the molecular level. In both cases blocks of information have been transferred between the interacting genes. In addition, nonhomologous flanking regions had been exchanged in one of these cases resulting in a reciprocal translocation. Here a conversion tract of at least 115 bp had been resolved by crossing-over (Szankasi *et al.*, 1986). This event can be explained in terms of the recombination model proposed by Meselon and Radding (1975).

C. Nature of the Process

The meiotic events resulting in information transfer between *sup3*, *sup9*, and *sup12* are not RNA mediated. This conclusion rests on the observation that *sup9-UGA,rX* alleles reduced in transcription are as proficient in exporting their active anticodon information to $sup3^+$ and $sup12^+$ as alleles with normal transcription levels (Willis *et al.*, 1984; Junker *et al.*, 1987).

Most likely the intergenic exchanges are in essence analogous to intragenic conversion and depend on direct contact of DNA segments followed by nonreciprocal transfer of information. The following findings are relevant. (1) The DNA sequences of *sup3* and *sup9* are known both in normal strains and the translocation strain discussed above (Section III,B). The breakpoints in the reciprocal translocation are at the *sup3* and *sup9* loci. Examination of these sequences leads to the conclusion that the translocation originated by a mitotic intergenic coconversion event associated with crossing-over. In this case evidence for direct DNA contact is strong. (2) That intergenic recombination and intragenic conversion have steps in common is suggested by the fact that the mutation *rec3-8* influences both processes (Thuriaux, 1985). (3) Junker *et al.* (1987) have

shown that the donor gene remains unchanged when dispersed serine tRNA genes are interacting. Thus a central aspect of conversion, the nonreciprocity of the information transfer, also holds for intergenic recombination. As a consequence the term intergenic conversion seems to be appropriate.

Although allelic conversion is strongly associated with crossing-over, the same has not been observed in intergenic events. Indeed, the translocation described above is the only example so far in which intergenic conversion is seen to be associated with crossing-over. If conversion events between dispersed genes were frequently associated with crossing-over, a high frequency of chromosome rearrangements would result. It is not known whether the cell has evolved mechanisms to suppress crossovers in this case or if the limited size of the homology region might be the reason for the paucity of associated crossovers.

Although the timing of intergenic conversion during the meiotic cycle is not known it appears that these events occur at the replicated (chromatid) stage rather than at the unreplicated (chromosome) stage. In addition, intergenic conversion does not seem to influence the frequency of crossing-over in the vicinity of a converted gene (Junker *et al.*, 1987).

In the leucine tRNA gene family (*sup8* and *sup10* coding for $tRNA^{Leu}_{UUA}$; Munz *et al.*, 1981) the frequency of intergenic conversion was much lower than in the serine tRNA gene family. In the latter family all members are flanked on their 3′ side by an identical initiator $tRNA^{Met}$ gene. Thus, the homology box is some 200 bp long. In contrast, no sequence similarity is found in the flanking regions of *sup8* and *sup10*. Thus, the difference in frequency of intergenic conversion between these families could be partly due to the different lengths of the homology regions. In addition, no other tRNA genes with sequences related to *sup8* have been found in *S. pombe*. Consequently, *sup10* must have a sequence rather dissimilar to *sup8* (Sumner-Smith *et al.*, 1984). This might be an even stronger reason for the low level of intergenic conversion in this family.

IV. EXPRESSION OF tRNA AND rRNA GENES IN *SCHIZOSACCHAROMYCES POMBE*

In parallel with the studies of suppression and intergenic conversion, the mechanism of tRNA biosynthesis was studied in detail. The fact that tRNA genes possess internal promoters (transcribed by RNA polymerase III) and that the processing of the resulting precursor tRNAs requires many different processing nucleases with interesting properties warranted

the full application of *in vitro* and *in vivo* genetic manipulation of tRNA genes to be applied to this process.

A. Structure and Location of Genes

The total number of tRNA genes in fission yeast has been estimated to be about 300 (Mao, 1980). First the sequences of phenylalanine tRNA (McCutchan *et al.*, 1978) and glutamate tRNA (Wong *et al.*, 1979) were determined. After the development of *in vitro* assays for UGA suppression (Kohli *et al.*, 1979) and UAA suppression (Hottinger *et al.*, 1984) suppressor tRNAs inserting serine or leucine were purified. The suppressor tRNAs were sequenced (Rafalski *et al.*, 1979; Wetzel *et al.*, 1979) and also the corresponding genes in their suppressor and wild-type forms (Hottinger *et al.*, 1982, 1984; Sumner-Smith *et al.*, 1984). The cloned serine suppressor tRNA genes are functional in *Sacch. cerevisiae* cells (Hottinger *et al.*, 1982). A number of other *S. pombe* tRNA genes were cloned with help of purified tRNA species and their nucleotide sequence determined. They include tRNA isoacceptor genes for the amino acids arginine, aspartate, glutamate, histidine, lysine, methionine, phenylalanine, and serine (Mao *et al.*, 1980, 1982; Gamulin *et al.*, 1983). Most of the tRNA genes are dispersed throughout the genome without recognizable pattern. A notable exception are the genes coding for the methionine initiator tRNA. Three of the four known genes are very closely linked, and also cotranscribed, with serine tRNA genes (Mao *et al.*, 1980). In contrast to prokaryotes, the occurrence of such dimeric precursors is rare in eukaryotes, as the tRNA genes contain internal transcription control regions (ICRs).

The genes coding for 5 S ribosomal RNA are dispersed throughout the genome of *S. pombe*. The nucleotide sequences of mature 5 S RNA (Komiya *et al.*, 1981) and of several 5 S genes have been described (Tabata, 1981; Mao *et al.*, 1982). The tandemly repeated DNA sequences coding for the other ribosomal RNAs are located on the right arm of chromosome III (Toda *et al.*, 1984). The organization of the ribosomal RNA repeat has been studied by restriction analysis and partial sequencing (Schaack *et al.*, 1982; Balzi *et al.*, 1985). A noteworthy characteristic of the sequences of the tRNAs or tRNA genes and of 5 S RNA is their strong divergence from the corresponding ones found in *Sacch. cerevisiae*. This may be another indication of the evolutionary distance of these two yeast species.

For the studies of tRNA biosynthesis described below the *sup3* or *sup9* genes were saturated with mutations. In addition to the *in vivo* created

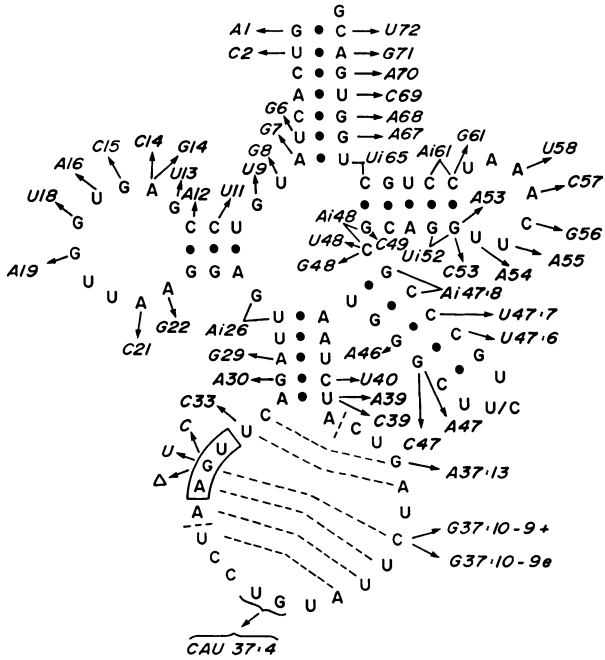


Fig. 2. Single-base substitutions in either the *sup3-e* or *sup9-e* $tRNA^{Ser}$ gene of *S. pombe*, shown on the tRNA cloverleaf form for *S. pombe*. The nomenclature for mutants consists of the substituting nucleotide followed by the position number of the substitution. An "i" is used to signify insertions which follow the position number given (e.g., Ai26 is an adenine insertion between positions 26 and 27). The suppressor anticodon is boxed. The 15-nucleotide intron is marked by the short dashed lines, starting after position 37 and ending before position 38, and is numbered 37:1 to 37:15.

second-site revertants (Section II,B) many mutants were created by *in vitro* mutagenesis (Nichols, 1987). The altered tRNA genes are shown in Fig. 2. Altogether, 59 genes containing single and 19 genes containing double mutations were constructed.

B. Biosynthesis of tRNA in *Schizosaccharomyces pombe*

As was demonstrated first in *E. coli* (for review, see Eggertsson and Söll, 1988) tRNA biosynthesis can be studied elegantly by analysis of genetically altered suppressor tRNA genes. In our original experiments the cloned *S. pombe* tRNA genes were transcribed in extracts of *Xenopus* oocytes where correct transcription and also correct accurate processing

of the precursor RNA including the splicing of introns is observed (Mao *et al.*, 1980). However, the recent experiments with the *S. pombe sup3/sup9* suppressor tRNA genes have been carried out in *Sacch. cerevisiae*. The great advantage of this system is that both *in vivo* and *in vitro* analysis is possible. *In vitro* studies require a well-functioning transcription/processing system, which became available (Klekamp and Weil, 1982). The *in vivo* experiments were possible when it was found that the nucleotide sequences of *S. pombe* and *Sacch. cerevisiae* tRNAs are very different (so as to allow the detection of *S. pombe* tRNA gene transcripts on a Northern blot of crude *Sacch. cerevisiae* RNA) and that *S. pombe* tRNA genes can be expressed in active form in *Sacch. cerevisiae* (Hottinger *et al.*, 1982). This allowed the introduction of *S. pombe* genes into *Sacch. cerevisiae* by transformation, the expression of these genes, and the analysis of the gene products in *Sacch. cerevisiae* by hybridization with the proper *S. pombe* DNA probe.

The mutant *sup3/sup9* tRNA genes were ideal for the analysis of the steps of biosynthesis of mature tRNA in *S. pombe*. They were used in *in vitro* and *in vivo* transcription/processing studies. Generally, there was excellent agreement between these two types of experiments. In addition, the suppressor efficiency of the mutants was measured by estimating the suppression of an opal mutation in an *ade* gene by analyzing the intensity of red coloration of the colonies. The results are presented in Table II. The effects of mutational changes in the serine tRNA genes on the efficiency of transcription were studied in great detail (Willis *et al.*, 1984, 1986b; Pearson *et al.*, 1985; Nichols, 1987). Two separated promoter elements (A-box ICR and B-box ICR) within the sequence coding for the mature tRNA were defined in agreement with data from other organisms. Mutations in these regions restrict transcription to varying degrees (see Table II). The initiator tRNA genes located downstream of the serine tRNA genes are not transcribed independently. Physical separation of the tRNA genes and transcription assays have led to the conclusion that efficient expression of the initiator tRNA genes can occur only in the dimeric organization. An isolated initiator tRNA gene was transcribed inefficiently (Hottinger-Werlen *et al.*, 1985). It was also found that cloned *Sacch. cerevisiae* tRNA genes are not expressed in *S. pombe in vivo* (Krupp *et al.*, 1985).

Analysis of the many second-site mutations in the serine suppressor tRNA genes also gave a wealth of information on the details of the mechanism of processing the primary transcripts. At first they are processed by ribonuclease P (RNase P), which cleaves at the 5′ end of mature tRNA. This endonucleolytic enzyme consists of RNA and protein subunits (Kline *et al.*, 1981). Two RNAs which copurify with RNase P activity

TABLE II

Mutant Alleles of *sup3-e* and *sup9-e* tRNA^{Ser} Genes of *S. pombe*[a]

Allele	Genomic locus	Base change	Gene transcription[b]	tRNA precursor processing defect	Suppression 1(− −) → 5(+ +)	Reference(s)[c]
A1	*sup3*	G → A		RNase P (very)	1	A, B
C2	*sup3*	U → C		RNase P (very)	1	A, B
G6	*sup3*	U → G		RNase P (very)	1	C, D
G7	*sup3*	A → G		Slightly ts	4	C
G8	*sup3*	U → G	Down	Splice, RNase P	1	C
U9	*sup3*	G → U	Down	Splice, RNase P	1	C
G10	*sup3*	U → G	Up (very)	RNase P (very)	1	C
U11	*sup3*	C → U	Down	RNase P	1	A
A12	*sup3*	C → A	Down	RNase P	1	C, D
U13	*sup3*	G → U	Up	Splice, RNase P	1	C, D
C14	*sup3*	A → C	Down	Splice, RNase P	1	C
G14	*sup3, sup9*	A → G	Down	Splice	1	A, E
C15	*sup3*	G → C	Down	RNase P, splice	1	C
A16	*sup3*	U → A	Down	Splice	3	C
U18	*sup3*	G → U	Down	RNase P (very)	1	C
A19	*sup3, sup9*	G → A	Down (very)	RNase P	1	A, E
C21	*sup3*	A → C	Down	RNase P, splice	1	C
G22	*sup3*	A → G		RNase P	1	A
Ai26	*sup3*	A insertion		Splice	1	A
G29	*sup3*	A → G		Splice	3	C
A30	*sup3, sup9*	G → A		RNase P, splice	1	A, E, F
C33	*sup3*	U → C		Splice	2	A
Δ35	*sup3*	C deletion		Splice	1	C, D
CAU37:4	*sup9*	UG → CAU		RNase P	3	E
G37:10-9+	*sup9*	C → G		RNase P	—	G
G37:10-9e	*sup9*	C → G		RNase P improved	5	G

(*continued*)

TABLE II (*Continued*)

Allele	Genomic locus	Base change	Gene transcription[b]	tRNA precursor processing defect	Suppression 1(−−) → 5(++)	Reference(s)[c]
A37:13	*sup9*	G → A		Splice	1	E
A37:13rep	*sup9*	CUAG → GCAA		Splice	1	C
A39	*sup3*	U → A		Splice (very)	1	C
C39	*sup9*	U → C		Splice	2	E
U40	*sup3*	C → U		No defect	5	C
A46	*sup9*	G → A		Splice	1	E
A47	*sup3*	G → A		Splice (very)	1	A
C47	*sup3*	G → C		Splice	1	A
U47:6	*sup3*	C → U		No defect	5	C
U47:7	*sup9*	C → U		Splice	1	E
Ai47:8	*sup3, sup9*	A insertion		Splice	1	A, E
G48	*sup3*	C → G	Down (very)	Splice	1	C
U48	*sup3*	C → U	Down (very)	Splice	1	A
Ai48	*sup3, sup9*	A insertion	Down (very)	RNase P	1	A, E
C49	*sup3*	G → C		RNase P, splice (very)	1	A
Ui52	*sup3*	U insertion	Down (very)		1	C
A53	*sup9*	G → A	Down (very)	RNase P	1	E, F
C53	*sup3*	G → C	Down (very)		1	C
A55	*sup3*	U → A	Down		1	C
G56	*sup3*	C → G	Down (very)		1	C
C57	*sup3*	A → C	Down (very)		1	C
U58	*sup3*	A → U	Down	RNase P	1	C
G61	*sup3*	C → G	Down	RNase P	1	C
Ai61	*sup3*	A insertion	Down		1	C, D
Ui65	*sup3*	U insertion		RNase P, splice	1	C
A67	*sup3*	G → A		RNase P improved	5	F
A68	*sup9*	G → A		RNase P	1	E
C69	*sup3*	U → C		RNase P	1	C, D

A70	*sup3*	G → A		RNase P	1	A
G71	*sup3*	A → G		No defect	5	B
U72	*sup3*	C → U		Slightly ts	3	B
Multiple Mutants						
A1U72	*sup3*			RNase P	2	B
C2G71	*sup3*			RNase P	5	B
G8C14	*sup3*		Down (very)	RNase P	1	C
C15G48	*sup3*		Down (very)		1	C
U18A55	*sup3*		Down	RNase P	1	C
A19G56	*sup3*		Down (very)		1	C
G29A67	*sup3*			Splice, RNase P	5	C
A30A67	*sup3*			RNase P, splice	2	F
A30A53A67	*sup3*		Down (very)		1	F
A53A67	*sup3*		Down (very)		1	F
Ai48Ui65	*sup3*		Down (very)		1	C
Ui52Ai61	*sup3*		Down (very)		1	C
C53G61	*sup3*		Down (very)		1	C
G8U13	*sup3*		Down	RNase P	1	C
G8A19	*sup3*		Down (very)		1	C
G8C21	*sup3*		Down	RNase P	1	C
G8A53	*sup9*		Down (very)		1	C
MAG	*sup3*	UC → AG (in Met)		Normal for serine	5	C
MUC	*sup3*	GG → UC (in Met)		Normal for serine	5	C

[a]The mutations are named as outlined in the legend to Fig. 2. Gene transcription is designated down (or up) if it varies by more than 20% from the wild-type level. The primary tRNA processing defect is shown for each allele where there is enough transcript formed to detect processing. A particularly strong phenotype is followed by the "(very)" designation. The range of suppression for each allele in *Sacch. cerevisiae* at 30°C is given from 1 → 5 (no activity to full activity), relative to the parental *sup3-e* or *sup9-e* opal suppressor tRNA.

[b](very) signifies a stronger phenotype.

[c]Reference key: (A) Pearson *et al.* (1985); (B) Nichols *et al.* (1988); (C) Nichols (1987); (D) I. Willis (unpublished results); (E) Willis *et al.* (1984); (F) Willis *et al.* (1986b); (G) Willis *et al.* (1986a).

have been isolated and characterized (Krupp *et al.*, 1985). They are the product of a single gene which is essential for viability of haploid *S. pombe* (Cherayil *et al.*, 1987). Many of the mutations produce tRNA precursors which can be cleaved only with difficulty by RNase P. Different mutations affect the other important processing step, the removal of the intron in the serine tRNA by the splicing enzyme(s) (Willis *et al.*, 1984, 1986b; Pearson *et al.*, 1985; Greer *et al.*, 1987).

All these investigations have led to the following picture of tRNA biosynthesis in *S. pombe*. The sequence of processing reactions of the dimeric tRNA precursor is initiated with a cleavage at the 5′ end by RNase P. Then the intact 3′ end is formed presumably by an exonuclease followed by addition of the 3′-terminal CCA sequence. As a final step the intron is excised. Analysis of the mutant precursor tRNAs, in addition, revealed these facts. (1) General mismatches in the tRNA sequence (in base-paired regions) lead to less efficient processing. This means that the mature coding region of the tRNA is folded, presumably in a tRNA-like structure, already in the precursor. The processing enzymes may then recognize this structure. (2) Base pairing between the anticodon bases and nucleotides of the intron sequence are desirable for accurate and facile processing *in vivo*. (3) RNase P appears to prefer certain sequences at the 5′ end of tRNAs, even though the enzyme is cleaving all tRNA precursors in the cell (Nichols *et al.*, 1988). (4) The binding and catalytic functions of *S. pombe* RNase P are separate. While the RNA is required for catalytic activity, the protein component(s) of the enzyme may facilitate substrate binding (Nichols *et al.*, 1988). (5) The recognition sites on the tRNA for the splicing endonuclease and splicing ligase differ to some extent (Greer *et al.*, 1987). Finally, information on the formation of modified nucleotides in the mature serine tRNAs has been obtained from the analysis of antisuppressor mutants (Heyer *et al.*, 1984; Grossenbacher *et al.*, 1986).

Further studies with these tRNA genes will lead to a better understanding of the enzymes/proteins involved in the process of tRNA biosynthesis and function. Extensive analysis of novel antisuppressor or allosuppressor mutants should reveal genes for transcription factors, RNA processing enzymes, modification enzymes, aminoacyl-tRNA synthases, or components of the ribosomal translation apparatus.

REFERENCES

Amstutz, H., Munz, P., Heyer, W.-D., Leupold, U., and Kohli, J. (1985). Concerted evolution of tRNA genes: Intergenic conversion among three unlinked serine tRNA genes in *S. pombe*. *Cell* **40**, 879–886.

Balzi, E., Di Pietro, A., Goffeau, A., Van Heerikhuizen, H., and Klootwijk, J. (1985). The RNA polymerase I initiation site and the external transcribed spacer of the fission yeast *Schizosaccharomyces pombe* ribosomal RNA genes. *Gene* **39,** 165–172.

Cherayil, B., Krupp, G., Schuchert, P., Char, S., and Söll, D. (1987). The RNA components of *Schizosaccharomyces pombe* RNase P are essential for cell viability. *Gene* **60,** 145–149.

Coddington, A., and Fluri, R. (1977). Characterization of the ribosomal proteins of *Schizosaccharomyces pombe* by two-dimensional polyacrylamide gel electrophoresis. Demonstration that a cycloheximide resistant strain, *cyh1,* has an altered 60S ribosomal protein. *Mol. Gen. Genet.* **158,** 93–100.

Egel, R., Kohli, J., Thuriaux, P., and Wolf, K. (1980). Genetics of the fission yeast *Schizosaccharomyces pombe*. *Annu. Rev. Genet.* **14,** 77–108.

Eggertsson, G., and Söll, D. (1988). Transfer RNA-mediated suppression of termination codons in *Escherichia coli*. *Microbiol. Rev.* **52,** 354–374.

Gamulin, V., Mao, J., Appel, B., Sumner-Smith, M., Yamao, F., and Söll, D. (1983). Six *Schizosaccharomyces pombe* tRNA genes including a gene for a $tRNA^{Lys}$ with an intervening sequence which cannot base-pair with the anticodon. *Nucleic Acids Res.* **11,** 8537–8546.

Greer, C. L., Söll, D., and Willis, I. (1987). Substrate recognition and identification of splice sites by the tRNA-splicing endonuclease and ligase from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7,** 76–84.

Grossenbacher, A.-M., Stadelmann, B., Heyer, W.-D., Thuriaux, P., Kohli, J., Smith, C., Agris, P. F., Kuo, K. C., and Gehrke, C. (1986). Antisuppressor mutations and sulfur-carrying nucleosides in transfer RNAs of *Schizosaccharomyces pombe*. *J. Biol. Chem.* **261,** 16351–16355.

Hawthorne, D. C., and Leupold, U. (1974). Suppressor mutations in yeast. *Curr. Top. Microb. Immunol.* **64,** 1–47.

Heyer, W.-D., Thuriaux, P., Kohli, J., Ebert, P., Kersten, H., Gehrke, C., Kuo, K. C., and Agris, P. F. (1984). An antisuppressor mutation of *Schizosaccharomyces pombe* affects the post-transcriptional modification of the "wobble" base in the anticodon of tRNAs. *J. Biol. Chem.* **259,** 2856–2862.

Heyer, W.-D., Munz, P., Amstutz, H., Aebi, R., Gysler, C., Schuchert, P., Szankasi, P., Leupold, U., Kohli, J., Gamulin, V., and Söll, D. (1986). Inactivation of nonsense suppressor transfer RNA genes in *Schizosaccharomyces pombe*. Intergenic conversion and hot spots of mutation. *J. Mol. Biol.* **188,** 343–353.

Hofer, F., Hollenstein, H., Janner, F., Minet, M., Thuriaux, P., and Leupold, U. (1979). The genetic fine structure of nonsense suppressors in *Schizosaccharomyces pombe*. I. *sup3* and *sup9*. *Curr. Genet.* **1,** 45–61.

Hottinger, H. R. (1980). Suppressors of frameshift mutations in *Schizosaccharomyces pombe*. Ph.D. thesis, University of Bern, Bern, Switzerland.

Hottinger, H., and Leupold, U. (1981). Putative frameshift suppressors in *Schizosaccharomyces pombe*. *Curr. Genet.* **3,** 133–143.

Hottinger, H., Pearson, D., Yamao, F., Gamulin, V., Cooley, L., Cooper, T., and Söll, D. (1982). Nonsense suppression in *Schizosaccharomyces pombe:* The *S. pombe sup3-e* $tRNA^{Ser}$ gene is active in *S. cerevisiae*. *Mol. Gen. Genet.* **188,** 219–224.

Hottinger, H., Stadelmann, B., Pearson, D., Frendewey, D., Kohli, J., and Söll, D. (1984). The *Schizosaccharomyces pombe sup3-i* suppressor recognizes ochre, but not amber codons *in vitro* and *in vivo*. *EMBO J.* **3,** 423–428.

Hottinger-Werlen, A., Schaack, J., Lapointe, J., Mao, J., Nichols, M., and Söll, D. (1985). Dimeric tRNA gene rearrangement in *Schizosaccharomyces pombe* allows increased expression of the downstream gene. *Nucleic Acids Res.* **13,** 8739–8747.

Janner, F., Vögeli, G., and Fluri, R. (1980). The antisuppressor *sin1* of *Schizosaccharomyces pombe* lacks the modification isopentenyladenosine in transfer RNA. *J. Mol. Biol.* **139,** 207–220.

Junker, A., Lehmann, E., and Munz, P. (1987). Genetic analysis of particular aspects of intergenic conversion in *Schizosaccharomyces pombe*. *Curr. Genet.* **12,** 119–126.

Klekamp, M. S., and Weil, P. A. (1982). Specific transcription of homologous class III genes in yeast soluble cell-extracts. *J. Biol. Chem.* **257,** 8432–8440.

Kline, L., Nishikawa, S., and Söll, D. (1981). Partial purification of RNase P from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **256,** 5058–5063.

Kohli, J. (1983). The genetics of modified nucleosides in transfer RNA. *In* "The Modified Nucleosides of Transfer RNA II" (P. Agris and R. A. Kopper, eds.), pp. 1–10. Liss, New York.

Kohli, J. (1987). Genetic nomenclature and gene list of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **11,** 575–589.

Kohli, J., Kwong, T., Altruda, F., Söll, D., and Wahl, G. (1979). Characterization of a UGA-suppressing serine tRNA from *Schizosaccharomyces pombe* with the help of a new *in vitro* assay system for eukaryotic suppressor tRNA. *J. Biol. Chem.* **254,** 1546–1551.

Kohli, J., Munz, P., Aebi, R., Amstutz, H., Gysler, C., Heyer, W.-D., Lehmann, L., Schuchert, P., Szankasi, P., Thuriaux, P., Leupold, U., Bell, J., Gamulin, V., Hottinger, H., Pearson, D., and Söll, D. (1984). Interallelic and intergenic conversion in three serine tRNA genes of *Schizosaccharomyces pombe*. *Cold Spring Harbor Symp. Quant. Biol.* **49,** 31–40.

Komiya, H., Miyazaki, M., and Takemura, S. (1981). The nucleotide sequence of 5S RNA from *Schizosaccharomyces pombe*. *J. Biochem. (Tokyo)* **89,** 1663–1666.

Krupp, G., Thuriaux, P., Willis, I., Gamulin, V., and Söll, D. (1985). First identification of an amber nonsense mutation in *Schizosaccharomyces pombe:* Major differences in the efficiency of homologous versus heterologous yeast suppressor tRNA genes. *Mol. Gen. Genet.* **201,** 82–87.

Krupp, G., Cherayil, B., Frendewey, D., Nishikawa, S., and Söll, D. (1986). Two RNA species copurify with RNase P from the fission yeast *Schizosaccharomyces pombe*. *EMBO J.* **5,** 1697–1703.

McCutchan, T., Silverman, S., Kohli, J., and Söll, D. (1978). Nucleotide sequence of phenylalanine transfer RNA from *Schizosaccharomyces pombe:* Implications for transfer RNA recognition by yeast phenylalanyl-tRNA synthetase. *Biochemistry* **17,** 1622–1628.

Mao, J. (1980). The structure of some *Schizosaccharomyces pombe* tRNA genes. Ph.D. thesis, Yale University, New Haven, Connecticut.

Mao, J., Schmidt, O., and Söll, D. (1980). Dimeric transfer RNA precursors in *S. pombe*. *Cell* **21,** 509–516.

Mao, J., Appel, B., Schaack, J., Sharp, S., Yamada, H., and Söll, D. (1982). The 5S RNA genes of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **10,** 487–500.

Meselson, M. S., and Radding, C. M. (1975). A general model for genetic recombination. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 358–361.

Munz, P., and Leupold, U. (1970). Characterization of ICR-170-induced mutations in *Schizosaccharomyces pombe*. *Mutat. Res.* **9,** 199–212.

Munz, P., Leupold, U., Agris, P., and Kohli, J. (1981). *In vivo* decoding rules in *Schizosaccharomyces pombe* are at variance with *in vitro* data. *Nature (London)* **294,** 187–188.

Munz, P., Amstutz, H., Kohli, J., and Leupold, U. (1982). Recombination between dis-

persed serine tRNA genes in *Schizosaccharomyces pombe*. *Nature* (*London*) **300,** 225–231.

Munz, P., Dorsch-Häsler, K., and Leupold, U. (1983). The genetic fine structure of nonsense suppressors in *Schizosaccharomyces pombe*. II. *sup8* and *sup10*. *Curr. Genet.* **7,** 101–108.

Nichols, M. (1987). Transfer RNA biosynthesis in yeast: Studies in gene transcription, RNA processing and suppression. Ph.D. thesis, Yale University, New Haven, Connecticut.

Nichols, M., Söll, D., and Willis, I. (1988). Yeast RNase P: Catalytic activity and substrate binding are separate functions. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 1379–1383.

Nurse, P., and Thuriaux, P. (1984). Temperature sensitive allosuppressor mutants of the fission yeast *S. pombe* influence cell cycle control over mitosis. *Mol. Gen. Genet.* **196,** 332–338.

Pearson, D., Willis, I., Hottinger, H., Bell, J., Kumar, A., and Leupold, U. (1985). Mutations preventing expression of *sup3* $tRNA^{Ser}$ nonsense suppressors of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **5,** 808–815.

Rafalski, A., Kohli, J., Agris, P., and Söll, D. (1979). The nucleotide sequence of a UGA suppressor serine tRNA from *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **6,** 2683–2695.

Schaack, J., Mao, J., and Söll, D. (1982). The 5.8S RNA gene sequence and the ribosomal repeat of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **10,** 2851–2864.

Sumner-Smith, M., Hottinger, H., Willis, I., Koch, T. L., Arentzen, R., and Söll, D. (1984). The *sup8* $tRNA^{Leu}$ gene of *Schizosaccharomyces pombe* has an unusual intervening sequence and reduced pairing in the anticodon stem. *Mol. Gen. Genet.* **197,** 447–452.

Szankasi, P., Gysler, C., Zehntner, U., Leupold, U., Kohli, J., and Munz, P. (1986). Mitotic recombination between dispersed but related tRNA genes of *Schizosaccharomyces pombe* generates a reciprocal translocation. *Mol. Gen. Genet.* **202,** 394–402.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **9,** 6429–6438.

Thuriaux, P. (1985). Direct selection of mutants influencing gene conversion in the yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **199,** 365–371.

Thuriaux, P., Minet, M., Hofer, F., and Leupold, U. (1975). Genetic analysis of antisuppressor mutants in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **142,** 251–261.

Thuriaux, P., Minet, M., Munz, P., Ahmad, A., Zbaeren, D., and Leupold, U. (1980). Gene conversion in nonsense suppressors of *Schizosaccharomyces pombe*. II. Specific marker effects. *Curr. Genet.* **1,** 89–95.

Toda, T., Nakaseko, Y., Niwa, O., and Yanagida, M. (1984). Mapping of rRNA genes by integration of hybrid plasmids in *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 93–98.

Wetzel, R., Kohli, J., Altruda, F., and Söll, D. (1979). Identification and nucleotide sequence of the *sup8-e* UGA-suppressor leucine tRNA from *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **172,** 221–228.

Willis, I., Hottinger, H., Pearson, D., Chisholm, V., Leupold, U., and Söll, D. (1984). Mutations affecting excision of the intron from an eukaryotic dimeric tRNA precursor. *EMBO J.* **3,** 1573–1580.

Willis, I., Frendewey, D., Nichols, M., Hottinger-Werlen, A., Schaack, J., and Söll, D. (1986a). A single base change in the intron of a serine tRNA affects the rate of RNase P cleavage *in vitro* and suppressor activity *in vivo* in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **261,** 5878–5885.

Willis, I., Nichols, M., Chisholm, V., Söll, D., Heyer, W.-D., Szankasi, P., Amstutz, H., Munz, P., and Kohli, J. (1986b). Functional complementation between mutations in

yeast suppressor tRNA gene reveals potential for evolution of tRNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7860–7864.

Wong, T.-W., McCutchan, T., Kohli, J., and Söll, D. (1979). The nucleotide sequence of the major glutamate transfer RNA from *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **6,** 2057–2068.

Young, P., and Fantes, P. A. (1984). Changed division response mutants function as allosuppressors. *In* "Growth, Cancer and the Cell Cycle" (P. Skeham and S. J. Friedman, eds.), pp. 221–228. Humana Press, Clifton, New Jersey.

4

Oncogene Homologs

S. A. NADIN-DAVIS,*,[1] A. NASIM,* Y. FUKUI,†,[2] AND M. YAMAMOTO†

*Division of Biological Sciences
National Research Council of Canada
Ottawa, Ontario, Canada K1A 0R6

†The Institute of Medical Science
University of Tokyo
Minato-Ku, Tokyo 108, Japan

[1]Present address: Agriculture Canada, Food Production and Inspection Branch, Nepean, Ontario, Canada K2H 8P9.

[2]Present address: The Rockefeller University, New York, New York 10021.

MOLECULAR BIOLOGY OF THE
FISSION YEAST

I. INTRODUCTION

For many years now, efforts to elucidate the molecular mechanisms of cancer have been in progress. Experimental evidence had suggested a genetic component to cancer (see Harris *et al.*, 1980), an idea now confirmed with the advent of recombinant DNA technology which permits isolation of cancer-causing genes or oncogenes (e.g., see Capon *et al.*, 1983). It has become abundantly clear that oncogenes and their normal counterparts "protooncogenes" have specific roles in the control of cell growth, cell division, and differentiation. Despite extensive characterization of many mammalian oncogenes, their precise roles in molecular terms are generally far from being fully understood. Recently, however, homologs of certain oncogenes have been found in the genomes of both *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). Since these simple eukaryotes are amenable to extensive biochemical and genetic manipulation, it should therefore be possible to determine the precise role of these yeast oncogene homologs relatively easily. Our hope is that the information obtained in these model systems helps us to understand the role of oncogenes in more complex eukaryotes.

Fission and budding yeasts exhibit many differences; for example, in their cell cycle, in the proportion of intron-containing genes in their genomes, and in the nature of their intron splicing signals (Russell and Nurse, 1986). Studies on *S. pombe* oncogenes may therefore define pathways of oncogene function rather different from those elucidated using *Sacch. cerevisiae*. The purpose of this chapter is to review briefly our understanding of the molecular mechanisms of oncogene function, particularly for the *ras* genes, and to indicate the importance of *S. pombe* as a model system for oncogene studies. This is a relatively new area of interest in *S. pombe*, certainly compared to many other aspects of *S. pombe* research such as meiosis and cell cycle. Thus, in order to place this work in perspective, it is informative to introduce the reader, albeit briefly, to the concept of mammalian oncogenes and to summarize for comparative purposes the progress made in studies on *Sacch. cerevisiae* oncogene homologs.

A. Oncogenes as Genetic Regulators of Cell Growth and Differentiation

Only a brief summary of general oncogene research is given here; further information is provided in several reviews (Weinberg, 1984; Bishop, 1985a; Hall, 1986; Barbacid, 1986). Presently about 40 different onco-

genes (cancer-causing genes) have been identified (Bishop, 1985b) by two independent approaches: transfection of DNA from tumor tissue into the established NIH 3T3 cell line (Cooper and Lane, 1984) and analysis of the cancer-causing genes of RNA tumor viruses (Bishop, 1985a). The discovery that these viral oncogenes are in reality viral copies of normal cellular genes was a landmark in cancer research first shown for the *src* gene of avian Rous sarcoma virus (Stehelin *et al.*, 1976) and now known to be true for all retroviral oncogenes. The two groups of oncogenes identified by these methods show significant but not complete overlap (Bishop, 1985b), thus the isolation of yet other oncogenes is likely. Normal protooncogenes are activated to oncogenes either by structural modifications resulting in an altered and often truncated protein product or by changes in expression of the gene through amplification or translocation (Weinberg, 1985).

Several oncogene products have been characterized, and many have protein kinase activity which is usually specific for tyrosine residues (Hunter, 1984). Indeed changes in protein phosphorylation are believed to play a central role in control of cell growth (Sefton, 1985). Since oncogenes cause uncontrolled cell growth and loss of differentiation, protooncogenes should be involved in control of these processes at some level (see Heldin and Westermark, 1984). Figure 1 depicts the normal growth control pathway for a typical generalized eukaryotic cell. All cells respond to external stimuli. In higher eukaryotes such stimuli are provided by several different growth factors which bind and activate appropriate cell surface receptors. Transducing proteins transfer these extracellular signals into changes in the levels of specific intracellular second messengers, e.g., cyclic AMP (cAMP), diacylglycerol (dG), and inositol trisphosphate (IP_3). These molecules, which control the activities of protein kinase A (cAMP) and protein kinase C (dG) and modulate Ca^{2+} ion levels (IP_3), thereby modulate indirectly many biochemical reactions in the cell (see Robison *et al.*, 1971; Pastan, 1972; Nishizuka, 1984; Berridge, 1986; Parker and Ullrich, 1986). When cell growth is an appropriate response to such signals, DNA synthesis is stimulated via nuclear regulatory proteins. Many of the oncogenes identified to date appear to act at some level within such environmental reception/signal transduction pathways. Our understanding of the role of the *ras* proteins in growth control pathways is now presented.

B. *ras* Genes

There are several reasons for the intense interest shown in the *ras* oncogene family. Activated *ras* genes have often been associated with

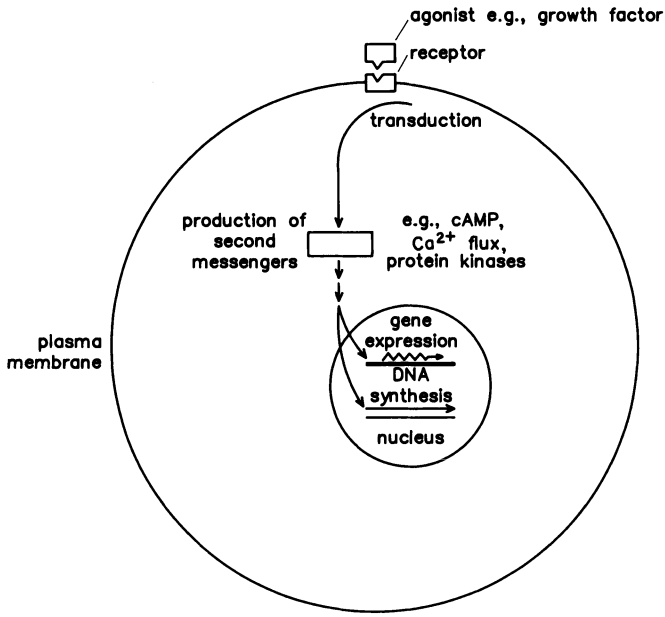


Fig. 1. Generalized model of the response of a eukaryotic cell to external growth stimuli.

human tumors, and activation is usually achieved by a single point mutation (Section I,B,1) although *ras* overexpression is also implicated in cell transformation (see Cooper and Lane, 1984). *ras* genes were first identified in Harvey and Kirsten forms of the murine sarcoma viruses but have since been identified by heterologous hybridization in the genomes of many diverse organisms (see Table I), thus demonstrating the high conservation of these genes over a wide evolutionary divide. It has been proposed that this structural homology is indicative of a highly conserved function, thus raising the expectation that analysis of *ras* gene function in simple eukaryotes could elucidate the function of the gene in more complex organisms. For general reviews on the *ras* gene family, see Lowy and Willumsen (1986) and Marshall (1986).

1. Mammalian ras Genes

The human genome contains three different functional *ras* genes, C-Ha-*ras1*, c-Ki-*ras2*, and N-*ras*, as well as two pseudogenes c-Ha-*ras2* and c-Ki-*ras1* (Hall, 1984; Marshall, 1986). The p21 proteins encoded by these genes and their viral counterparts share the highly conserved structure illustrated in Fig. 2; they are 188 or 189 amino acids in size and have a

TABLE I

***ras* Genes in Different Organisms**

Organism	Number of *ras* genes identified	References
Human	3: Ha-*ras1*, Ki-*ras2*, N-*ras*	Shimizu *et al.* (1983)
	2 pseudogenes: Ha-*ras2*, Ki-*ras1*	Hall (1984)
Rat	4: 2 Ha-*ras*, 2 Ki-*ras*	DeFeo *et al.* (1981), Ellis *et al.* (1981)
Mouse	4: 2 Ha-*ras*, 2 Ki-*ras*	Ellis *et al.* (1982)
Drosophila	3: D-*ras1*, D-*ras2*, D-*ras3*	Neuman-Silberberg *et al.* (1984), Mozer *et al.* (1985)
Goldfish	3 including 1 Ki-*ras*	Nemoto *et al.* (1986)
Dictyostelium	1: Dd-*ras*	Reymond *et al.* (1984)
Saccharomyces cerevisiae	2: SC-*RAS1*, SC-*RAS2*	DeFeo-Jones *et al.* (1983), Powers *et al.* (1984)
Schizosaccharomyces pombe	1: SP-*ras1*	Fukui and Kaziro (1985), Nadin-Davis *et al.* (1986a)

molecular weight of 21,000 (see Dhar *et al.*, 1982; Capon *et al.*, 1983). The very highly conserved 80 amino acid amino-terminal domain is believed to be responsible for the guanine nucleotide-binding and GTPase activities of p21 (McCormick *et al.*, 1985; Jurnak, 1985). The next 80 amino acid domain exhibits a lower level of conservation and is followed by a short variable region of about 20 amino acids. The 5 amino acid carboxy-terminal domain includes a highly conserved cysteine residue required for normal localization of p21 to the cytoplasmic side of the plasma membrane and for functional activity (Willumsen *et al.*, 1984a); this cysteine residue may permit membrane association by providing a site for fatty

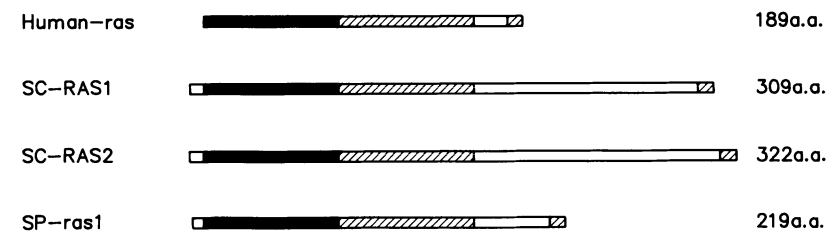


Fig. 2. Schematic comparison of the structure of the protein products of the human and yeast *ras* genes. Solid bars represent sequences showing a high degree of homology, hatched bars depict sequences with limited levels of homology, and open bars indicate those sequences with no significant homology. The number of amino acid (a.a.) residues in each protein is indicated on the right.

acid attachment (Willumsen *et al.*, 1984b). Other highly conserved residues include Gly-12, Ala-59, Glu-61, and Glu-63; changes at these sites can activate the oncogenic potential of p21 (Fasano *et al.*, 1984) usually with a concomitant substantial reduction in GTPase activity (McGrath *et al.*, 1984; Levinson, 1986). The human T_{24} bladder carcinoma *ras* gene has a single point mutation in the codon for residue 12 thereby changing the encoded amino acid to valine (Capon *et al.*, 1983).

The p21 protein is thought to shuttle between inactive (GDP-bound) and active (GTP-bound) forms with the inherent GTPase activity of the protein limiting its activation (Gibbs *et al.*, 1985). Similarities between p21, transducin, and mammalian G proteins in terms of sequence similarity, localization to the plasma membrane, guanine nucleotide binding, and GTP hydrolysis (Hurley *et al.*, 1984; Lochrie *et al.*, 1985) suggest the widely accepted view that p21 proteins function as membrane-transducing agents (Masters and Bourne, 1986). Unfortunately, there is no consensus on the identity of the detector and effector molecules which might be coupled by p21 in mammalian tissues. One report suggests that EGF (epidermal growth factor) receptor function is linked to p21 (Kamata and Feramisco, 1984), but *ras* could also transduce signals from other growth receptors, e.g., bradykinin and PDGF (platelet-derived growth factor) (Parries *et al.*, 1987). There is conflicting experimental evidence bearing on the identity of the *ras* effector pathway(s). Some studies indicate that p21 stimulates adenylate cyclase (Franks *et al.*, 1985; Spina *et al.*, 1987) although perhaps by an indirect route (Franks *et al.*, 1987a,b), others suggest an inhibitory effect of *ras* (Carchman *et al.*, 1974), and still others report no role for *ras* in direct adenylate cyclase modulation and control of cAMP levels (Beckner *et al.*, 1985; Levitzki *et al.*, 1986). *ras* may function in the coupling of growth factor receptors to inositol phospholipid turnover and thus in protein kinase C activation (Wakelam *et al.*, 1986; Wolfman and Macara, 1987; Parries *et al.*, 1987; Kamata *et al.*, 1987; Lacal *et al.*, 1987).

Obviously a clear picture of the molecular basis of mammalian p21 function has not yet emerged. It is quite conceivable that different *ras* gene products employ different transducing systems (Bourne and Sullivan, 1986). More consistent and detailed results have been obtained from studies on the *ras* genes of the yeast *Sacch. cerevisiae*.

2. *Saccharomyces cerevisiae RAS Genes*

Two genes with homology to viral *ras* probes were identified and cloned from *Sacch. cerevisiae* (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984); these genes, SC-*RAS1* and SC-*RAS2*, encode proteins of 309 and 322

amino acids, respectively, both of which exhibit many of the structural and biochemical properties of mammalian p21 (Dhar *et al.*, 1984; Fujiyama *et al.*, 1986) (see Fig. 2). The larger size of the SC-*RAS* proteins is due to a substantially extended variable region not found in any other *ras* protein so far studied.

The role in *Sacch. cerevisiae* of *ras* genes in growth control and the molecular basis of their function have been thoroughly reviewed previously (Tatchell, 1986; Fasano, 1986), and only a brief summary is presented here for comparative purposes. The SC-*RAS2* gene is recognized as being involved in the environmental sensing mechanism of the budding yeast cell, especially in nutrient detection (Tatchell *et al.*, 1985; Toda *et al.*, 1985). Genetic and biochemical data have established that *RAS2* transduces such information into changes in the activity of membrane-bound adenylate cyclase, thereby altering intracellular levels of the second messenger cAMP (Toda *et al.*, 1985; Broek *et al.*, 1985; Cannon *et al.*, 1986) (see Fig. 3). It remains to be resolved whether the SC-*RAS2* protein interacts with adenylate cyclase directly (Uno *et al.*, 1985, 1986) or indirectly (Kataoka *et al.*, 1985b), but there is no doubt that the primary

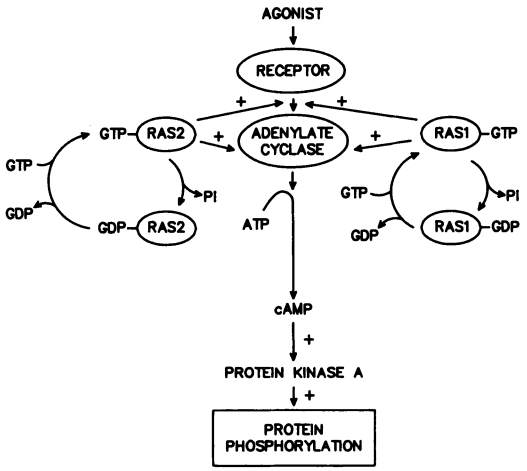


Fig. 3. Model of *ras* gene function in *Sacch. cerevisiae*, indicating the primary role of the SC-*RAS* proteins in stimulation of adenylate cyclase activity. Normal *RAS* proteins cycle between an active GTP-bound form and an inactive GDP-bound form owing to their inherent GTPase activity. It is presently unclear whether the active *RAS* protein stimulates adenylate cyclase directly or indirectly via an intermediate. The net effect, however, is that *ras* transduces an extracellular signal (of nutrient levels) into an increase in the activity of cAMP-dependent protein kinase (protein kinase A), thereby increasing phosphorylation of its protein substrates.

role of SC-*RAS2* is GTP-dependent modulation of adenylate cyclase, possibly in a manner analogous to that for the G proteins of mammalian tissues (Bourne *et al.*, 1986).

Kaibuchi *et al.* (1986) proposed that the SC-*RAS1* protein regulates glucose-induced changes in phosphoinositol metabolism and thus control of a protein kinase C activity. Support for this function has not been forthcoming, however, and others propose that SC-*RAS1* plays a secondary role in modulation of adenylate cyclase by functioning in a manner identical to that determined for SC-*RAS2*, albeit at a reduced level (Broek *et al.*, 1985; Marshall *et al.*, 1987) (see Fig. 3). Some overlap in *RAS1* and *RAS2* function is indeed suggested by the fact that cells require only one functional *RAS* gene to remain viable (Tatchell *et al.*, 1984; Kataoka *et al.*, 1984).

Although the variable region of mammalian *ras* appears functionally unimportant (Willumsen *et al.*, 1985), the long variable region of SC-*RAS* probably interacts with the regulatory *CDC25* gene product (Marshall *et al.*, 1987). It thus remains to be seen to what extent the mode of *ras* action as defined in studies on *Sacch. cerevisiae* will apply to *ras* function in other systems. The discovery of the SP-*ras1* gene of fission yeast, which, as described below, probably operates in a pathway not involving adenylate cyclase, is providing a useful alternative system for studying modes of *ras* function. It should be borne in mind that a degree of functional diversity among the *ras* gene family now appears inevitable in view of the identification of many genes which exhibit significant sequence similarity to *ras* genes.

C. Superfamily of *ras*-Related Genes

It is becoming increasingly clear that the *ras* genes comprise a subgroup within a much larger family of *ras*-related genes (not to be confused with the *ral* genes of *S. pombe*). Most of these *ras*-related genes were isolated quite accidentally and their importance realized only when sequencing studies indicated significant homology to the *ras* genes. All of these *ras*-related genes encode proteins close in size to p21, they are most conserved in their amino-terminal regions, they have a typical *ras* structure, and they all probably bind GTP.

A good example is the *YPT1* gene of *Sacch. cerevisiae*, which was originally identified as an open reading frame localized between the actin and tubulin genes of this yeast (Gallwitz *et al.*, 1983). This gene was not observed by hybridization techniques since it exhibits a lower overall level of conservation to mammalian *ras* than the two SC-*RAS* genes. The

essential *YPT1* gene encodes a 206 amino acid GTP-binding protein which appears to be required for normal microtubule organization (Schmitt *et al.*, 1986) and it is thus distinct in function from the SC-*RAS* genes. The *YPT1* protein is characterized by a serine residue at a position equivalent to residue 12 of *ras* proteins and by a Cys–Cys sequence at the carboxy terminus in place of the conserved Cys–xxx sequence of *ras* proteins. The *ypt* protein is highly conserved since *YPT1* homologs and related genes have been identified in higher eukaryotes (Haubruck *et al.*, 1987; Touchot *et al.*, 1987). The *Sacch. cerevisiae SEC4* gene product is also structurally related to the *YPT1* protein (Salminen and Novick, 1987) but functions in the secretion pathway.

Three *S. pombe* genes have been shown to be closely homologous to *YPT1*, namely, *ypt1*, *ypt2*, and *ypt3*. Their deduced products all carry the serine and cysteine–cysteine residues characteristic of the *YPT1* product. D. Gallwitz and co-workers have identified the *S. pombe ypt2* gene, which lacks introns and encodes a 201 amino acid product (personal communication). This product is 53% homologous to *Sacch. cerevisiae YPT1*. S. Miyake and M. Yamamoto have identified the *ypt1* and *ypt3* genes (unpublished data). Characterization of the *ypt1* gene is not yet complete, but its overall amino acid identity with *YPT1* is nearly 70% and that with mouse *ypt1* (Haubruck *et al.*, 1987) is nearly 75%. Presumably this gene is the counterpart of *Sacch. cerevisiae YPT1*. The *ypt3* gene encodes a 214 amino acid product and has two introns. This gene is only 45% homologous to *YPT1*. Thus, it is clear that several *ras*-related genes are present in the *S. pombe* genome.

Other *ras*-related genes reported include the *rho* gene class, which has been identified in many diverse species including humans, *Drosophila*, *Aplysia*, and budding yeast (Madaule and Axel, 1985; Madaule *et al.*, 1987) and the simian *ral* sequence (Chardin and Tavitian, 1986). Even the prokaryote *Escherichia coli* contains a gene, *era*, which encodes a GTP-binding protein weakly homologous to SC-*RAS* (Ahnn *et al.*, 1986). However, the biological function of these genes is currently unclear.

It has been known for many years that both prokaryotic and eukaryotic cells contain many GTP-binding proteins which are involved in such diverse cell processes as signal transduction, protein synthesis, and microtubule assembly, with GTP hydrolysis acting as a common mechanism for limiting the functional activity of these proteins (Hughes, 1983; Spiegel, 1987). The *ras* and *ras*-related genes described here appear to encode several species of such proteins with very distinct and diverse roles. It is likely that members of this *ras* superfamily will eventually be shown to act as regulators in the transduction of many different extracellular or intracellular signals through different receptor/effector systems thereby pro-

viding a communication network within the cell. Certainly, further functional studies of *ras*-related genes should help to broaden our view of the significance of the *ras* sequences. The discovery of *ras*-related genes in *E. coli* appears significant and may enable us to assess a primordial structure and function of *ras* proteins.

II. *SCHIZOSACCHAROMYCES POMBE ras*

A. SP-*ras1*$^+$ Structure

There are two independent reports of the identification and cloning of a *ras* homolog from fission yeast (Fukui and Kaziro, 1985; Nadin-Davis *et al.*, 1986a). Probes consisting of either SC-*RAS* or viral *ras* sequences hybridized, under low stringency conditions, to a 4.0 kilobase (kb) *Sal*I and a 2.5-kb *Eco*RI fragment of *S. pombe* genomic DNA. Both fragments were cloned from partial genomic libraries by hybridization screening using SC-*RAS* probes. In both instances the *ras*-homologous region was localized to a 1.5-kb *Eco*RI–*Pst*I genomic fragment, and subsequent DNA sequence analysis indicated that despite minor differences the two independently isolated clones represented the same gene, hereafter referred to as *ras1*.

The predicted protein product of the *ras1* gene differed slightly in these two reports owing to a difference in the precise size and location of a small intron in the *ras1* gene which must separate the initiating AUG codon and the coding region. Fukui and Kaziro (1985) predicted a 67-bp intron and a 219 amino acid protein, whereas Nadin-Davis *et al.* (1986a) predicted an 86-bp intron and a 214 amino acid product. Subsequent analysis of two independently isolated cDNA clones indicated that the former prediction was correct (Y. Fukui and M. Yamamoto, unpublished results). Thus, when aligned with the human p21 sequence, the SP-*ras* protein has a five amino acid amino-terminal extension so that residue 6 of this protein is equivalent to residue 1 of p21. The confirmed splicing signals in *ras1* correspond well to the consensus splicing signals suggested for fission yeast by Kaufer *et al.* (1985). Variations in the nucleotide sequence of the *ras1* gene between the two groups cause two other differences in the predicted amino acid sequence in the less conserved region of the protein (see Nadin-Davis *et al.*, 1986a).

It is clear that the *ras1* gene is a true *ras* homolog when its product structure is compared with other *ras* proteins (see Fig. 2). Amino acids 6–

85 of the SP-*ras* protein are highly conserved to the amino-terminal domain of other *ras* proteins, and a significant but lower homology is observed for the next 80 amino acids; this is followed by a variable region that terminates in a short section which includes a cysteine residue equivalent to Cys-186 of human p21. The amino acid residues equivalent to the potentially oncogenic residues 12, 59, 61, and 63 of mammalian p21 all correspond to the normal amino acids found in the *ras* protooncogene. The high level of homology of the SP-*ras* protein to other *ras* proteins would indicate that it has similar biochemical properties, namely, guanine nucleotide binding and GTP hydrolysis. Although such properties have not been demonstrated directly, the presence in the *ras1* protein of the conserved sequence Asn-Lys-Cys-Asp, which is found in other *ras* proteins and is known to be the guanine nucleotide binding site of *E. coli* EF-Tu, is most significant (Fukui and Kaziro, 1985; Arai *et al.*, 1980). A significant feature of the *ras1* protein is that in size it is much closer to mammalian p21 than the SC-*RAS* proteins because it does not have the long variable region characteristic of the products of budding yeast *RAS* genes (see Fig. 2).

B. SP-*ras1*$^+$ Functions in Sexual Differentiation

Expression of the *ras1*$^+$ gene during vegetative growth was indicated by Northern analysis of RNA prepared from cells in the log phase of growth (Fukui and Kaziro, 1985; Nadin-Davis *et al.*, 1986a). It has been shown, however, that *ras1* is not essential for vegetative growth of fission yeast (Fukui *et al.*, 1986a). Null alleles of this gene were constructed and introduced initially into diploids thereby producing a diploid strain heterozygous at the *ras* locus (see Chapter 7 by Russell, this volume, for details on gene disruption/replacement). Subsequent sporulation of these diploids yielded viable *ras1*$^-$ haploids. Haploid *ras1*$^-$ cells grow vegetatively at rates comparable to those observed for *ras1*$^+$ cells in complete medium and, like wild-type cells, *ras1*$^-$ strains grow on media containing maltose or glycerol as the sole carbon source (Fukui *et al.*, 1986a). The only obvious defect of vegetatively growing *ras1*$^-$ strains is their deformed and swollen shape (see Fig. 4), which is particularly apparent on entry of the cells into stationary phase (Fukui *et al.*, 1986a; Nadin-Davis *et al.*, 1986b).

Although *ras1*$^+$ function is not essential for vegetative growth, this gene is required for normal sexual differentiation of fission yeast. As reviewed in Chapter 2 by Egel (this volume), on nitrogen starvation haploid *S.*

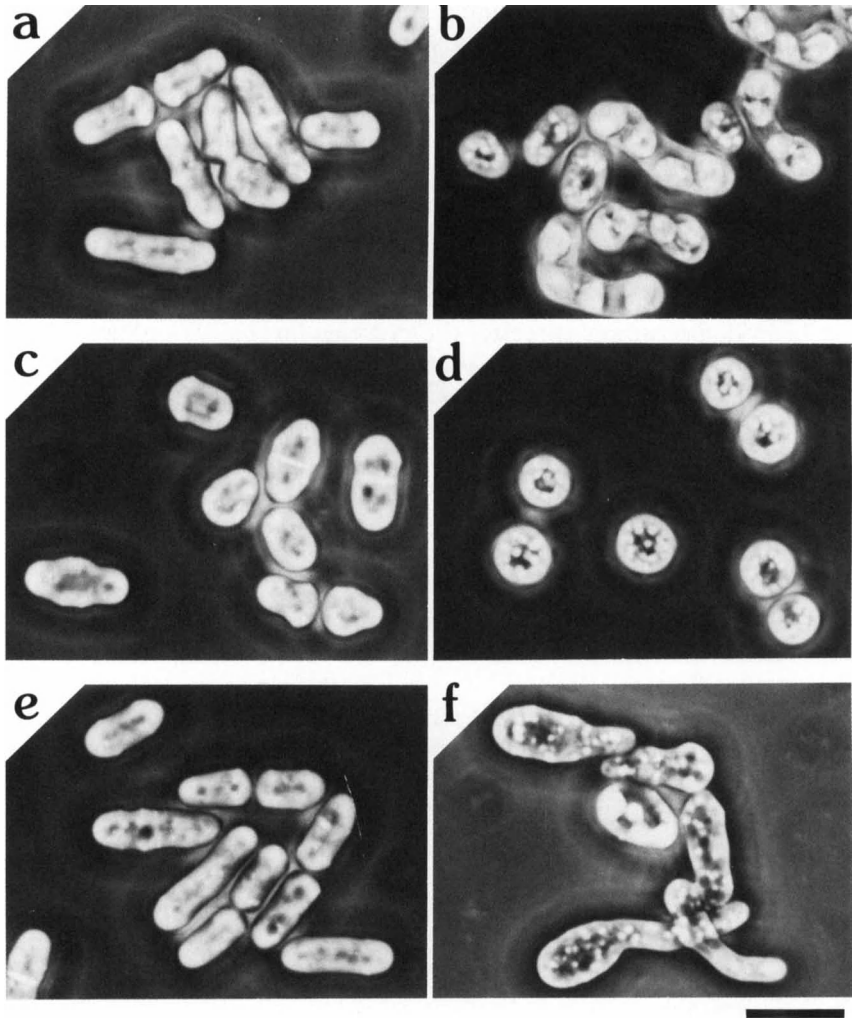


Fig. 4. Morphology of several *S. pombe* strains containing different alleles of *ras1*. The strains in these phase-contrast micrographs are as follows: (a,b) h^{90} *ras1*$^{+}$; (c,d) h^{90} *ras1*$^{-}$; (e,f) h^{90} *ras1*$_{\text{Val-17}}$(N). a, c, and e illustrate rapidly growing cells, while b, d, and f show cells that have been nitrogen starved for 2 days. Bar, 10 μm.

pombe strains of opposite mating type conjugate to produce zygotes which subsequently form four-spored asci. Diploid strains heterozygous at the mating type loci can sporulate without prior conjugation to form azygotic four-spored asci. Haploid *ras1*$^{-}$ strains fail to mate with cells of

opposite mating type and are completely sterile even when one mating partner is *ras1*$^+$ (Fukui *et al.*, 1986a; Nadin-Davis *et al.*, 1986b) (see Fig. 4). Even cell agglutination, the first event of the mating process, is reported to be absent if one partner has a nonfunctional *ras1* gene (Fukui *et al.*, 1986a). These studies on haploid cells indicated an essential role for *ras1* in mating, but since sporulation is dependent on conjugation in haploids, the role of *ras1* in sporulation had to be evaluated in diploids. h^+/h^- or h^{90} diploids, homozygous for *ras1*$^-$, were able to sporulate but only at a level much below normal, and the resulting asci had a very rounded, abnormal shape (Fukui *et al.*, 1986a; Nadin-Davis *et al.*, 1986b). Clearly *ras1*$^+$ functions in the processes of conjugation and sporulation in *S. pombe*, but the gene is essential only for the mating process. The sterile phenotype conferred by the *ras1* null allele is similar to that conferred by the *ste5* mutation described by Girgsdies (1982), and in fact these two genes are allelic (see Section II,C).

Studies on the physiological effects of *ras1* missense mutations support the conclusion that *ras1*$^+$ functions in sexual differentiation. In mammals, a Gly → Val change at the twelfth residue of p21 has dramatic consequences on the role of the protein in the cell as does the equivalent change at residue 19 of the SC-*RAS2* protein (Toda *et al.*, 1985; Broek *et al.*, 1985), and the effect of such a mutation in SP-*ras* has been studied. The SP-*ras*$_{\text{Val-17}}$ mutation (Fukui *et al.*, 1986a) [denoted as SP-*ras*$_{\text{Val-12}}$ mutation in Nadin-Davis *et al.* (1986b)] is equivalent to p21 Val-12. When this mutation is introduced into a haploid h^+ strain it has no observed influence on cell growth (Nadin-Davis *et al.*, 1986b). The *ras*$_{\text{Val-17}}$ strain has a slightly fatter but almost normal morphology throughout the log phase of growth and in stationary phase. The only phenotype associated with h^+ strains carrying the *ras1*$_{\text{Val-17}}$ allele was observed on their mating to a h^- *ras1*$^+$ strain. Many cells in the mating mix produced very elongated processes which appeared to be overextended unfused conjugation tubes, and the successful production of zygotes and zyogotic asci was greatly impaired although not entirely prevented (Nadin-Davis *et al.*, 1986b) (see Fig. 4). Production of these elongated conjugation tubes, which is also seen on starvation of h^{90} *ras1*$_{\text{Val-17}}$ strains, appears to be restricted to h^+ cells since such structures are not observed on mating of h^+ *ras1*$^+$ and h^- *ras1*$_{\text{Val-12}}$ strains (S. Nadin-Davis, unpublished data). Similar processes have been observed during the response of h^+ *ras1*$^+$ cells to a putative mating pheromone, the h^- factor (Fukui *et al.*, 1986b) (see p. 110). However, the *ras1*$_{\text{Val-17}}$ allele does not appear to interfere with sporulation. h^{90} diploids with either a *ras1*$_{\text{Val-17}}$/*ras*$^+$ or *ras1*$_{\text{Val-17}}$/*ras1*$_{\text{Val-17}}$ genotype sporulate well azygotically but also exhibit abortive conjugation between cells

of opposite mating type, and clearly the $ras1_{Val\text{-}17}$ allele interferes with sexual differentiation of fission yeast primarily at the level of mating (Nadin-Davis *et al.*, 1986b).

The above results indicate that the $ras1_{Val\text{-}17}$ allele constructed by Nadin-Davis *et al.* (1986b) is dominant over the wild type. Another $ras1_{Val\text{-}17}$ allele was constructed, and conflicting results have been obtained (Y. Fukui and M. Yamamoto, unpublished results). Cells carrying this allele [$ras1_{Val\text{-}17}$(F)] behave exactly in the same manner as, but always less extensively than, those carrying the allele of Nadin-Davis *et al.* [$ras_{Val\text{-}17}$(N)]. However, the $ras_{Val\text{-}17}$(F) allele is recessive to the wild type in a mating assay. The reason for this difference is unclear but may reflect the different genomic organization of the flanking regions in the two constructs. Other missense mutations of $ras1^+$, $ras1_{Thr\text{-}64}$, and $ras1_{Leu\text{-}66}$ (equivalent to $ras_{Thr\text{-}59}$ and $ras1_{Leu\text{-}61}$ of mammalian p21) have also been constructed. The $ras1_{Thr\text{-}64}$ allele is a weaker homolog of $ras1_{Val\text{-}17}$(N); it also substantially reduces conjugation efficiency, but in this case no extended conjugation tubes are observed (Nadin-Davis and Nasim, 1988). The $ras1_{Leu\text{-}66}$ allele has a stronger phenotype than $ras1_{Val\text{-}17}$(F) (Y. Fukui and M. Yamamoto, unpublished results). Curiously, $ras1_{Thr\text{-}64}$ behaves recessively and $ras1_{Leu\text{-}66}$ dominantly. Hence, degree of dominance of an activated mutation may only reflect its overall strength in *S. pombe*.

Analysis of haploid $ras1^-$ strains has provided support for the existence of a pheromone, secreted by h^- cells regardless of their *ras* genotype, that causes spatially separate h^+ $ras1^+$, but not h^+ $ras1^-$, cells to respond by cell elongation (Fukui *et al.*, 1986b) (see Chapter 2 by Egel, Section IV,B,2, this volume). These observations suggest at least two alternative roles for *ras* in the mating process. The *ras1* protein might transduce extracellular mating pheromone signals into an appropriate mating response, but this would not readily explain the role of *ras* in h^- cells. Alternatively *ras* could be involved in the nutritional sensing apparatus of the cell which prepares both h^+ and h^- cells to receive specific mating signals.

C. Mapping of *ras1*

Schizosaccharomyces pombe strains carrying *Sacch. cerevisiae LEU2* immediately downstream to *ras1* were constructed to aid the mapping of *ras1*, since strains defective in *ras1* itself are sterile and difficult to handle in crosses (Lund *et al.*, 1987). *Sacch. cerevisiae LEU2* confers a leu^+ phenotype to *S. pombe* $leu1^-$ strains (Beach and Nurse, 1981). The use of such strains in random spore and tetrad analysis following haploidization

revealed some linkage of integrated *LEU2* to *arg3* and very tight linkage to *pro2*. It was concluded that *ras1* resides in the vicinity of *pro2* on *S. pombe* chromosome II, which was confirmed by linkage analysis employing *ras1*-disrupted strains directly in crosses by cell fusion (Lund *et al.*, 1987).

Furthermore, it became evident that *ras1* is allelic with one of the sterility genes, *ste5*, described previously by Girgsdies (1982); the mutants of *ste5* have the same cell morphology as *ras1*$^-$ strains and are mating deficient (Lund *et al.*, 1987). These studies indicated that the map position previously assigned to *ste5* was erroneous. In view of the context of this chapter, we shall continue to refer to the *ras1* gene, although, since the *ste5* mutation was identified earlier, it may technically be correct to refer to this gene as *ste5*.

D. Human *ras* Functions in Fission Yeast

To examine whether *S. pombe ras1*$^+$ is a good model for *ras* gene studies, the ability of a human *ras* sequence to complement *ras1*$^-$ strains of fission yeast was assessed (Nadin-Davis *et al.*, 1986b). *Schizosaccharomyces pombe* expression vectors containing either normal (Gly-12) or activated (Val-12) forms of human *ras* cDNA were transformed into a haploid sterile h^{90} *ras1*$^-$ strain. Although clearly not as efficient as SP-*ras*, the human *ras* Gly-12 sequence did restore cell shape and mating ability to a significant proportion of cells since conjugants and zygotic asci were readily observed microscopically. The human *ras* Val-12 sequence produced a weaker phenotype, but significant numbers of cells exhibited the elongated conjugation-defective forms typical of SP-*ras1*$_{\text{Val-17}}$ strains and ascus formation was an exceedingly rare event. Homozygous h^{90} *ras1*$^-$ diploids carrying either human *ras* plasmid sporulated well azygotically; thus human *ras* is more effective in compensating for the sporulation defect than the cell shape and conjugation defects of *ras1*$^-$ strains of fission yeast.

This demonstration of considerable functional equivalence of *S. pombe* and human *ras* genes is encouraging for the future role of fission yeast as a model organism for *ras* studies, but it also introduces a paradox. Human *ras* has also been shown to function in budding yeast (Kataoka *et al.*, 1985a). However, the available evidence (see Sections I,B,2 and II,E) suggests that *ras* has a different mechanism of action in the two yeasts. How then can human *ras* complement *ras*$^-$ strains of both yeasts? The evidence might argue in favor of the hypothesis proposed by Birchmeier *et al.* (1985) that homologous *ras* genes have evolved different biological

functions through association with other conserved proteins or protein domains that in turn interact with alternative cellular pathways. Heterologous complementation might therefore not necessarily imply strict functional equivalence. We should remember that neither yeast may be representative of most eukaryotes with respect to *ras* function, but it appears certain that together they will permit us to identify alternative mechanisms of *ras* function which may aid us in understanding the probable diverse roles of *ras* in higher eukaryotes.

E. Does SP-*ras1*$^+$ Modulate Adenylate Cyclase?

Since the SC-*RAS* genes are involved in the regulation of initiation of meiosis through control of adenylate cyclase and cAMP levels (see Matsumoto *et al.*, 1983, 1986; Toda *et al.*, 1985; Section I,B,2), several experiments have investigated the possibility that SP-*ras1*$^+$ exerts its effect on meiosis and sporulation of fission yeast through adenylate cyclase modulation. Several of the genes involved in the triggering of meiosis in *S. pombe* have been identified (see Chapter 2 by Egel, this volume). One gene, *pat1*$^+$, which is allelic to *ran1*$^+$, is of particular interest. Haploid strains with a temperature-sensitive *pat1* mutation can, at the restrictive temperature, sporulate in the absence of the usual requirements for nutritional and mating-type information although most of the resulting spores are inviable (Iino and Yamamoto, 1985a; Nurse, 1985). The *pat1*$^+$ gene product is believed to inhibit meiosis, probably by interaction with a positive effector encoded by the *mei2* gene; normally nutritional and mating-type signals would reduce *pat* activity below a certain point, thus permitting the *mei2* protein to elicit premeiotic DNA synthesis (Iino and Yamamoto, 1985b; Yamamoto, 1986). The primary interest here in the *pat1*$^+$ gene, which encodes a protein kinase (McLeod and Beach, 1986), stems from the observation that *pat1* (*ts*) mutations are suppressed by high cAMP concentrations (Beach *et al.*, 1985). It was therefore reasoned that if SP-*ras1*$^+$ modulates adenylate cyclase, like SC-*RAS2*, the SP-*ras1*$_{\text{Val-17}}$ allele should overstimulate this enzyme, thereby raising cAMP levels and thus possibly suppressing a *pat1* (*ts*) allele. It has been observed, however, that neither *ras1*$^-$ nor *ras1*$_{\text{Val-17}}$(N) alleles suppress the *pat1* (*ts*) mutation (Nadin-Davis *et al.*, 1986b).

Other pieces of evidence also suggest that SP-*ras1* does not modulate adenylate cyclase. The structural gene for *Sacch. cerevisiae* adenylate cyclase, *CYR1*, is expressed in fission yeast to produce high levels of cAMP (Beach *et al.*, 1985) with the consequence that cell growth con-

tinues after cell division has ceased and grossly elongated cells are produced (Nadin-Davis *et al.*, 1986b). High cAMP levels therefore disrupt entry of cells into stationary phase. It was also shown that cAMP prevents efficient mating and meiosis (Watanabe *et al.*, 1988). Although SP-*ras1* and cAMP are both implicated in the mitotic/meiotic switching mechanism of *S. pombe*, the observation that SP-$ras1_{Val\text{-}17}$(N) and elevated cAMP do not cause substantially similar phenotypes again suggests that one is not directly controlled by the other.

Besides these suggestive data, the strongest evidence for the above inference was provided by the demonstration that $ras1^+$, $ras1^-$, and $ras1_{Val\text{-}17}$(F) strains all contained similar cAMP levels and comparable adenylate cyclase activities under various nutritional conditions (Fukui *et al.*, 1986a). The available evidence thus strongly hints that adenylate cyclase modulation is not the molecular basis of *ras* function in fission yeast.

F. *ral* and *byr*: Two Classes of Genes Implicated in SP-$ras1^+$ Pathway

If SP-$ras1^+$ does not modulate adenylate cyclase, it is of great interest to determine what cellular pathways are regulated by this gene in fission yeast. Two independent studies currently underway to analyze SP-*ras1* function are aimed at identification of genes which interact with SP-*ras* and thereby function either as upstream (potential detector) or downstream (potential effector) components in this pathway.

In the first instance, Fukui and Yamamoto (1988) isolated a series of *ral* (*ras*-like) mutants which have a similar phenotype to ras^- mutants but which are not allelic to the *ras1* gene. They define four complementation groups (*ral1* to *ral4*). A defect in any one of these genes is recessive and causes the same phenotype as $ras1^-$: ral^- mutants are deformed in cell morphology, are unable to mate, do not recognize the putative h^- mating factor, but yet retain a potential to secrete it. Sporulation of h^+/h^- ral^-/ral^- diploids is generally poor, as is typical in a $ras1^-/ras1^-$ strain. These observations suggest that the *ras1* and *ral* gene products possibly function in the same regulatory system. Evidence supporting this idea is that a high copy number of either the $ras1^+$ or the activated $ras1_{Val\text{-}17}$(F) allele partially restores mating in *ral1* and *ral2* mutants. The *ral1*, *ral2*, and *ral3* genes have been cloned by complementation (Fukui and Yamamoto, 1988). The mating deficiency of *ral1* mutants is suppressed partially by plasmid-borne *ral2* and *ral3*. These results apparently establish a functional link among *ras1*, *ral1*, and *ral3*. Complementation tests of *ral4* by

such plasmids are not conclusive because this mutant is leaky in mating. All *ral3* isolates are leaky too, and this leakiness appears inherent since strains which bear disrupted *ral3* exhibit the same phenotype.

Among the *ral* genes, *ral2* is of particular interest. If the $ras1_{Val\text{-}17}$(F) mutation is superimposed, $ral2^-$ cells resume sensitivity to the mating factor and become phenotypically close to a $ral2^+$ $ras1_{Val\text{-}17}$(F) strain. Furthermore, among various host–plasmid combinations except self complementation, only *ral2* mutants carrying $ras1^+$ or $ras1_{Val\text{-}17}$(F) plasmids recovered normal cell morphology. Thus, the *ral2* function must be closely related to the *ras1* activity. Unfortunately, no remarkable feature is recognized in the deduced product of *ral2*, whose complete nucleotide sequence has been determined (Fukui and Yamamoto, 1989).

In an alternative approach a screening for genes acting downstream of *ras* was performed (Nadin-Davis and Nasim, 1988). A sterile h^{90} $ras1^-$ strain was transformed with a *S. pombe* genomic library, and the resulting transformants were screened for sporulation. The procedure yielded four sporulation-competent colonies carrying four different plasmids (pFL20-1, -2, -3, and -4). Analysis of these plasmids indicated that pFL20-1 carried the wild-type *ras1* gene, while pFL20-3 and -4 contained the mating-type loci *mat1-M* and *mat1-P*, respectively. The gene products of these loci and their function in sexual differentiation have been described elsewhere (see Chapter 2 by Egel, this volume; Kelly *et al.*, 1988), and studies investigating the interrelationship between the *ras1* gene and the mating-type loci are discussed below. A previously uncharacterized gene was contained on pFL20-2 (Nadin-Davis and Nasim, 1988). This plasmid did not restore mating to h^{90} $ras1^-$ haploids, but when introduced into $ras1^-$ strains of either h^+ or h^- mating type these cells were capable of mating with $ras1^+$ cells of opposite mating type at low efficiency to produce a very small number of zygotic asci. Furthermore, pFL20-2 restored efficient sporulation to h^{90} $ras1^-$ diploids.

The gene responsible for these phenotypes, which was named *byr1* (bypass of *ras*), was localized to a 2.7-kb region. Sequencing of this DNA fragment has identified a 1020-bp open reading frame encoding a putative protein of 340 amino acids. This predicted protein has significant homology to several protein kinases and is therefore likely to have kinase activity itself. The close functional relationship of the *ras1* and *byr1* genes has been further substantiated by investigating the consequences of loss of *byr1* function. $byr1^-$ strains are viable but are completely unable to either mate or sporulate (Nadin-Davis and Nasim, 1988). Thus, it is apparent that these two genes are both operating in the sexual differentiation pathway and that at least some *ras1* functions are probably mediated by *byr1*.

By constructing diploid strains through the appropriate cell fusions, it has been possible to investigate the allelism of *byr1* to several of the *ste* genes identified previously (Thuriaux *et al.*, 1980; Girgsdies, 1982; Michael and Gutz, 1987). The sporulation defect was complemented in all strains except those containing both the *byr1*$^-$ and *ste1*$^-$ mutations. Furthermore plasmid-borne *byr1*$^+$ restores normal sexual differentiation to *ste1*$^-$ strains, and the *byr1* and *ste1* genes map to the same region of the genome. These data indicate that these two genes are allelic (Nadin-Davis and Nasim, 1989). Thus both *ras1* and *byr1* are members of the *ste* gene family, suggesting that further characterization of the other members of this gene family will be most helpful in defining the precise molecular basis of *ras1* function in *S. pombe*.

The finding that *mat1-M* or *mat1-P* could overcome the sporulation defect of *ras1*$^-$ strains hinted that their expression might be controlled by the *ras1* gene. Analysis of transcript levels of the four mating-type genes (Mc, Mi, Pc, and Pi) upon nitrogen starvation of strains carrying wild-type or mutant forms of either *ras1* or *byr1* do indicate that *ras* and *byr* are involved in the induction of the Mi and Pi transcripts, especially the latter, in response to starvation. It appears that the role of the *ste* genes in meiosis is probably in signal transduction of extracellular nutrient levels into the appropriate expression of the mating-type genes. This would be consistent with the role of *ras* determined for other organisms.

G. Comparison of SC-*RAS* and SP-*ras* Genes

This chapter has indicated that although there are some similarities there are very major differences between the *ras* genes of budding and fission yeasts. (1) Difference in structure and gene number: only one SP-*ras* gene was identified by heterologous hybridization compared to two SC-*RAS* genes. The SP-*ras1* gene does not have a counterpart to the long variable SC-*RAS* regions, and its product is thus much closer in size to that of mammalian p21. (2) Differences in physiological function: the SC-*RAS* genes are strongly implicated to function in the cell's nutritional sensing mechanism, and SP-*ras* may also be involved in environmental sensing of nutrients (Section II,B). However, loss of *ras* gene function has different consequences in the two organisms. Loss of SC-*RAS2* function causes cells to sporulate because of hypersensitivity to nutritional stress whereas loss of SP-*ras* function prevents mating and causes very inefficient sporulation. Furthermore, the SC-$RAS2_{Val\text{-}19}$ allele interferes with sporulation whereas the SP-$ras1_{Val\text{-}17}$ allele primarily affects mating. The

fact that SP-*ras1* plays no obvious role in cell growth also distinguishes it from the SC-*RAS* genes. (3) Differences in molecular function: SC-*RAS* function is mediated at least primarily by the second messenger cAMP. No specific second messenger component has yet been identified for SP-*ras*, but the above observations indicate that cAMP is unlikely to play such a role (Section II,E). However, the predicted kinase activity of the *byr1* gene product suggests that one of the far-reaching effects of SP-*ras* will be modulation of protein phosphorylation, an effect which is also true for SC-*RAS*.

III. FUTURE POTENTIAL FOR ONCOGENE STUDIES IN YEASTS

The presence of *ras* genes in yeasts hinted at the possibility of finding other oncogene homologs in these simple eukaryotes. Unfortunately, to date attempts to identify other oncogene homologs by direct genome screening using mammalian or viral oncogene sequences have had very limited success. Certainly, *myc* probes have not thus far identified *myc* homologs in the *S. pombe* genome (Stephen and Nasim, unpublished data). Furthermore, although Prakash and Seligy (1985) have evidence for the existence of *mos* and *abl* sequences in *S. pombe*, these potential oncogene homologs have not yet been confirmed by cloning and sequence analysis. Similarly, screening of the *Sacch. cerevisiae* genome with oncogenes other than *ras* has not yet identified confirmed homologs to these sequences. Although Sarid and Leder (1988) have identified a budding yeast gene containing a region homologous to human c-*myc*, it would be premature to class this as a yeast *myc* gene.

It is certainly possible that the *ras* genes occupy a unique niche within the oncogene group. The relative ease with which it has been shown that *ras*-related sequences are present ubiquitously in living organisms strongly suggests that ancestral *ras*-like genes existed in some of the very earliest organisms; *ras* and *ras*-related genes may even represent some of the earliest "cellular controlling" elements. The development of multicellular organisms clearly resulted in the need for higher levels of controls, some of which may have been provided by the evolution of novel genetic controlling elements, that is, other oncogenes. Thus, the possibility remains that unicellular yeasts lack homologs to many mammalian oncogenes since these simpler organisms never evolved requirements for them. It is also possible, however, that other yeast oncogene homologs do exist but that they are not as highly conserved as the *ras* genes, as suggested by the isolation of a *myc*-like gene from budding yeast. Different

strategies might therefore be required to detect such genes. One possible approach would be to screen yeast cell extracts with antibodies directed against highly conserved epitopes of mammalian oncogenes.

Since protein sequences tend to be more highly conserved than DNA sequences owing to redundancy within the genetic code, screening for conserved proteins or protein domains may be a more sensitive assay for other yeast oncogene homologs. For example, Dahl *et al.* (1987) have detected a protein kinase antigenically related to the v-*src* pp60 product which is involved in cell cycle control. They have not, however, demonstrated as yet that this peptide is a true *src* protein homolog. Domain conservation has also been noted between the *jun* and *fos* mammalian oncogene proteins and the *Sacch. cerevisiae* GCN4 regulatory protein (Vogt *et al.*, 1987). However, the homologous regions are restricted to those responsible for DNA binding, and they may represent domains conserved to perform a very general function in the same way that the amino-terminal region of the *ras*-related proteins is conserved for binding and hydrolyzing GTP. It is therefore unlikely that the GCN4 gene is functionally related to the *jun* and *fos* oncogenes, even though these genes may be evolutionarily related.

In view of the current limited success in detecting oncogene homologs in yeasts, the usefulness of yeasts as models for mammalian oncogene function remains to be established. However, because of the difficulties encountered in studying oncogenes directly in mammalian systems, the potential of model organisms such as yeasts is clearly worth thorough investigation. Even the detection of mammalian oncogenes by present assays has significant limitations; for example, transformation of the established NIH 3T3 cell line by DNA from tumor tissue successfully identifies oncogenes in only 20% of all tumors tested (Bishop, 1987). In fact, several known oncogenes are inefficient in this test, and this is explained on the basis that the immortal NIH 3T3 cell line is already abnormal and thereby allows identification of only those oncogenes involved in the later stages of cell transformation (Cooper and Lane, 1984). Indeed, several human cancers appear to be due to recessive genetic lesions which require more sophisticated assays to permit their detection (Bishop, 1987). The best example of such a phenomenon is the retinoblastoma gene; functional loss of both alleles of this gene is associated with a high incidence of the disease (see Murphree and Benedict, 1984). It is postulated that this gene encodes a regulatory product which suppresses tumor formation.

The existence of highly conserved mammalian anticancer genes or antioncogenes is supported by interspecies cell fusions between transformed and nontransformed cell types (Sager and Craig, 1985). Furthermore, the

tumor-suppressing action of these genes often operates at the post-transcriptional level, thus hinting at the likelihood of a direct interaction between the products of certain oncogenes and antioncogenes. Although present methods for detecting mammalian oncogenes are limited, the methodical detection and isolation of antioncogenes, which could be invaluable in terms of human cancer therapy, will clearly require more sophisticated procedures than currently available. Model lower eukaryotes such as *S. pombe* may provide less direct but ultimately worthwhile systems for identifying novel but key regulatory components of mammalian cells.

Since no gene operates within the cell in isolation, analysis of the components interacting with known yeast oncogene homologs might help identify equivalent components of mammalian cells which could themselves have either oncogenic or antioncogenic potential. The *byr1* gene is a possible example (see Section II,F). A gene involved in posttranslational modification of *RAS*, the *DPR1* gene of *Sacch. cerevisiae* (which is allelic to the *RAM* gene), is another example (Fujiyama *et al.*, 1987). Interestingly, a *dpr1* gene has also been detected in *S. pombe* (F. Tamanoi, personal communication). The *CDC25* gene product, a regulatory protein which interacts with the variable region of SC-*RAS* proteins, is another potential oncogene homolog. Although human *ras* proteins lack such a variable region, it is possible that in human cells separate proteins fulfill equivalent functions of the SC-*RAS* carboxy termini and the *CDC25* protein (Marshall *et al.*, 1987). Indeed, many of the *CDC* genes of *Sacch. cerevisiae* or the *cdc* genes of *S. pombe*, which are involved in progression of the yeast cell through the cell division cycle, could quite possibly be related to growth control genes of higher eukaryotes, that is, oncogenes and antioncogenes (see Baserga *et al.*, 1986).

Many of the *cdc* genes of *S. pombe* have been well characterized (see Chapter 5 by Fantes, this volume). For example, several authors had noted that both the *cdc2* gene of *S. pombe* and its *Sacch. cerevisiae* equivalent, *CDC28*, code for protein kinases homologous to several protein kinases encoded by vertebrate oncogenes (Hindley and Phear, 1984; Lorincz and Reed, 1984; Wheals, 1986). In fact, two independent groups have now shown that human cells contain a *cdc2* homolog to *S. pombe cdc2* (Lee and Nurse, 1987; Draetta *et al.*, 1987). The ability of the cDNA copy of this human *cdc2* sequence to complement a *cdc2(ts)* mutation in *S. pombe* strongly suggests that certain fundamental aspects of cell cycle control are conserved between fission yeast and humans (Lee and Nurse, 1987). It remains a most exciting possibility that further characterization of other fission yeast genes, particularly those involved in fundamental or regulatory functions, for example, *cdc* and *rad* genes, could ultimately permit detection of novel mammalian genes involved in regulatory pro-

cesses. Such genes could have either oncogenic potential or antioncogenic properties.

IV. CONCLUDING REMARKS

In the 1980s there has been tremendous development in the methodology for genetic manipulation of fission yeast. Once protocols for transformation of this yeast were available the way was opened for development of vectors and the techniques of gene disruption and replacement in *S. pombe* (see Chapter 7 by Russell, this volume). The combination of this recombinant DNA technology with classic fission yeast genetics and biochemical studies makes this organism a versatile model system for analysis of a wide range of cellular functions including cell growth control, sexual differentiation, DNA repair, and a variety of metabolic pathways. The characterization of the genes responsible for effecting these diverse cellular processes is starting to provide us with a basis for understanding the fundamental mechanisms involved in cell control.

It is rather less clear, however, what functional relationship a fission yeast gene might have with a structurally closely related mammalian gene given that these two organisms are far apart on the evolutionary scale. As discussed in this chapter, the observation of apparent functional homologies between "structurally equivalent genes" of widely diverged species can be most deceptive. Nature has found ways of adapting different *ras* and *ras*-related genes for the performance of very diverse functions while retaining their essential structural similarities. It is far too early to predict whether oncogene research in *S. pombe* will help to clarify specific aspects of mammalian oncogene function, but certainly the discovery of "*ras*-like" homologs in fission yeast adds a new and exciting dimension to the study of this organism. If the potential for discovery of other novel mammalian oncogenes or regulatory elements can be realized in this yeast, it will become an increasingly useful and versatile model eukaryote.

REFERENCES

Ahnn, J., March, P. E., Takiff, H. E., and Inouye, M. (1986). A GTP-binding protein of *Escherichia coli* has homology to yeast RAS proteins. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8849–8853.

Arai, K., Clark, B. F. C., Duffy, L., Jones, M. D., Kaziro, Y., Laursen, R. A., L'Italien, J., Miller, D. L., Nagarkatti, S., Nakamura, S., Nielsen, K. M., Petersen, T. E., Taka-

hashi, K., and Wade, M. (1980). Primary structure of elongation factor Tu from *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1326–1330.

Barbacid, M. (1986). Mutagens, oncogenes and cancer. *Trends Genet.* **2,** 188–192.

Baserga, R., Kaczmarek, L., Calabretta, B., Battini, R., and Ferrari, S. (1986). Cell cycle genes as potential oncogenes. *In* "Cell Cycle and Oncogenes" (W. Tanner and D. Gallwitz, eds.), pp. 3–12. Springer-Verlag, Berlin and New York.

Beach, D., and Nurse, P. (1981). High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature* (*London*) **290,** 140–142.

Beach, D., Rodgers, L., and Gould, J. (1985). *RAN1*$^+$ controls the transition from mitotic division to meiosis in fission yeast. *Curr. Genet.* **10,** 297–311.

Beckner, S. K., Hattori, S., and Shih, T. Y. (1985). The *ras* oncogene product p21 is not a regulatory component of adenylate cyclase. *Nature* (*London*) **317,** 71–72.

Berridge, J. M. (1986). Inositol lipids and cell proliferation. *In* "Oncogenes and Growth Control" (P. Kahn and T. Graf, eds.), pp. 147–153. Springer-Verlag, Berlin and New York.

Birchmeier, C., Broek, D., Toda, T., Powers, S., Kataoka, T., and Wigler, M. (1985). Conservation and divergence of *RAS* protein function during evolution. *Cold Spring Harbor Symp. Quant. Biol.* **50,** 721–726.

Bishop, J. M. (1985a). Viral oncogenes. *Cell* **42,** 23–38.

Bishop, J. M. (1985b). Trends in oncogenes. *Trends Genet.* **1,** 245–249.

Bishop, J. M. (1987). The molecular genetics of cancer. *Science* **235,** 305–311.

Bourne, H. R., and Sullivan, K. A. (1986). Mammalian G proteins: Models for *ras* proteins in transmembrane signalling? *Cancer Surv.* **5,** 257–274.

Bourne, H. R., Casperson, G. F., Walker, N., Sullivan, K., and Medynski, D. C. (1986). Adenylate cyclase and GTP-binding proteins in yeast and vertebrate cells. *UCLA Symp. Mol. Cell. Biol.* **33,** 81–100.

Broek, D., Saniy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J., and Wigler, M. (1985). Differential activation of yeast adenylate cyclase by wild-type and mutant *RAS* proteins. *Cell* **41,** 763–769.

Cannon, J. F., Gibbs, J. B., and Tatchell, K. (1986). Suppression of the *RAS2* mutation of *Saccharomyces cerevisiae*. *Genetics* **113,** 247–264.

Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* (*London*) **302,** 33–37.

Carchman, R. A., Johnson, G. S., Pastan, I., and Scolnick, E. M. (1974). Studies on the levels of cyclic AMP in cells transformed by wild-type and temperature-sensitive Kirsten sarcoma virus. *Cell* **1,** 59–64.

Chardin, P., and Tavitian, A. (1986). The *ral* gene: A new *ras*-related gene isolated by the use of a synthetic probe. *EMBO J.* **5,** 2203–2208.

Cooper, G. M., and Lane, M.-A. (1984). Cellular transforming genes and oncogenes. *Biochim. Biophys. Acta* **738,** 9–20.

Dahl, C., Biemann, H.-P., and Dahl, J. (1987). A protein kinase antigenically related to pp60$^{v\text{-}src}$ possibly involved in yeast cell cycle control: Positive *in vivo* regulation by sterol. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 4012–4016.

DeFeo, D., Gonda, M. A., Young, H. A., Chang, E. H., Lowy, D. R., Scolnick, E. M., and Ellis, R. W. (1981). Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3328–3332.

DeFeo-Jones, D., Scolnick, E. M., Koller, R., and Dhar, R. (1983). *Ras*-related gene se-

quences identified and isolated from *Saccharomyces cerevisiae*. *Nature* (*London*) **306,** 707–709.

Dhar, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D., and Scolnick, E. (1982). Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. *Science* **217,** 934–937.

Dhar, R., Nieto, A., Koller, R., DeFeo-Jones, D., and Scolnick, E. M. (1984). Nucleotide sequence of two ras^H related genes isolated from the yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **12,** 3611–3618.

Draetta, G., Brizuela, L., Potashkin, J., and Beach, D. (1987). Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by $cdc2^+$ and $suc1^+$. *Cell* **50,** 319–325.

Ellis, R. W., DeFeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R., and Scolnick, E. M. (1981). The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. *Nature* (*London*) **292,** 506–511.

Ellis, R. W., DeFeo, D., Furth, M. E., and Scolnick, E. M. (1982). Mouse cells contain two distinct *ras* gene mRNA species that can be translated into a p21 *onc* protein. *Mol. Cell. Biol.* **2,** 1339–1345.

Fasano, O. (1986). *RAS* genes and growth control in the yeast *Saccharomyces cerevisiae*. *In* "Oncogenes and Growth Control" (P. Kahn and T. Graf, eds.), pp. 200–208. Springer-Verlag, Berlin and New York.

Fasano, O., Aldrich, T., Tamanoi, F., Taparowsky, E., Furth, M., and Wigler, M. (1984). Analysis of the transforming potential of the human H-*ras* gene by random mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4008–4012.

Franks, D. J., Whitfield, J. F., and Durkin, J. P. (1985). The mitogenic/oncogenic p21 Ki-*RAS* protein stimulates adenylate cyclase activity early in the G_1 phase of NRK rat kidney cells. *Biochem. Biophys. Res. Commun.* **132,** 780–786.

Franks, D. J., Whitfield, J. F., and Durkin, J. P. (1987a). Viral p21 Ki-*RAS* protein: A potent intracellular mitogen that stimulates adenylate cyclase activity in early G_1 phase of cultured rat cells. *J. Cell Biochem.* **33,** 87–94.

Franks, D. J., Whitfield, J. F., and Durkin, J. P. (1987b). A viral k-*ras* protein increases the stimulability of adenylate cyclase by cholera toxin in NRK cells. *Biochem. Biophys. Res. Commun.* **147,** 596–601.

Fujiyama, A., Samiy, N., Rao, M., and Tamanoi, F. (1986). Biochemistry of yeast *RAS1* and *RAS2* proteins. *UCLA Symp. Mol. Cell. Biol.* **33,** 125–149.

Fujiyama, A., Matsumoto, K., and Tamanoi, F. (1987). A novel yeast mutant defective in the processing of *ras* proteins: Assessment of the effect of the mutation on processing steps. *EMBO J.* **6,** 223–228.

Fukui, Y., and Kaziro, Y. (1985). Molecular cloning and sequence analysis of a *ras* gene from *Schizosaccharomyces pombe*. *EMBO J.* **4,** 687–691.

Fukui, Y., and Yamamoto, M. (1988). Isolation and characterization of *Schizosaccharomyces pombe* mutants phenotypically similar to $ras1^-$. *Mol. Gen. Genet.* **215,** 26–31.

Fukui, Y., and Yamamoto, M. (1989). In preparation.

Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986a). Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. *Cell* **44,** 329–336.

Fukui, Y., Kaziro, Y., and Yamamoto, M. (1986b). Mating pheromonelike diffusible factor released by *Schizosaccharomyces pombe*. *EMBO J.* **5,** 1991–1993.

Gallwitz, D., Donath, C., and Sander, C. (1983). A yeast gene encoding a protein homologous to the human c-*has*/*bas* protooncogene product. *Nature* (*London*) **306,** 704–707.

Gibbs, J. B., Sigal, I. S., and Scolnick, E. M. (1985). Biochemical properties of normal and oncogenic *ras* p21. *Trends Biochem. Sci.* **10,** 350–353.

Girgsdies, O. (1982). Sterile mutants of *Schizosaccharomyces pombe*: Analysis by somatic hybridization. *Curr. Genet.* **6,** 223–227.

Hall, A. (1984). The *ras* gene family. *In* "Oxford Surveys on Eukaryotic Genes" (N. MacLean, ed.), Vol. 1, pp. 111–144. Oxford Univ. Press, London and New York.

Hall, A. (1986). Oncogenes. *Genet. Eng.* **5,** 61–116.

Harris, C. C., Mulvihill, J. J., Thorgeirsson, S. S., and Minna, J. D. (1980). Individual differences in cancer susceptibility. *Ann. Intern. Med.* **92,** 809–825.

Haubruck, H., Disela, C., Wagner, P., and Gallwitz, D. (1987). The *ras*-related *ypt* protein is an ubiquitous eukaryotic protein: Isolation and sequence analysis of mouse cDNA clones highly homologous to the yeast *YPT1* gene. *EMBO J.* **6,** 4049–4053.

Heldin, C.-H., and Westermark, B. (1984). Growth factors: Mechanism of action and relation to oncogenes. *Cell* **37,** 9–20.

Hindley, J., and Phear, G. A. (1984). Sequence of the cell division gene *cdc2* from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinases. *Gene* **31,** 129–134.

Hughes, S. M. (1983). Are guanine nucleotide binding proteins a distinct class of regulatory proteins? *FEBS Lett.* **164,** 1–8.

Hunter, T. (1984). The proteins of oncogenes. *Sci. Am.* **251,** 70–79.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., and Gilman, A. G. (1984). Homologies between signal transducing G proteins and *ras* gene products. *Science* **226,** 860–862.

Iino, Y., and Yamamoto, M. (1985a). Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. *Mol. Gen. Genet.* **198,** 416–421.

Iino, Y., and Yamamoto, M. (1985b). Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2447–2451.

Jurnak, F. (1985). Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene proteins. *Science* **230,** 32–36.

Kaibuchi, K., Miyajima, A., Arai, K. I., and Matsumoto, K. (1986). Possible involvement of *RAS*-encoded proteins in glucose-induced inositol phospholipid turnover in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8172–8176.

Kamata, T., and Feramisco, J. R. (1984). Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of *ras* oncogene proteins. *Nature* (*London*) **310,** 147–150.

Kamata, T., Sullivan, N. F., and Wooten, M. W. (1987). Reduced protein kinase C activity in a *ras*-resistant cell line derived from Ki-MSV transformed cells. *Oncogene* **1,** 37–46.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J., and Wigler, M. (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell **37,** 437–445.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. (1985a). Functional homology of mammalian and yeast *RAS* genes. *Cell* **40,** 19–26.

Kataoka, T., Broek, D., and Wigler, M. (1985b). DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. *Cell* **43,** 493–505.

Kaufer, N. F., Simanis, V., and Nurse, P. (1985). Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. *Nature* (*London*) **318,** 78–80.

Kelly, M., Burke, J., Smith, M., Klar, A., and Beach, D. (1988). Four mating-type genes control sexual differentiation in the fission yeast. *EMBO J.* **7,** 1537–1547.

Lacal, J. C., Fleming, T. P., Warren, B. S., Blumberg, P. M., and Aaronson, S. A. (1987).

Involvement of functional protein kinase C in the mitogenic response to the H-*ras* oncogene product. *Mol. Cell. Biol.* **7,** 4146–4149.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* (*London*) **327,** 31–35.

Levinson, A. D. (1986). Normal and activated *ras* oncogenes and their encoded products. *Trends Genet.* **2,** 81–85.

Levitzki, A., Rudick, J., Pastan, I., Vass, W. C., and Lowy, D. R. (1986). Adenylate cyclase activity of NIH 3T3 cells morphologically transformed by *ras* genes. *FEBS Lett.* **197,** 134–137.

Lochrie, M. A., Hurley, J. B., and Simon, M. I. (1985). Sequence of the alpha subunit of photoreceptor G protein: Homologies between transducin, *ras*, and elongation factors. *Science* **228,** 96–99.

Lorincz, A. T., and Reed, S. I. (1984). Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. *Nature* (*London*) **307,** 183–185.

Lowy, D. R., and Willumsen, B. M. (1986). The *ras* gene family. *Cancer Surv.* **5,** 275–289.

Lund, P. M., Hasegawa, Y., Kitamura, K., Shimoda, C., Fukui, Y., and Yamamoto, M. (1987). Mapping of the *ras1* gene of *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **209,** 627–629.

McCormick, F., Clark, B. F. C., LaCour, T. F. M., Kjeldgaard, M., Norskov-Lauritsen, L., and Nyborg, J. (1985). A model for the tertiary structure of p21, the product of the *ras* oncogene. *Science* **230,** 78–82.

McGrath, J. P., Capon, D. J., Goeddel, D. V., and Levinson, A. D. (1984). Comparative biochemical properties of normal and activated human *ras* p21 protein. *Nature* (*London*) **310,** 644–649.

McLeod, M., and Beach, D. (1986). Homology between the *ran1*$^+$ gene of fission yeast and protein kinases. *EMBO J.* **5,** 3665–3671.

Madaule, P., and Axel, R. (1985). A novel *ras*-related gene family. *Cell* **41,** 31–40.

Madaule, P., Axel, R., and Myers, A.M. (1987). Characterization of two members of the *rho* gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 779–783.

Marshall, C. J. (1986). The *ras* gene family. *In* "Oncogenes and Growth Control" (P. Kahn and T. Graf, eds.), pp. 192–199. Springer-Verlag, Berlin and New York.

Marshall, M. S., Gibbs, J. B., Scolnick, E. M., and Sigal, I. S. (1987). Regulatory function of the *Saccharomyces cerevisiae RAS* C-terminus. *Mol. Cell. Biol.* **7,** 2309–2315.

Masters, S. B., and Bourne, H. R. (1986). Role of G proteins in transmembrane signaling: Possible functional homology with the *ras* proteins. *In* "Oncogenes and Growth Control" (P. Kahn and T. Graf, eds.), pp. 184–191. Springer-Verlag, Berlin and New York.

Matsumoto, K., Uno, I., and Ishikawa, T. (1983). Initiation of meiosis in yeast mutants defective in adenylate cyclase and cyclic AMP-dependent protein kinase. *Cell* **32,** 417–423.

Matsumoto, K., Uno, I., and Ishikawa, T. (1986). Role of cyclic AMP in cell division. *UCLA Symp. Mol. Cell. Biol.* **33,** 101–111.

Michael, H., and Gutz, H. (1987). Sterility (*ste*) genes of *Schizosaccharomyces pombe*. *Yeast* **3,** 5–9.

Mozer, B., Marlor, R., Parkhurst, S., and Corces, V. (1985). Characterization and developmental expression of a *Drosophila ras* oncogene. *Mol. Cell. Biol.* **5,** 885–889.

Murphree, A. L., and Benedict, W. F. (1984). Retinoblastoma: Clues to human oncogenesis. *Science* **223,** 1028–1033.

Nadin-Davis, S. A., and Nasim, A. (1988). A gene which encodes a predicted protein kinase can restore some functions of the *ras* gene in fission yeast. *EMBO J.* **7,** 985–993.

Nadin-Davis, S. A., and Nasim, A. (1989). Submitted.

Nadin-Davis, S. A., Yang, R. C. A., Narang, S. A., and Nasim, A. (1986a). The cloning and characterization of a *RAS* gene from *Schizosaccharomyces pombe*. *J. Mol Evol.* **23,** 41–51.

Nadin-Davis, S. A., Nasim, A., and Beach, D. (1986b). Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. *EMBO J.* **5,** 2963–2971.

Nemoto, N., Kodama, K.-I., Tazawa, A., Masahito, P., and Ishikawa, T. (1986). Extensive sequence homology of the goldfish *ras* gene to mammalian *ras* genes. *Differentiation* **32,** 17–23.

Neuman-Silberberg, F. S., Scheizter, E., Hoffmann, F. M., and Shilo, B.-Z. (1984). The *Drosophila ras* oncogenes: Structure and nucleotide sequence. *Cell* **37,** 1027–1033.

Nishizuka, Y. (1984). Protein kinases in signal transduction. *Trends Biochem. Sci.* **9,** 163–166.

Nurse, P. (1985). Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. *Mol. Gen. Genet.* **198,** 497–502.

Parker, P. J., and Ullrich, A. (1986). Protein kinase C. *In* "Oncogenes and Growth Control" (P. Kahn and T. Graf, eds.), pp. 154–162. Springer-Verlag, Berlin and New York.

Parries, G., Hoebel, R., and Racker, E. (1987). Opposing effects of a *ras* oncogene on growth factor-stimulated phosphoinositide hydrolysis: Desensitization to platelet-derived growth factor and enhanced sensitivity to bradykinin. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 2648–2652.

Pastan, I. (1972). Cyclic AMP. *Sci. Am.* **227,** 97–105.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* proteins. *Cell* **36,** 607–612.

Prakash, K., and Seligy, V. L. (1985). Oncogene related sequences in fungi: Linkage of some to actin. *Biochem. Biophys. Res. Commun.* **133,** 293–299.

Reymond, C. D., Gomer, R. M., Mehdy, M. C., and Firtel, R. A. (1984). Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian *ras* protein. *Cell* **39,** 141–148.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971). "Cyclic AMP." Academic Press, New York.

Russell, P., and Nurse, P. (1986). *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*: A look at yeasts divided. *Cell* **45,** 781–782.

Sager, R., and Craig, R. W. (1985). Anti-oncogenes and the suppression of tumor formation. *In* "Cancer Cells, Vol. 3: Growth Factors and Transformation" (J. Feramisco, B. Ozanne, and C. Stiles, eds.), pp. 95–100. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Salminen, A., and Novick, P. J. (1987). A *ras*-like protein is required for a post-golgi event in "yeast secretion." *Cell* **49,** 527–538.

Sarid, J., and Leder, P. (1988). Sequence analysis of a yeast genomic DNA fragment sharing homology with the human c-*myc* gene. *Nucleic Acids Res.* **16,** 4725.

Schmitt, H. D., Wagner, P., Pfaff, E., and Gallwitz, D. (1986). The *ras*-related *YPT1* gene product in yeast: A GTP-binding protein that might be involved in microtubule organization. *Cell* **47,** 401–412.

Sefton, B. M. (1985). Oncogenes encoding protein kinases. *Trends Genet.* **1,** 306–308.

Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., Kamata, T., Feramisco, J.,

Staunezer, E., Fogh, J., and Wigler, M. H. (1983). Three human transforming genes are related to the viral *ras* oncogenes. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 2112–2116.

Spiegel, A. M. (1987). Signal transduction by guanine nucleotide binding proteins. *Mol. Cell. Endocrinol.* **49,** 1–16.

Spina, A., DiDonato, A., Colella, G., and Illiano, G. (1987). Increased adenylate cyclase activity in rat thyroid epithelial cells expressing viral *RAS* genes. *Biochem. Biophys. Res. Commun.* **142,** 527–535.

Stehelin, D., Varmus, H. E., Bishop, J. M., and Vogt, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature (London)* **260,** 170–173.

Tatchell, K. (1986). *Ras* gene and growth control in *Saccharomyces cerevisiae*. *J. Bacteriol.* **166,** 364–367.

Tatchell, K., Chaleff, D. T., DeFeo-Jones, D., and Scolnick, E. M. (1984). Requirement of either of a pair of *ras*-related genes of *Saccharomyces cerevisiae* for spore viability. *Nature (London)* **309,** 523–527.

Tatchell, K., Robinson, L. C., and Breitenbach, M. (1985). *Ras2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3785–3789.

Thuriaux, P., Sipiczki, M., and Fantes, P. A. (1980). Genetical analysis of a sterile mutant by protoplast fusion in the yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **116,** 525–528.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, *RAS* proteins are controlling elements of adenylate cyclase. *Cell* **40,** 27–36.

Touchot, N., Chardin, P., and Tavitian, A. (1987). Four additional members of the *ras* gene superfamily isolated by an oligonucleotide strategy: Molecular cloning of YPT-related cDNAs from a rat brain library. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 8210–8214.

Uno, I., Mitsuzawa, H., Matsumoto, K., Tanaka, K., Oshima, T., and Ishikawa, T. (1985). Reconstitution of the GTP-dependent adenylate cyclase from products of the yeast *CYR1* and *RAS2* genes in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7855–7859.

Uno, I., Matsumoto, K., and Ishikawa, T. (1986). Functions of the *cyr1* and *ras* genes in yeast. *UCLA Symp. Mol. Cell. Biol.* **33,** 113–123.

Vogt, P. K., Bos, T. J., and Doolittle, R. F. (1987). Homology between the DNA-binding domain of the GCN4 regulatory protein of yeast and the carboxyl-terminal region of a protein coded for by the oncogene *jun*. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3316–3319.

Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J., and Hall, A. (1986). Normal $p21^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. *Nature (London)* **323,** 173–176.

Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C., and Yamamoto, M. (1988). The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *EMBO J.* **7,** 761–767.

Weinberg, R. A. (1984). Cellular oncogenes. *Trends Biochem. Sci.* **9,** 131–133.

Weinberg, R. A. (1985). The action of oncogenes in the cytoplasm and nucleus. *Science* **230,** 770–776.

Wheals, A. E. (1986). Oncogene homologues in yeast. *BioEssays* **3,** 108–112.

Willumsen, B. M., Christensen, A., Hubbert, N. L., Papageorge, A. G., and Lowy, D. R. (1984a). The p21 *ras* C-terminus is required for transformation and membrane association. *Nature (London)* **310,** 583–586.

Willumsen, B. M., Norris, K., Papageorge, A. G., Hubbert, N. L., and Lowy, D. R. (1984b). Harvey murine sarcoma virus p21 *ras* protein: Biological and biochemical significance of the cysteine nearest the carboxy terminus. *EMBO J.* **3,** 2581–2585.

Willumsen, B. M., Papageorge, A. G., Hubbert, N., Bekesi, E., Kung, H.-F., and Lowy, D. R. (1985). Transforming p21 *ras* protein: Flexibility in the major variable region linking the catalytic and membrane-anchoring domains. *EMBO J.* **4,** 2893–2896.

Wolfman, A., and Macara, I. G. (1987). Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in *ras*-transformed fibroblasts. *Nature* (*London*) **325,** 359–361.

Yamamoto, M. (1986). Regulation of meiosis in *Schizosaccharomyces pombe*. *Microbiol. Sci.* **3,** 234–237.

5

Cell Cycle Controls

PETER A. FANTES

Department of Zoology
University of Edinburgh
Edinburgh EH9 3JT, Scotland

MOLECULAR BIOLOGY OF THE
FISSION YEAST

I. INTRODUCTION

Interest in control of the cell cycle predates the discoveries in the 1950s and 1960s that established its ground rules: in bacteria, the mode of DNA replication and how it is integrated into the cell division cycle, and in eukaryotes, the existence of discrete DNA replicative and segregation phases and the intervening G_1 and G_2 periods (Howard and Pelc, 1953). Many of the early investigations used cell types particularly suited for the straightforward microscopic observation of mitosis and cell division: plant meristems and invertebrate eggs were much used. The use of radiolabeled thymidine allowed the time of DNA synthesis in cells in tissues and in culture to be investigated (Quastler and Sherman, 1959) and the effects of various physical and chemical treatments tested. The natural synchrony of *Physarum polycephalum* plasmodia made it a favorite system for investigating the effects of such treatments on the time of mitosis (reviewed by Sachsenmaier, 1981). Following on from this, the response to various stimuli was quantitated in several laboratories, and on this basis "black box" models for the control of mitosis were proposed (Tyson, 1984; Fantes *et al.*, 1975). This sort of approach has continued although the major stumbling block has been the inability to define precisely the molecular target(s) of the usually nonspecific stimuli such as blockage of protein synthesis for short periods or heat shock.

The process of mitosis itself has been studied for many decades, progress being closely related to the availability of techniques for examining cells at various cycle stages and for interfering with mitosis using chemical or physical agents. The involvement of microtubules in the mitotic spindle has been clear for some time, but investigation of their precise behavior by direct experiment has only recently been made possible using antibodies in conjunction with sophisticated labeling and microinjection techniques (Mitchison *et al.*, 1986).

The substrates for mitosis, and for S phase events including DNA replication, are the chromosomes. A combination of biochemical analysis and electron microscopy has yielded detailed information about the structure of chromosomes during interphase and when condensed at metaphase. Progress in investigating how chromosomal elements such as centromeres

and telomeres function in the cell has been slower, largely because of the difficulty in identifying or specifically affecting the properties of individual components.

Once DNA replication had been demonstrated to be semiconservative in eukaryotic as well as prokaryotic cells (Taylor, 1963), interest in the machinery of DNA synthesis grew. The biochemical pathways of precursor synthesis were amenable to classic enzymological methods, and specific inhibitors of both precursor metabolism and the assembly of nucleotides into growing DNA chains became available. These approaches have led to a good understanding of the mechanism of DNA replication (Adams *et al.*, 1986). In contrast, the question of what initiates DNA replication, and what controls exit from G_1 and entry into the S phase, is not easily amenable to this sort of analysis, yet this control is of central importance to our understanding of cell cycle controls.

Related to the control of DNA replication is the phenomenon of quiescence. Mammalian cells can be pushed into a quiescent state by starvation for serum or for certain amino acids (Pardee *et al.*, 1978). This observation has diverted attention away from the way cells progress through the cycle itself to the related question of what controls transitions between the cycle and quiescence. Research into this area has expanded enormously in the 1980s, and the mechanisms by which mammalian cells respond to proliferation stimuli are understood at least in part. This work has depended on the sophisticated biochemical techniques now available and also on the discovery and analysis of oncogenes (Bishop, 1983) and their products.

The controls over proliferation and the timing of mitosis and the mechanisms of DNA replication and mitosis have remained major areas of interest for cell biologists. Some areas have grown more rapidly than others, and it is not trivial to point out that most progress has been made in systems where specific elements can be easily manipulated. In many cases, the absence of any specific inhibitor or probe has hindered investigation; one example is the contrast between our understanding of the mechanism of DNA replication, which can be studied biochemically, and how it is initiated, which (until recently) could not be.

A. *Schizosaccharomyces pombe*

The fission yeast *Schizosaccharomyces pombe* was chosen for cell cycle studies by Mitchison in the 1950s largely because of its linear growth pattern and symmetrical mode of division (Mitchison, 1957). At about the same time Leupold started a genetic analysis of the organism (1950) and

constructed linkage maps. Both these lines of investigation were initiated before the distinction between eukaryotic and prokaryotic cells was clear. Nevertheless, the reasons for which *S. pombe* was originally taken up have proved invaluable in subsequent investigations. Chapter 6 by Mitchison (this volume) discusses the occurrence and significance of metabolic and morphological processes that are periodic in the *S. pombe* cell cycle, while Robinow and Hyams (Chapter 8, this volume) describe the cytology of *S. pombe*.

Cell Cycle

Cells of *S. pombe* are hemispherically capped cylinders, which grow by length extension alone. Nuclear division, or mitosis, occurs at about three-fourths of the time between divisions and is followed by the formation of a septum and its cleavage to form two daughter cells of nearly equal size (Mitchison, 1970; Mitchison, Chapter 6, this volume). The G_1 phase is short, normally being complete by the time of cell cleavage. The S phase is also short, lasting less than one-tenth of the cycle (Nasmyth *et al.*, 1979).

Mitosis in *S. pombe* is typical of higher eukaryotes in that the mitotic spindle is present for only a short time during mitosis (McCully and Robinow, 1971; Hiraoka *et al.*, 1984) and the spindle pole body is undivided until the spindle appears (see Robinow and Hyams, Chapter 8, this volume). There is no protracted period when cells possess a short spindle such as is found in budding yeast (Byers, 1981). As argued elsewhere (Nurse, 1985b), the cell cycles of *S. pombe* and higher eukaryotes are more similar to one another than either is to *Saccharomyces cerevisiae*. *Schizosaccharomyces pombe* chromosomes condense during mitosis (Robinow, 1977; Umesono *et al.*, 1983b; Nasmyth and Nurse, 1981). Under appropriate conditions three chromosomes are seen, in agreement with the number of genetic linkage groups (Kohli *et al.*, 1977) and the electrophoretic karyotype (Smith *et al.*, 1987).

B. Cell Cycle Mutants

The study of the cell cycle was revolutionized when Hartwell and colleagues isolated mutants of the budding yeast *Sacch. cerevisiae* that were conditionally defective in cell cycle progress (reviewed by Pringle and Hartwell, 1981). In *S. pombe*, similar mutants were first obtained by Nurse *et al.* (1976), and many more have since been isolated (see Section II,A and Table I). At the permissive temperature, such cell cycle mutants

grow and progress through the cell cycle essentially normally, but on shift to the restrictive (usually higher) temperature cells accumulate at a specific cell cycle stage. The use of such mutants to investigate the cell cycle avoids the two types of criticism sometimes leveled at other experimental approaches. Observations at the biochemical level that the quantity or nature of a molecule changes (or does not change) during the cell cycle are not themselves informative about whether the molecule or the changes are important to cell cycle progress. On the other hand, observations made by the use of nonspecific agents such as chemical inhibitors, heat, or irradiation that overtly affect cell cycle progress may be difficult to interpret, since it is often unclear whether the agent has one or many targets within the cell, or what those targets might be.

Cell cycle mutants have been isolated in a number of organisms, but it is largely in the yeasts *Sacch. cerevisiae* and *S. pombe* that they have proved such powerful tools for investigating the cell cycle. Through the use of cell cycle mutants the final time of action in the cell cycle of a specific gene product (the transition point of the gene) can be determined (Nurse *et al.*, 1976; Fantes, 1983). In addition detailed dependency "maps" of the cell cycle can be constructed (Pringle and Hartwell, 1981; Nurse *et al.*, 1976; Fantes, 1982). Perhaps the main contribution of classical genetics to cell cycle research has been the identification of major control points within the cell cycle. The so-called start point in both yeasts that controls entry into the cycle and is a commitment point for developmental alternatives (Pringle and Hartwell, 1981) was identified in *S. pombe* by Nurse and Bisset (1981). A second control point, regulating entry into mitosis, was also identified in *S. pombe* (Nurse, 1975; Fantes and Nurse, 1977; Nurse and Thuriaux, 1980). These two cycle controls are discussed in detail in Sections III,A and V,A.

C. Molecular Genetics of Cell Cycle

Schizosaccharomyces pombe is an excellent organism for genetic analysis (Munz *et al.*, Chapter 1, this volume) and can be transformed by circular or linear DNA molecules. These techniques and how they may be exploited are reviewed by Russell (Chapter 7, this volume).

Cell cycle mutants are invaluable tools for investigating the pathways of the cell cycle, and for perturbing the cells in a reasonably defined way for physiological analysis. It was hoped in the mid-1970s that it would be possible to identify the products of the normal genes using established methods such as enzymatic assays or two-dimensional gel electrophoresis of proteins. By comparing the mutant and wild-type behavior, it was

thought, it might be possible to determine the nature of the gene product. This was successful in some cases, mainly where something was previously known of the biochemistry involved (Nasmyth, 1979a). As a general method, however, this approach was not successful (D. P. Dickinson, 1981), and it became clear that some other means of investigating the mutants would be needed.

In the late 1970s the transformation of *Sacch. cerevisiae* by plasmids was reported (Hinnen *et al.*, 1978), and before long a similar system for *S. pombe* had been developed (Beach and Nurse, 1981). This in turn allowed the construction of gene libraries in which inserts of yeast DNA were carried in shuttle vectors that were selectable and able to be propagated in *S. pombe* and *Escherichia coli* (Beach *et al.*, 1982b). Such libraries could be screened by transformation into a yeast strain carrying a conditional cell cycle mutation, and plasmid recovered from colonies in which the cycle defect was rescued. This has been the major route to cloning cell cycle genes in yeast, though others have been employed. In some cases genes have been cloned by other methods in which conditional lethal mutants are not required, and here the availability of *in vitro* mutagenesis and gene transplacement techniques (Rothstein, 1983) has allowed "reverse genetics" to be performed. The availability of cloned genes has led to identification of the gene products and their analysis both *in vivo* and *in vitro*. These techniques are discussed in Chapter 7 by Russell (this volume).

D. Perspectives

The properties of many cell cycle mutants have been reviewed previously (Fantes, 1984a), and after a brief summary of them in Section II,A, I shall refer to them individually where relevant. The broad category of cell cycle genes includes genes encoding proteins that have a major, through not exclusively, cell cycle role and DNA sequences involved in chromosome behavior that are not strictly genes. Approaches to the cloning of cell cycle and related genes are outlined in Sections II,C and II,D.

The remainder of this chapter deals with the genes and other elements involved in the sequence of cell cycle stages. G_1 and start controls are described in Section III, and S phase genes (including *ars* elements) in Section IV. Section V describes the G_2 system which controls entry into mitosis, where the ability to clone and to manipulate genes has been exceptionally useful. Genes involved in the events of mitosis, including those encoding cytoskeletal components, are discussed in Section VI. Section VI also includes a summary of our current knowledge of the

elements needed for chromosome maintenance, centromeres, and telomeres.

II. CELL CYCLE MUTANTS AND GENES

A. Cell Cycle Mutants

The number of cell cycle genes, in the broadest sense, that have been identified in *S. pombe* now approaches 50, compared to 70 or so in *Sacch. cerevisiae*. This figure includes genes defined mutationally, generally by temperature-sensitive conditional lethal mutations: these are "classical" cell cycle genes, often referred to as *cdc* genes. The properties of many of these have been reviewed previously (Fantes, 1984a; also see Table I). Also included are a significant minority of genes identified by other methods. The latter class is rather diverse, but can be broadly grouped into (1) genes encoding highly conserved proteins such as histones and cytoskeletal elements, mainly isolated by their DNA sequence similarity to heterologous probes; (2) mutations or cloned sequences that suppress or otherwise interact with known cell cycle mutations, but often confer no other major phenotypic change; (3) mutations or sequences that affect cycle controls, without evidence for any specific genetic interaction. The mutations and cloned suppressors in the latter two classes have in several cases identified hitherto unknown genetic elements involved in particular cell cycle steps. The genes discussed in this chapter, which are referred to by the broad term "cell cycle genes" are presented in Table I, with an outline of their properties.

Classical *cdc* mutations have been identified that are conditionally defective in each stage of the cell cycle. Of these, three (*cdc10, cdc20,* and *cdc2*) have confirmed G_1 defects (Nasmyth and Nurse, 1981; Nurse and Bissett, 1981). Another gene, *cdc22,* is probably a G_1 mutation (Nasmyth and Nurse, 1981), but J. R. Dickinson (1981) presents evidence for an S phase defect. This issue is discussed by Gordon and Fantes (1986), who favor the G_1 interpretation. Several genes involved in DNA replication have been identified: in particular, *cdc17* encodes DNA ligase (Nasmyth, 1979a). Other genes involved in some way in DNA replication are *cdc21, cdc23,* and *cdc24: cdc18* and *cdc19* control steps in either S phase or mitosis (Nasmyth and Nurse, 1981). The only gene likely to be controlling a mid-G_2 event is *cdc6* (Nurse *et al.,* 1976).

Another group of genes are required for mitosis: *cdc13* (Nasmyth and Nurse, 1981), *cdc16* (Minet *et al.,* 1979), *ben4* (Roy and Fantes, 1982), the

TABLE I

***cdc* and Similar Genes**

Gene	Defect	Special properties	Cloned	Reference(s)[a]
Classical *cdc* mutants				
cdc1	G_2/M		Yes	*1, 2*
cdc2	G_1 and G_2/M	Start gene; some alleles *wee;* protein kinase	Yes	*1, 3–6*
cdc3	Septum			*1*
cdc4	Septum			*1*
cdc6	G_2			*1*
cdc7	Septum			*1*
cdc8	Septum			*1*
cdc9		Renamed *wee1:* see *wee1* for details		
cdc10	G_1	Start gene	Yes	*1, 3, 7*
cdc11	Septum			*1*
cdc12	Septum			*1*
cdc13	M	Chromosomes condense; septum forms	Yes	*1, 8–9a*
cdc14	Septum			*1*
cdc15	Septum			*1*
cdc16	Septum	Continues to initiate septa		*10*
cdc17	S	Encodes DNA ligase	Yes	*8, 11, 12*
cdc18	S/M?			*8*
cdc19	S/M?			*8*
cdc20	G_1			*8*
cdc21	S			*8*
cdc22	Probably G_1	Transcript level periodic	Yes	*8, 13*
cdc23	S			*8*
cdc24	S			*8*
cdc25	G_2/M	Mitotic control	Yes	*14, 15*
cdc27	G_2/M		Yes	*8, 16*
cdc28	G_2/M			*8*
cid2–4	CK?	Colcemid resistant		*17*
cut1	M	Septum forms	Yes	*18*
cut2–8	M	Septum forms		*18*
nuc1	M	Aberrant nucleus		*18*
nuc2	M	Aberrant nucleus		*19, 19a*
pat1/ran1	G_1	Controls switch between mitotic and other pathways; predicted protein kinase	Yes	*20–22*
top1	No phenotype	Encodes topoisomerase I		*23, 24*
top2	M	Encodes topoisomerase II; aberrant nucleus, septum forms	Yes	*23–25*
wee1	M	Mitotic control element, predicted protein kinase	Yes	*4, 5, 14, 26*

(*continued*)

TABLE I (*Continued*)

Gene	Interacts with	Special properties	Cloned	Reference(s)[a]
Interacting elements				
cdr1) *cdr2*)	Mitotic control	Altered starvation response; interact with *cdc2, cdc25*		*27*
mcs1–6	*cdc2.3w/wee1*	Suppress "mitotic catastrophe" of *cdc2.3w/wee1*		*9*
nim1	*cdc25*	Suppresses *cdc25* in multicopy; mitotic control element; predicted protein kinase	Yes	*28*
suc1	*cdc2*	Chromosomal and plasmid-mediated suppression of *cdc2*	Yes	*29, 30*
suc22	*cdc22*	Suppresses *cdc22* in multicopy	Yes	*13*
win1	*wee1/cdc25*	Reverses suppression of *cdc25* by *wee1*		*31*

Gene	Protein product	Comments	Cloned	Reference(s)[a]
Cytoskeletal elements				
nda2	α-Tubulin	Mutant phenotypes allele-specific; MBC/TBZ supersensitive, *cs* mitosis	Yes	*32, 33*
—	α-Tubulin	Mutationally silent	Yes	*32, 33*
nda3/ben1	β-Tubulin	Mutant phenotypes allele-specific: MBC/TBZ resistant or supersensitive, *cs* mitosis	Yes	*34*
ben4	Unknown	Existing mutants resistant to MBC and cold-sensitive for mitosis		*35*
—	Actin		Yes	*36*
cal1	Calmodulin	Null allele lethal	Yes	*37*

[a]References: (*1*) Nurse *et al.* (1976); (*2*) S. Moreno and P. Nurse (personal communication); (*3*) Nurse and Bissett (1981); (*4*) Nurse and Thuriaux (1980); (*5*) Fantes (1981); (*6*) Simanis and Nurse (1986); (*7*) Aves *et al.* (1985); (*8*) Nasmyth and Nurse (1981); (*9*) Booher and Beach (1987); (*9a*) Booher and Beach (1988); (*10*) Minet *et al.* (1979); (*11*) Nasmyth (1979a); (*12*) Barker *et al.* (1987); (*13*) Gordon and Fantes (1986); (*14*) Fantes (1979); (*15*) Russell and Nurse (1986); (*16*) D. A. Hughes and P. A. Fantes (unpublished); (*17*) Sackett and Lederberg (1986); (*18*) Hirano *et al.* (1986); (*19*) Yanagida *et al.* (1986); (*19a*) Hirano *et al.* (1988); (*20*) Iino and Yamamoto (1985a,b); (*21*) Nurse (1985a); (*22*) Beach *et al.* (1985); (*23*) Uemura and Yanagida (1984); (*24*) Uemura and Yanagida (1986); (*25*) Uemura *et al.* (1987); (*26*) Russell and Nurse (1987a); (*27*) Young and Fantes (1987); (*28*) Russell and Nurse (1987b); (*29*) Hayles *et al.* (1986a); (*30*) Hayles *et al.* (1986b); (*31*) Ogden and Fantes (1986); (*32*) Toda *et al.* (1984); (*33*) Adachi *et al.* (1986); (*34*) Hiraoka *et al.* (1984); (*35*) Roy and Fantes (1982); (*36*) Mertins and Gallwitz (1987); (*37*) Takeda and Yamamoto (1987).

nine *cut* and two *nuc* genes (Hirano *et al.*, 1986; Yanagida *et al.*, 1986), and the major tubulin genes *ben1/nda3* (referred to herein as *nda3;* Yamamoto, 1980; Hiraoka *et al.*, 1984) and *nda2* (Toda *et al.*, 1984). Mutations in *cdc1, cdc2,* and *cdc25* are probably best classified as late G_2 rather than mitotic defects, as discussed in Sections V,A,2 and VI,A.

B. Dependency Relationships in Cycle

Early work with *cdc* mutants (Nurse *et al.*, 1976) showed that DNA replication and mitosis form a mutually interdependent pathway: arresting DNA replication prevents the next mitosis from taking place, while after mitotic arrest the subsequent S phase does not take place. This conclusion was not unexpected, since the interdependence had been previously demonstrated by use of chemical inhibitors (Mitchison, 1974). An additional conclusion, that the DNA replication–mitosis cycle (the nuclear cycle) could occur independently of septation and later stages in division, was also drawn. Septation is dependent on the completion of some, though not all steps in mitosis (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; Hirano *et al.*, 1986). According to this oversimplified picture there should be three archetypal classes of cell cycle mutants. Mutants in G_1 or S phase should arrest after block with a single nucleus with the 1*c* (unreplicated) DNA content. Mutants in G_2 or mitosis should arrest as uninucleate cells with 2*c* DNA content. In neither case should septa form in arrested cells. Mutants defective in septation should continue through rounds of DNA replication and mitosis, forming multinucleate cells. All three of these phenotypes have been observed, but a considerable number of mutants do not conform to this pattern, and these are discussed in the appropriate sections. Deviation from these archetypal phenotypes is indicated in Table I.

A high proportion of the mutants isolated in earlier screens (Nurse *et al.*, 1976) have defects in septum formation, classified as "early cell plate" or "late cell plate" mutants. These have not been extensively studied apart from their cytological effects (Streiblova *et al.*, 1984), and none of the genes has so far been cloned. They are not further discussed here; however, a discussion of septation mutants is presented by Robinow and Hyams (Chapter 8, this volume).

C. Cloning Cell Cycle Genes by Complementation

The major route to cloning genes for which conditional lethal mutations are available has been by complementation (Russell, Chapter 7, this vol-

ume). In many cases, the plasmid recovered from transformed cells carries a functional copy of the gene that is defective in the recipient strain. In a substantial minority of cases, however, sequences other than the gene of interest have been recovered. Although the isolation of such extragenic suppressor sequences during screening can be disheartening, several new genetic elements have been discovered in this way. Methods for distinguishing between cloned sequences that carry the authentic gene and those that do not are discussed by Russell (Chapter 7, this volume). In general it is found that the cloned gene of interest complements mutant defects when present as a single, integrated copy. In contrast, extragenic suppressors are often only able to suppress the defect when present in multiple copies (Gordon and Fantes, 1986; Russell and Nurse, 1987b; Hayles *et al.*, 1986a).

In at least one case it has proved impossible to clone a gene by complementation of a mutant defect, despite its presence in the library used. The normal β-tubulin gene, *nda3*, was cloned by an indirect route, and it was subsequently shown to be lethal when present on a multicopy plasmid (Hiroaka *et al.*, 1984). The general point that overexpression can be lethal may be important for the isolation of other genes, particularly those having a structural or similar role where an excess of protein may be deleterious.

D. Cloning by Other Methods

The products of certain genes are known to be highly conserved through evolution: the histones, tubulins, actin, calmodulin, and certain other essential proteins vary remarkably little across a wide range of organisms. Homology at the protein level does not imply correspondingly high similarity of nucleotide sequences because of redundancy of the genetic code. Nevertheless, screening by hybridization to a heterologous probe has proved successful for several genes such as those encoding the histones (Matsumoto and Yanagida, 1985) and the actin gene (Mertins and Gallwitz, 1987). In contrast, screening for the *S. pombe* calmodulin gene using a chick cDNA probe resulted in the isolation of several clones that showed hybridization, but the sequence of hybridizing subclones did not encode calmodulin (D. A. Hughes, personal communication). The authentic calmodulin gene was cloned by using synthetic oligonucleotides whose sequences were chosen on the basis of the expected protein sequence and the *S. pombe* codon bias (Takeda and Yamamoto, 1987). A similar case is that of the human homolog to the *S. pombe cdc2* gene (Lee and Nurse, 1987). Attempts to clone the gene by hybridization failed, yet

a functional sequence was isolated by a radically different approach (Section V,D,1,c).

Cloning by other methods has been tried in certain cases. The inactivation of certain genes can allow a growth advantage under special circumstances. In principle the gene of interest could be "tagged" by integrative inactivation by transformation with a suitable library. This approach has been tried (unsuccessfully: Ogden and Fantes, 1986) for the isolation of the *wee1* gene (Section V,D,2). The *wee1* gene was eventually cloned by a different approach (Russell and Nurse, 1987a).

E. Why Clone Cell Cycle Genes?

The reason for wanting to clone a cell cycle gene depends very much on the gene concerned. In all cases it allows the abundance of transcript to be determined, and questions about the effect of cell cycle stage or developmental state to be asked. However, the cloning of a gene allows much deeper probing into the cellular role of the protein product of the gene.

Proteins of major cellular importance, such as those comprising the cytoskeleton and the histones, have been much investigated *in vitro* using sophisticated biochemical techniques. Nevertheless, the *in vivo* functions of such proteins, and other cellular components with which they interact, are often not clearly defined. This is because, however sophisticated the biochemistry, the cellular importance and role of a component cannot be determined by *in vitro* experiments alone. Some way is needed of investigating the effect *on cells* of experimental manipulations. *In vitro* mutagenesis allows defined changes to be made to the expression and nature of the protein produced. In yeasts, gene transplacement allows the normal copy of a gene to be replaced with a modified version, and the effect on cellular behavior examined (Rothstein, 1983; Shortle *et al.*, 1984). Genes can be reintroduced after deletion or disruption of the coding sequence, which determines whether the gene is essential (e.g., Adachi *et al.*, 1986). More informative is the ability to make point mutations, some of which are likely to yield temperature-conditional phenotypes, invaluable in physiological analysis (Shortle *et al.*, 1984). The modification of sequences involved in gene expression may also be used to determine whether a particular pattern of expression during the cell cycle is important to the cell.

The primary DNA sequence of a gene provides information about the evolutionary conservation of the protein sequence (e.g., Hiraoka *et al.*, 1984), given that the nature and general properties of the protein are understood beforehand. The DNA sequence can be compared with

known protein or cDNA sequences and information gained about the presence of introns (Hiraoka *et al.*, 1984; Hindley and Phear, 1984).

The reasons for cloning *cdc* and similar genes are somewhat different. In general, the cellular phenotype conferred by conditional mutations is known, but the molecular basis of the defect and the biochemical role of the normal product are unknown. Cloning a gene of this type of course allows the effects of disruption, and of any variations in transcript level, to be investigated, but perhaps a more important reason is that the availability of a clone allows access, direct or indirect, to the protein product (e.g., Hindley and Phear, 1984; Simanis and Nurse, 1986). The amino acid sequences of proteins encoded by other genes have been predicted by DNA sequence analysis and have been used for comparison with sequence databases; these are discussed at appropriate places in this chapter. The products of several cloned genes show no homology with previously identified proteins. A particular problem with sequence comparisons of *S. pombe* genes is the frequent presence of introns, which are usually quite short. Although consensus intron processing sequences have been compiled for *S. pombe* (Barker *et al.*, 1987; Kaufer *et al.*, 1985; Russell, Chapter 7, this volume), they are based on the rather small number of introns so far identified. Some processing sites, therefore, may not be detected simply by DNA sequence searches. Knowledge of the DNA sequence is virtually essential if the production of the gene product *in vitro* or in *E. coli* is desired.

III. START AND G_1 CONTROLS

A. Start

1. Start in Saccharomyces cerevisiae

Any review of the concept of "start" in other cells needs first to consider the budding yeast *Sacch. cerevisiae*, for which the idea was originally proposed. Hartwell and colleagues (1974) postulated the existence of a unique point in the G_1 phase of *Sacch. cerevisiae* at which cells made decisions about their future. This proposal was strengthened by a series of studies in several laboratories (reviewed by Pringle and Hartwell, 1981). The start point of the cycle is now taken to mean the stage beyond which cells are committed to the mitotic cycle, or to stationary phase, or to the pathways leading to meiosis and sporulation (diploids) or conjugation (haploids). The availability of a diffusible factor (α-factor) secreted by

cells of α mating type which was found to arrest *a* cells at start greatly facilitated these studies. Reciprocal shift experiments showed that the α-factor-sensitive step and the step controlled by the *CDC28* gene were functionally interdependent (Hereford and Hartwell, 1974). The point of α-factor or *CDC28* arrest has frequently been used as a definition of start.

"Start" was originally proposed as the single point in the cell cycle at which the decision about the future development of the cell was made and beyond which a cell was irreversibly committed to that route (Hartwell *et al.*, 1974). Once cells have passed the *CDC28* step in the mitotic cycle they are unable to conjugate (Reid and Hartwell, 1977), and the *CDC28* and α-factor steps are functionally interdependent (Hereford and Hartwell, 1974). Criticisms of start as a unitary concept have been made (Nurse, 1981), for two sorts of reasons. First, cells arrested at start by different treatments have different morphologies and differ in other ways (Nurse, 1981). A second and more serious criticism is that commitment to meiosis in diploid strains does not appear to take place until a slightly later stage in the cell cycle, after the cell has executed the *CDC4* step (Hirschberg and Simchen, 1977). An alternative view, that start is a less well-defined part of G_1 where there is a degree of developmental plasticity, has been proposed (Nurse, 1981).

The traverse of start is the major rate-limiting step in cell cycle progress in *Sacch. cerevisiae* (Carter, 1981). At slow growth rates the G_1 period, or, more precisely, the prestart part of G_1, is extended disproportionately compared with the other cycle phases. Start is therefore not only the point where the mitotic cycle is initiated, and beyond which cells are committed to mitosis, but also the major rate-limiting event of the *Sacch. cerevisiae* cycle.

2. *Start in Schizosaccharomyces pombe*

In *S. pombe* "start" is less well understood. In fast exponential growth it appears not to be rate limiting for cycle progress, this role being taken by the control over mitosis (Nurse and Fantes, 1981; Section V). In small cells produced by starvation, and during the outgrowth of spores (Nurse and Thuriaux, 1977), a cell size control over the initiation of S phase becomes more important, and it is likely but not proven that this control acts at start. In slowly growing cells, the prestart period is lengthened, while the poststart interval that includes part of G_1, S, G_2, and M remains fairly constant (Nasmyth, 1979b).

The experimental approaches taken in *Sacch. cerevisiae* to investigate commitment to mitosis, conjugation, or meiosis have not been used to the same extent in *S. pombe* largely because its life cycle is not so readily

amenable to the manipulations required. One particular difficulty has been the lack of any diffusible mating factor comparable to α-factor. This has prevented the synchronization of cells at start by any means other than the use of conditional lethal *cdc* mutants and precluded the sort of dependency analysis around start described by Hartwell (1974). Recent reports supporting the existence of such a factor (Fukui *et al.*, 1986; Leupold, 1987) do not describe its physical isolation, which would be a necessary prerequisite for its experimental use.

Evidence for the existence of start in *S. pombe* derives almost entirely from the variation in ability of cells at different cycle stages to conjugate. Conjugation occurs essentially only after starvation for nitrogen (Egel and Egel-Mitani, 1974), perhaps because certain mating-type gene transcripts are produced in response to nitrogen deprivation (McLeod *et al.*, 1987). Nurse and Bissett (1981) investigated the ability of a range of *cdc* mutants to conjugate after arrest under nitrogen starvation at the restrictive temperature of 36°C. Conjugation with cells of opposite mating type was tested at the slightly lower temperature of 33°C required for conjugation. Mutants of 11 genes required for the nuclear cycle were tested in this way, and only *cdc10* and *cdc2* were able to mate at frequencies comparable to that of a cdc^+ control. This showed that *cdc10*, previously classified as controlling a G_1 function (Nasmyth and Nurse, 1981), was in some sense a start gene. The observation with *cdc2* was more surprising, since *cdc2* mutants had previously been characterized as defective in an early step in mitosis (Nurse and Thuriaux, 1980; see also Section V). The existence of a second cycle block point in G_1 was, however, confirmed by taking *cdc2* cells into G_1 by nitrogen starvation and reinoculating into growth medium at the restrictive temperature. It was also shown that the ability of *cdc2* cells to mate at the restrictive temperature was much enhanced at a time corresponding to G_1 in synchronous culture (Nurse and Bissett, 1981).

The study by Nurse and Bissett (1981) shows that cells arrested at the G_1 block of *cdc2* and *cdc10* mutants are not yet committed to the mitotic cycle when presented with conjugation as the alternative. After this point cells become committed to mitosis and are unable to conjugate. However, the decision point for diploid cells presented with a choice between mitosis and meiosis may be different, as found in *Sacch. cerevisiae* (Hirschberg and Simchen, 1977).

a. *cdc10* Gene. The *cdc10* gene was cloned by complementation, the nature of the cloned sequence confirmed by directed integration and mapping, and the primary DNA sequence determined (Aves *et al.*, 1985). No introns were predicted by comparison with a limited set of *S. pombe* consensus sequences, and a single continuous open reading frame of 2.3

kilobases (kb) corresponding to a 85 kilodalton (kDa) protein was identified. The predicted secondary structure of the *cdc10* protein is consistent with its being a soluble protein. The 3′ half of the gene was able to complement the *cdc10-129* defect weakly, perhaps suggesting some type of interallelic complementation, which would imply that the functional protein is an oligomer. Alternatively the 3′ half alone may encode a partially functional protein.

The major *cdc10* transcript of 2.7 kb was found not to be regulated during the cell cycle (Aves *et al.*, 1985). Changes were observed in synchronous cultures in minor RNA species homologous to *cdc10*, but the significance of these is unclear. No changes in the major transcript were observed during entry into stationary phase, nor during G_1 arrest induced by nitrogen deprivation.

While the role of *cdc10* in controlling entry into the cell cycle at start has been established, the mechanism by which this is mediated remains unknown. The level of major transcript is insensitive to either the cycle stage or the growth state, implying that the control must operate post-transcriptionally. One possibility is that the *cdc10* gene product controls the expression of genes downstream to the start control, such as the *cdc22* gene which shows periodic changes in transcript level (Section III,D). Alternatively the *cdc10* gene product may not be regulatory, in the sense of rate limiting, at all: other elements may fulfill this role, and the *cdc10* product may simply be part of the start machinery.

Intriguingly, limited sequence similarity of the *cdc10* gene with the *SWI6* gene of *Sacch. cerevisiae* has been reported (Breeden and Nasmyth, 1987). The latter gene is implicated in the transcriptional regulation of the periodically expressed *HO* gene, and it is possible that the *cdc10* gene is also involved in the transcriptional regulation of other cell cycle genes. The homology is limited to certain regions, however, and *SWI6* does not complement *cdc10* (P. A. Fantes, unpublished observation).

b. *cdc2* Gene. The *cdc2* gene is one of the most intriguing *S. pombe* genes so far identified. It has central roles in the control of start, control of cell size, and entry into mitosis, and has functional homologs in both *Sacch. cerevisiae* and human cells (Nurse and Bissett, 1981; Nurse and Thuriaux, 1980; Lee and Nurse, 1987; Draetta *et al.*, 1987). Discussion of its significance for mitosis, and how it interacts with other mitotic control elements, is deferred until Section V. Its role in the start control is discussed in this section.

The wild-type $cdc2^+$ gene was cloned by complementation of a temperature-sensitive lethal *cdc2* mutant, and the *Sacch. cerevisiae CDC28* gene

was also found to complement *cdc2* mutations of *S. pombe* (Beach *et al.*, 1982a). Since at least one of the *cdc2* mutations had been shown to be conditionally defective in G_1 and G_2 (Nurse and Bissett, 1981), this implied that the *S. pombe* and *Sacch. cerevisiae* genes both controlled functions required in G_1 and G_2. The existence of an arrest point in the *Sacch. cerevisiae* cycle later than start for *CDC28* mutations has been reported by Piggott *et al.* (1982), and this presumably corresponds to the G_2 arrest point of *S. pombe cdc2* (Nurse, 1985b).

The complementing region of *cdc2* was identified (Durkacz *et al.*, 1985), and shown to correspond with the region previously sequenced by Hindley and Phear (1984). The primary DNA sequence of the *cdc2* gene was compared with that of *Sacch. cerevisiae CDC28* (Lorincz and Reed, 1984). Substantial homology (62% amino acid identity) was found within the reading frame, provided the existence of four postulated introns in the *S. pombe* gene was taken into account (Hindley and Phear, 1984). The sequences of both genes showed homology with a number of protein kinases, particularly in the region of the ATP binding site and around potential sites of phosphorylation (Hindley and Phear, 1984; Nurse, 1985b). Subsequent isolation of the proteins has shown that they both indeed have protein kinase activity and can be phosphorylated (Reed *et al.*, 1985; Simanis and Nurse, 1986).

The existence of the predicted introns in *cdc2* was confirmed in two ways. First, S_1 nuclease mapping of the major 1.6-kb transcript was consistent with the predicted intron positions. The fourth intron, which was short, contained no stop codon, and might therefore have been used differentially as a control mechanism, was shown to be spliced out normally (Durkacz *et al.*, 1986). Second, the introns were removed *in vitro* by oligonucleotide-directed mutagenesis (Booher and Beach, 1986). The resulting gene was functional in *S. pombe* and also in *Sacch. cerevisiae*. This is of particular interest since the genomic *S. pombe* $cdc2^+$ gene does not complement *CDC28* mutations. The two yeasts are known to have different conserved splicing sequences (Kaufer *et al.*, 1985; Barker *et al.*, 1987), so that the introns in the *S. pombe* gene cannot be properly processed in *Sacch. cerevisiae*. In contrast, the same gene after removal of introns is functional.

The central importance of the *cdc2* gene to the *S. pombe* cell cycle made it a good candidate for a periodically expressed gene. However, the *cdc2* transcript was found to show no changes in level during the cell cycle, or on entry into stationary phase by exhaustion of nutrients (Durkacz *et al.*, 1986). Curiously, a gene adjacent to the *cdc2* gene, designated *cdc2L*, did show cell cycle-related changes in transcript level with a peak around G_1 or S phase. *cdc2L* shows considerable homology to his-

tone H2A genes, particularly that of chicken H2A.F (J. Hindley and P. Nurse, personal communication), and its periodicity in transcript level is similar to that of known histones (Section IV,B). This suggests a cell cycle role for *cdc2L*.

Interest in the *cdc2* gene has progressed to the protein product. The DNA sequence of the coding region predicted that it should be a 34-kDa protein and that it might be a phosphoprotein with protein kinase activity (Hindley and Phear, 1984). These predictions have been borne out by the identification and isolation of the $cdc2^+$ product in two laboratories.

To obtain antibody preparations against the *cdc2* protein, Simanis and Nurse (1986) used synthetic peptides corresponding to portions of the predicted protein. Antisera against two peptides were active against the same *S. pombe* protein, designated p34, and this was shown to be present at higher levels when the *cdc2* gene was overexpressed from a strong promoter. This showed that the antisera recognized the authentic $cdc2^+$ product. The antisera were used to immune precipitate p34 after labeling cells with either ^{35}S or ^{32}P. The precipitate was analyzed by one-dimensional gel electrophoresis and autoradiography, and the same band was labeled by either ^{35}S or ^{32}P, showing that p34 is a phosphoprotein.

In a separate study, Draetta *et al.* (1987) obtained monoclonal antibodies against the *cdc2* protein expressed in *E. coli*. Using two-dimensional electrophoresis they showed that multiple forms of the *cdc2* protein were present in extracts of *S. pombe*, indistinguishable in molecular weight but differing in charge. In a further study (Potashkin and Beach, 1988), both phosphorylated and unphosphorylated forms were identified, which may be significant in terms of cell cycle control (Section V).

Simanis and Nurse (1986) demonstrated that the p34 protein has protein kinase activity. They also isolated the corresponding protein from several *cdc2* mutant strains by immune precipitation and showed that in all cases the enzymatic activity was either temperature sensitive or reduced in absolute level. It is therefore likely that the protein kinase activity associated with the *cdc2* product is essential for cell cycle function.

Specific *in vitro* mutants of the *cdc2* gene predicted to interfere with protein kinase activity were examined by Booher and Beach (1986). Mutations of Lys to Arg at the predicted ATP binding site (position 33) abolished the ability to complement a *cdc2* mutation, but a similar change at position 34 did not. This is consistent with the prediction based on other protein kinases that Lys-33 should be directly concerned with ATP binding. Putative phosphorylation sites were also modified and the *in vivo* effects tested. The Thr residue at position 167, a potential phosphorylation site, was found to be essential, and changing an Ala residue to Val in a neighboring, highly conserved region abolished activity.

While the properties of these *in vitro* mutations are consistent with the predicted protein kinase activity, they cannot alone be taken as conclusive evidence for it. The known temperature-sensitive mutations of *cdc2* map throughout the gene (Nurse and Thuriaux, 1980; A. Carr, S. MacNeill, and P. Nurse, personal communication), and these by definition encode *cdc2* products that are defective *in vivo*. Some of these mutations may be at the ATP binding or phosphorylation sites, but several must be elsewhere. Thus, while all mutations that are predicted to affect protein kinase activity abolish function *in vivo,* there are other ways of inactivating the *in vivo* function.

The physiological significance of phosphorylation of the *cdc2* protein kinase was investigated by Simanis and Nurse (1986). They showed that, as for the *cdc2* transcript, there are no systematic changes in level of the *cdc2* protein or in the extent of its phosphorylation in synchronous culture. However, nitrogen starvation of cells, which allows one rapid division followed by G_1 accumulation, caused a decrease in the extent of p34 phosphorylation, while the level of total p34 remained unchanged. The decrease in phosphorylation was associated with a loss of p34 protein kinase activity in cell extracts. Reinoculation into fresh medium caused an increase in protein kinase activity after a lag: the kinetics are consistent with the increase being required for reentry into the cell cycle. The correlation of phosphorylation state with protein kinase activity is striking, and suggests that the activity may be regulated by phosphorylation. This attractive idea is by no means proven, though, and loss of phosphorylation might be an effect rather than a cause of loss of kinase activity, if autophosphorylation were involved.

One important question is whether the changes induced by nitrogen starvation are due to G_1 accumulation or to some other effect of the treatment such as the severe inhibition of cellular growth. Phosphate deprivation also results in the accumulation of some cells in G_1 (Bostock, 1970), and p34 dephosphorylation is observed (V. Simanis and P. Nurse, personal communication). Does dephosphorylation occur during the G_1 phase of the cycle in growing cells? *Schizosaccharomyces pombe* cells normally have a very short G_1, and transient dephosphorylation might be missed in synchronous cultures, as discussed by Simanis and Nurse (1986). However, *wee1* cells have an extended G_1 (see Section III,C), and dephosphorylation of p34 should be seen in *wee1* synchronous cultures. *cdc10* arrested cells might also be expected to dephosphorylate. Observations by V. Simanis and P. Nurse (personal communication) show that p34 is still phosphorylated under these conditions. This suggests that being in the G_1 phase is insufficient to bring about complete p34 dephosphorylation. It therefore seems likely that complete dephosphorylation of

the *cdc2* gene product occurs as a response to the cessation of growth rather than being directly related to cell cycle stage.

It may be that more subtle changes in phosphorylation pattern occur during the normal G_1 phase, perhaps detectable by separation of the various forms of the protein (see above). The possible significance of p34 phosphorylation is discussed further (Section IV) in relation to the two cycle steps for which $cdc2^+$ is required.

B. Developmental Control

Schizosaccharomyces pombe cells of appropriate mating type will conjugate when starved of nitrogen to form a diploid zygote. Normally the diploid nucleus will enter the meiotic pathway, forming four haploid nuclei which are subsequently surrounded by spore walls within the zygote. These processes are discussed in detail in Chapter 2 by Egel (this volume). "Start" is the point at which the pathways to mitosis and conjugation diverge, and, by analogy with *Sacch. cerevisiae* (Hirschberg and Simchen, 1977), the choice between mitosis and meiosis in diploid cells may be made at a similar cycle stage. It is therefore pertinent here to discuss the controls determining entry to these pathways. Since conjugation and karyogamy are normally followed immediately by meiosis, a functional connection between control of the two processes might be expected. As will be seen, the two "decision" points, namely, between mitosis and conjugation for haploids and between mitosis and meiosis for diploids, share at least one genetic element and are therefore discussed together.

As discussed in the previous section, mutants in two cell cycle genes *cdc2* and *cdc10* are able to conjugate from their arrest points provided nitrogen is limiting. Using a different approach to the control, Nurse (1985a) isolated mutants from a homothallic strain that were able to conjugate in rich medium, by direct visual selection. Two of the eight mutations defined a single gene *ran1:* these alleles are temperature sensitive. In a separate study, Iino and Yamamoto (1985a) isolated mutations that suppressed the defect of *mat2-102,* a mating-type mutation that allows conjugation but not meiosis (Egel, 1973). Both heat- and cold-sensitive mutations were obtained, and all mapped to the same locus, *pat1*. The similarities between *ran1* and *pat1* mutants described below suggested that they might be allelic. This was confirmed (Iino and Yamamoto, 1985a; Nurse, 1985a), and the locus is now designated *pat1* (Kohli, 1987).

pat1 mutants are derepressed both for conjugation and for meiosis and sporulation. Of the two normal requirements for conjugation, starvation and the presence of cells of opposite mating type, only the former is

relieved, since conjugation is not observed unless cells of both mating types are present. The effect on meiosis is more profound: the starvation requirement for meiosis and sporulation is either reduced or abolished in *pat1* mutants. More strikingly, the requirement for mating-type heterozygosity is abolished, and even haploid cells of single mating type undergo what is presumably an attempt at meiosis. Small and very variable spores are formed within the parent haploid cell. These spores have very low viability, presumably because very few have a complete chromosome set.

The recessive nature of *pat1* mutations suggested that the *pat1*$^+$ gene acts as an inhibitor of conjugation and sporulation. Further work (Iino and Yamamoto, 1985b) showed that *pat1* mutants, when shifted to the restrictive temperature, undergo premeiotic DNA synthesis and meiotic recombination, and that the *pat1*$^+$ product represses a step early in the normal meiotic pathway. The *pat1*$^+$ product was proposed to be inactivated as a prerequisite for meiosis. This idea was pursued by Beach *et al.* (1985) who proposed a model in which the *pat1* gene product is an inhibitor of conjugation and meiosis and is itself inhibited by the combined action during G_1 of the products of *matP*$^+$ and *matM*$^+$ (expressed mating-type genes) and of *mei3*$^+$. The importance of *mei3*$^+$ in particular is stressed by McLeod *et al.* (1987), and McLeod and Beach (1988) have demonstrated physical interaction *in vitro* between the *pat1* and *mei3* products. This may not be a complete picture, however, since the *mei2*$^+$ gene also appears to be involved in the switch between mitosis and meiosis (Iino and Yamamoto, 1985b; M. Yamamoto, personal communication). McLeod and Beach (1986) reported significant homology of the *pat1* DNA sequence with protein kinases, consistent with the suppression of *pat1* phenotype by stimulation of cAMP-dependent protein kinase (Beach *et al.*, 1985).

How do these studies on the control of entry into meiosis bear on the question of start? Much of the evidence rests on studies by Beach *et al.* (1985), who investigated the ability of cells arrested in G_1 by *cdc2* or *cdc10* to undergo premeiotic DNA synthesis. Double mutants carrying a *pat1* temperature-sensitive allele and either *cdc2*, *cdc10*, or no *cdc* allele were first nitrogen starved at the permissive temperature to arrest the majority of the cells in G_1. To challenge the ability of cells to progress down the meiotic pathway, complete medium was restored at the restrictive temperature. The *cdc10* strain showed no premeiotic DNA synthesis and limited sporulation, the latter presumably caused by entry into meiosis directly from mitotic G_2. The *cdc*$^+$ and *cdc2* strains, however, showed both premeiotic DNA replication and sporulation. The simplest explanation of these observations is that the G_1 *cdc2* arrest point is earlier in the cycle than the *cdc10* arrest point and that start is not a single-step process. An alternative explanation is that both *cdc2* and *cdc10* arrested cells are in the same prestart state but that the *cdc10*$^+$ product is required again

during early stages of the meiotic pathway, for instance, for premeiotic S phase. It is also conceivable that the *pat1* mutation has a direct effect on the function of *cdc2* or *cdc10,* altering the apparent structure of the pathway control.

In an attempt to extend their conclusion to a more normal situation, Beach *et al.* (1985) devised a set of conditions that allowed reasonably synchronous passage of sporulation-competent diploids through premeiotic DNA replication, meiosis, and sporulation. *cdc2* and *cdc10* diploids were tested for their ability to enter the meiotic pathway from their respective arrested states. The results were generally in favor of the *cdc10* arrest point being earlier than the G_1 *cdc2* arrest point. However, the possibility that the treatment applied to the cells to prepare them for meiosis might affect their subsequent behavior cannot be ruled out. It is curious that while $cdc2^+$ is required for mitotic DNA synthesis, it is apparently not required for premeiotic DNA synthesis. This behavior is paralleled by that of *Sacch. cerevisiae* cells carrying a *cdc7* mutation (Schild and Byers, 1978), but *CDC7* acts after start whereas *S. pombe* $cdc2^+$ is apparently prestart.

In summary, the evidence for the existence of start in *S. pombe* still rests heavily on the "conjugation-challenge" experiments of Nurse and Bissett (1981). The traverse of start defined in this way requires the $cdc2^+$ and $cdc10^+$ gene products, and haploid cells that have not completed one of these gene-controlled steps are still uncommitted to mitosis and can enter the conjugation pathway. The available evidence suggests that entry into meiosis cannot begin until the *cdc10* step has been completed, while the evidence for *cdc2* requirement remains uncertain. Parallel experiments, similar to those of Hirschberg and Simchen (1977) with *Sacch. cerevisiae,* to investigate commitment to mitosis in diploids presented with the possibility of the meiotic pathway have not yet been carried out. There may be differences between cells arrested at the *cdc10* and G_1 *cdc2* blocks with respect to their meiotic capacities, but the question of commitment itself has not been addressed.

C. Cell Size Control

Experiments with *Sacch. cerevisiae* showed that the attainment of a critical cell size, or some cellular parameter closely correlated with size, acted as a major control point early in the cycle (Johnston *et al.,* 1977). Further studies demonstrated that the control acted very close to start (Carter, 1981). In *S. pombe* the importance of cell size in cycle control was realized at about the same time (Nurse, 1975; Fantes, 1977; Fantes

and Nurse, 1977). However, the major size control in *S. pombe* acts at mitosis, as discussed in Section V,A. The existence of a second size-related control that acts in G_1 was inferred from a number of conditions in which small cells were investigated (Nurse, 1975; Fantes and Nurse, 1978; Nasmyth, 1979b). The two size controls and their significance has been reviewed previously (Nurse and Fantes, 1981; Fantes, 1984a), and is mentioned only briefly here.

The size that cells must attain for entry into S phase is not great, and under normal conditions cells are born above the critical size. The existence of a size requirement is revealed only in cells made substantially smaller than normal, either mutationally (Nurse, 1975; Nasmyth *et al.*, 1979) or by physiological manipulation (Nurse and Thuriaux, 1977; Nasmyth, 1979b). The G_1 size control appears to act coincidentally with completion of the *cdc10* step at start (Nasmyth, 1979b), and it is possible that the execution of start itself requires a critical cell size. In *wee1* mutants the size requirement is expressed, and the question arose of what coordinates growth and division in *wee* mutants. From a combination of genetic and physiological manipulations, it was concluded that the size control over S phase worked in conjunction with a requirement for a minimum G_2 period to control the time of mitosis and division (Fantes and Nurse, 1978; Nurse and Fantes, 1981). There are thus two mechanisms able to control cell size at division, and consequently the time of division. The mitotic size control is expressed in normal growth, while a backup control using the G_1 size control and the minimum G_2 "timer" are expressed in small cells.

Cooper's "continuum" model of the cell cycle proposes (Cooper, 1979) that the G_1 interval in general is solely a period of growth, S phase being initiated purely in response to the attainment of a critical cell size. This idea runs contrary to the conventional view of the cell cycle, in proposing that the events from the initiation of S phase (start, in the case of yeasts) up to cell division constitute a sequence rather than a true cycle. While cell size is undoubtedly a major factor in regulating the transit of cells from G_1 to S phase in both yeasts, it seems unlikely that cell growth is the sole requirement for this.

According to the continuum model, the prestart period in *Sacch. cerevisiae* is devoid of any cycle-specific events (Cooper, 1979; Singer and Johnston, 1981); similar suggestions have been made for *S. pombe* (Singer and Johnston, 1985). Start should thus be able to occur without any requirement for the completion of the previous cycle, provided growth to the critical size can occur. However, *S. pombe* cells arrested in mitosis with benomyl are unable to pass the *cdc10* arrest point in the subsequent cycle during arrest, although substantial growth takes place

(Fantes, 1982). This implies that the start event (requiring *cdc10* function) is dependent on completion of events in the previous cycle and that growth alone is insufficient.

D. Other G_1 Events

The G_1 interval requires the function of at least two genes, *cdc20* and *cdc22*, in addition to the start genes *cdc2* and *cdc10*. At a physiological level mutants in *cdc20* and *cdc22* are indistinguishable (Nasmyth and Nurse, 1981). *cdc22* at least is not a start gene since cells are unable to conjugate from the *cdc22* arrest point (Nurse and Bissett, 1981). It seems very likely that both genes control steps subsequent to start, though the only direct evidence is that histone gene transcription, which begins in G_1, is prevented in *cdc10* arrested cells but not in *cdc20* or *cdc22* cells (Matsumoto *et al.*, 1987a). The biochemical functions of *cdc20* and *cdc22* are currently unknown: *cdc22* has been reported to have an S phase rather than a G_1 function (J. R. Dickinson, 1981), though the balance of evidence is heavily in favor of G_1 (Gordon and Fantes, 1986). The *cdc22* gene has been cloned (Gordon and Fantes, 1986), and a detailed molecular analysis is now underway.

cdc22 and suc22

The *cdc22* gene encodes a transcript of 3.3 kb, sufficient to encode a protein of greater than 100 kDa. Of great interest is the observation that the transcript level shows striking cell cycle changes in level (Gordon and Fantes, 1986), with a maximum around the G_1/S period. This is to date the only periodic transcript in *S. pombe* other than those for the histone genes (Matsumoto *et al.*, 1987a), and the mechanism responsible for the periodicity is currently under study (K. Campbell, M.-J. Fernandez-Sarabia, C. B. Gordon, and P. A. Fantes, unpublished observations). The question of whether the periodicity is caused by periodic transcription or by periodic changes in stability, both of which have been reported for histone genes in *Sacch. cerevisiae* (Lycan *et al.*, 1987), needs to be addressed.

A DNA sequence, designated *suc22*, was isolated that suppresses the conditional lethal defect of two *cdc22* mutant alleles, but only when the sequence is present on a multicopy plasmid. Two transcripts from this suppressor sequence have been identified. One, of 1.5 kb, is present constitutively; this transcript does not show periodic changes in level in synchronous culture (Gordon and Fantes, 1986). A larger transcript of 2.4 kb is highly induced by certain types of cell cycle arrest (C. B. Gordon, M.-J. Fernandez-Sarabia, and P. A. Fantes, unpublished observations).

Cells held in G_1 or S phase by *cdc* mutant arrest have a high level of 2.4-kb transcript, while cells arrested by most G_2 or mitotic mutations have a low level (C. B. Gordon, M.-J. Fernandez-Sarabia, and P. A. Fantes, unpublished observations).

The relationship between the two *suc22* transcripts is largely unknown, although they appear to overlap and are transcribed from the same strand (M.-J. Fernandez-Sarabia, unpublished observations). An important question is the mechanism of suppression of *cdc22* defects by extra copies of *suc22*. The *cdc22* gene is not overexpressed under conditions of *cdc22* suppression, excluding the possibility that *suc22* is a transcriptional regulator of *cdc22* expression (M.-J. Fernandez-Sarabia and P. A. Fantes, unpublished observations). The induction of the larger *suc22* transcript after specific cell cycle arrest may be connected with the periodic transcript changes of *cdc22*.

IV. S PHASE

A number of mutants with S phase defects have been described (Nasmyth and Nurse, 1981). Of these, none shows the physiological behavior expected of a simple defect in any function directly involved in DNA chain elongation. The majority show more complex phenotypes which are at present difficult to interpret (reviewed in Fantes, 1984a).

A. *cdc17* and DNA Ligase

The *S. pombe cdc17* gene encodes DNA ligase, as shown by direct enzymatic assay (Nasmyth, 1977, 1979a; Johnston *et al.*, 1986) and by comparison of the nucleotide sequence with that of the *Sacch. cerevisiae* ligase gene (Barker *et al.*, 1987). One *cdc17* mutant investigated accumulates Okazaki fragments at the restrictive temperature and shows increased sensitivity to ultraviolet radiation, consistent with the roles of DNA ligase in DNA replication and repair (Nasmyth, 1977).

The $cdc17^+$ gene has been cloned (Johnston *et al.*, 1986) and characterized at the molecular level (Barker *et al.*, 1987). The DNA sequence predicts 53% amino acid similarity with the *Sacch. cerevisiae* enzyme, allowing for two predicted introns whose presence was confirmed. A short region containing the putative ATP binding site also showed homology with bacteriophage DNA ligases. The 2.8-kb transcript is present at all stages of the cell cycle at essentially the same level, and the level of ligase activity is also constant. This is unexpected as the corresponding *Sacch. cerevisiae* gene *CDC9* shows marked periodicity both in transcript

level and in enzymatic activity (White *et al.*, 1986). As pointed out by White *et al.* (1986) this is particularly surprising since the genes encode the same function, and the *Sacch. cerevisiae* gene can complement ligase mutations in *S. pombe* (Barker and Johnston, 1983).

B. Histone Genes

The histones are small, basic proteins present in all eukaryotic chromatin. The four core histones, H2A, H2B, H3, and H4, are assembled into nucleosome core particles around which DNA is wrapped. A fifth histone, H1, is thought to stabilize individual nucleosomes and may play a role in the higher order structure of chromatin.

In neither *Sacch. cerevisiae* nor *S. pombe* has a histone of the H1 class or a gene that might encode one been identified (Certa *et al.*, 1984; Matsumoto and Yanagida, 1985), and discussion of histone genes is therefore restricted to those encoding the four core histones. Nine histone genes have so far been identified and cloned in *S. pombe*, by hybridization to heterologous probes from *Sacch. cerevisiae* or *Drosophila*. There are two genes for histone H2A, one for H2B, and three each for H3 and H4 (Matsumoto and Yanagida, 1985; Choe *et al.*, 1985). This arrangement is strikingly different from that in *Sacch. cerevisiae*, which has two genes for each of the core histones. The arrangement of the genes in *S. pombe* is as follows: H3 and H4 genes are arranged in three units, each containing one H3 and one H4 gene, which are transcribed divergently. The units are dispersed in the genome. There is one similar unit containing the H2B gene and one of the H2A genes. The remaining H2A gene is unpaired and is genetically unlinked to the H2A–H2B pair. There are DNA sequence differences between the various genes coding for each histone, as well as differences between the predicted proteins in the case of H2A (Matsumoto and Yanagida, 1985; Choe *et al.*, 1985). As mentioned earlier (Section III,A,2,b) the *cdc2L* gene shows considerable homology to H2A genes, particularly that of chicken H2A.F (J. Hindley and P. Nurse, personal communication). Its transcript level varies in a periodic manner during the cell cycle (Durkacz *et al.*, 1986), with a pattern similar to that of known histone transcripts, discussed below. It is possible that the *cdc2L* gene encodes a hitherto unidentified histone species.

Transcription of the histone genes is largely restricted to the late G_1 and S phases in many cell types (Schumperli, 1986), and *S. pombe* is no exception. Aves *et al.* (1985) observed major changes in the level of H2A1 transcript in phase with the cell cycle, with a maximum level at about the time of the S phase. In a more detailed study, Matsumoto *et al.* (1987a) investigated the control of histone H2B transcript level during the cell cycle. Wild-type cells showed a striking periodicity similar to that ob-

served for H2A1. Imposing a *cdc10* start block on cells presynchronized by size separation abolished the rise in H2B transcript level, indicating that completion of start is required for the increase. With mutants defective in other G_1 functions, *cdc20* and *cdc22*, a rise in H2B transcript level was observed, though this was considerably slower than that seen in cdc^+ cells. The transcript level rose to a broad peak and then fell slowly. Thus, histone gene expression appears to occur despite the absence of DNA synthesis, showing that expression of the histone genes is switched on in the cell cycle before the initiation of DNA replication.

Mutants in *cdc17* (which encodes DNA ligase) and *cdc24* are defective in the process of DNA replication, but on shift to the restrictive temperature synthesize the amount of DNA corresponding to one round of normal replication (Nasmyth and Nurse, 1981). Similar patterns of H2B transcript level were seen in arrested *cdc17* and *cdc24* cells as for *cdc20* and *cdc22*, though the pattern of rise and fall varies between the four mutants.

The cell cycle control of histone gene transcript level in *S. pombe* is rather similar to that of *Sacch. cerevisiae*, where arrest at start prevents any increase in histone transcript level while arrest later in the cycle allows transcription (Hereford *et al.*, 1981). In *S. pombe* the rise normally observed in H2B transcript level is abolished in the *cdc10* start mutant, and this is very likely due to the absence of transcription. Rises in level after arresting in G_1 after start or in the S phase occur, but at a slower rate than normal (Matsumoto *et al.*, 1987a). There is no obvious explanation for this, but posttranscriptional mechanisms that affect the stability of the mRNAs after G_1 or S phase block may play a role. Likewise, the slow fall in mRNA levels after G_1 or S phase blocks may be a consequence of increased message stability. Stability control of histone mRNAs has been proposed to be involved in their cell cycle regulation in *Sacch. cerevisiae* (Hereford *et al.*, 1981).

DNA sequence analysis of the five cloned histone gene units in *S. pombe* has revealed the presence of a 17-base sequence 5′ to each gene with only one base showing variation (Matsumoto and Yanagida, 1985). In the case of the H3–H4 and H2A–H2B pairs, the conserved sequence is in the intergenic region. Within the 17-base sequence there is a tandem repeat of 6 bases with no variation. It is tempting to think that the presence of this element is directly responsible for the periodic and coordinate expression of the genes. Whether this or similar elements are present 5′ to other cell cycle-regulated genes such as *cdc2L* and *cdc22* remains to be seen.

C. *ars* Elements and DNA Replication

DNA replication in *Sacch. cerevisiae*, and presumably in *S. pombe*, is typical of eukaryotic cells: replication is initiated along the chromosomes

at many points, each controlling the replication of part of the DNA molecule. An appealing hypothesis is that initiation occurs at specific sites defined by their DNA sequence, but this has not been demonstrated for any eukaryote (other than certain viruses), and apparent lack of sequence specificity has been observed for the replication of DNA sequences injected into *Xenopus* eggs (Harland and Laskey, 1980; Mechali and Kearsey, 1984). Eggs may be special cases, however, and it seems likely that discrete replication origins do exist in other systems. If so, then the *ars* elements (for autonomous replication sequence) identified in yeasts are good candidates for origins.

From the earliest reports of transformation of yeast by introduced plasmids (Hinnen *et al.*, 1978; Struhl *et al.*, 1979), it became clear that two types of transformants could be obtained. In one type, the plasmid integrated into a site on one of the chromosomes, often by homologous recombination, and was inherited stably through mitosis and meiosis. In the other type, the plasmid was maintained as an autonomous extrachromosomal element and was not stably inherited. The ability to confer autonomous replication could be narrowed down to a specific DNA sequence (an *ars*) in most cases (Kearsey, 1984). Comparison of *ars* elements from different sources reveals the presence of a "core" region of 14 bp which is present in the vast majority of *ars* elements and within which point mutations often have a very deleterious effect on transformation frequency (Kearsey, 1983; see Williamson, 1985, for review). Initially it was thought that *ars* elements conferred three properties on plasmids carrying them: (1) high-frequency transformation, (2) high copy number, and (3) moderate mitotic stability. It now seems that this view is oversimplified, and that the three properties can be separated in some cases. This issue, and that of whether *ars* sequences are real chromosomal DNA replication origins, is discussed critically by Williamson (1985).

In *S. pombe* the situation is somewhat more complex (Maundrell *et al.*, 1985; Wright *et al.*, 1986). Transformants of the types found in *Sacch. cerevisiae* are also observed in *S. pombe*, and the properties of both plasmids and transformants are analogous. However, plasmids lacking both an *ars* sequence and homology to the genome are capable of transforming *S. pombe* at reasonably high frequency (Maundrell *et al.*, 1985). Analysis of these transformants revealed that nonhomologous recombination between the introduced plasmid and the genome had resulted in the acquisition of genomic sequences by the plasmid. In at least some cases the resulting plasmid contained an *ars* element, as judged by the ability of the recovered plasmid to retransform *S. pombe* (Wright *et al.*, 1986).

Further analysis of several *ars* elements has established a consensus sequence of 11 bases (Maundrell *et al.*, 1986). An unusual *ars* element has been reported whose activity is not associated with the short consensus

sequence. Rather, the activity is delocalized along the 1.8-kb fragment, and the consensus-like sequence can be deleted without loss of *ars* function (Johnston and Barker, 1987).

V. G_2 PHASE AND CONTROL OVER MITOSIS

The G_2 phase in *S. pombe* under "normal" conditions, that is, wild-type cells growing at moderate to high growth rates, is the longest cell cycle phase, occupying about three-quarters of the cycle. There is a major control point at the end of G_2 regulating entry into mitosis, and the mitotic control of *S. pombe* is one of the best understood cell cycle control systems among eukaryotes. Three genes identified originally by mutation play major roles in the control, and they are summarized first in Section V,A. Observations based on classical genetics are followed by description of the cloning and molecular analysis of the genes (Section V,B). Interactions among the major elements are discussed in Section V,C. Other elements concerned with the mitotic control are considered in Section V,D. In Fig. 1 a tentative model is presented for the mechanism of mitotic control, which attempts to include the majority of elements discussed in the text.

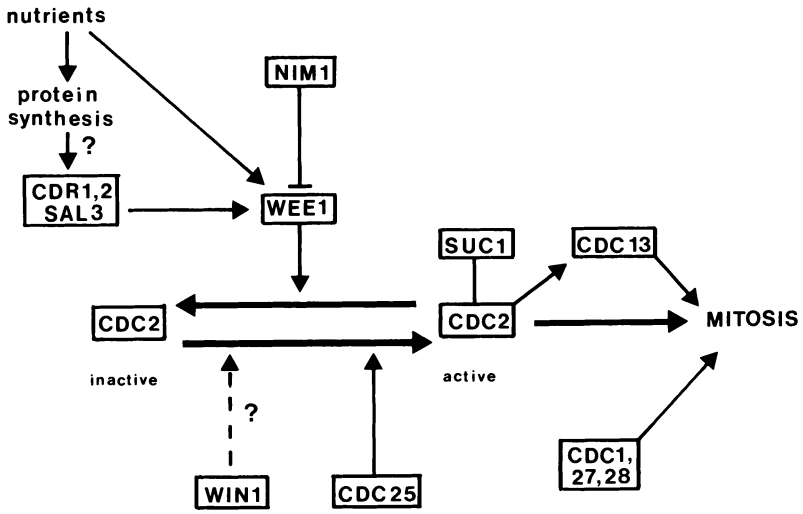


Fig. 1. Elements of mitotic control. Boxed symbols represent the products of the respective genes.

> There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. (Adams, 1980a.)

A. Major Elements of Mitotic Control

Evidence for the existence of a major cell cycle control point in G_2 originated in the isolation by Nurse (1975) of a cell size mutant, originally designated *cdc9-50* but subsequently renamed *wee1-50*. This mutant is temperature sensitive, dividing at nearly the wild-type size at 25°C but at only half that size at 35°C. The doubling time at each temperature is close to that of wild-type cells. By shifting growing cultures between the two temperatures, the product of the *wee1* gene was shown to act shortly before mitosis. Thus, cells growing at 25°C (large cell conditions) shifted to 35°C (small cell conditions) exhibited a transient acceleration in their rate of entry into mitosis, showing a shortening of G_2. The reverse shift produced a transient delay to mitosis owing to a lengthened G_2 (Nurse, 1975; Thuriaux *et al.*, 1978).

A different way of altering cell division size was provided by varying the composition of the growth medium: poorer media reduced cell size. Shifts between different media led to essentially the same conclusion as the temperature shifts with *wee1-50*, in that mitosis was delayed during the transition between poor and richer medium while a transient acceleration was observed during shifts in the opposite direction (Fantes and Nurse, 1977). Total deprivation of a nitrogen source causes an initial stimulation of mitosis and cell division, showing strikingly how the normal balance between growth and division can be disturbed (Fantes, 1984b). The kinetic similarities between *wee1-50* temperature shifts and nutritional shifts with the wild-type suggested that the same underlying mechanism might be responsible for both phenomena. Consistent with this, *wee1* cells do not respond to nutritional shift (Fantes and Nurse, 1978), suggesting that nutritional status is monitored intracellularly through the *wee1* product.

Further small cell or *wee* mutants were identified by direct cell size selection (Thuriaux *et al.*, 1978; Nurse and Thuriaux, 1980). The majority mapped to the *wee1* locus, and most were not temperature conditional but manifested *wee* phenotype at any temperature. The frequency at which *wee1* mutations were obtained, the existence of a nonsense suppressible mutation, and the dominance relationships led to the conclusions that *wee1* mutants resulted from loss of function and that the wild-type product therefore acted as a regulatory inhibitor of mitosis (Nurse and Thuriaux, 1980).

One mutant, originally designated *wee2-1*, was shown to map very close to or within the *cdc2* locus. The *cdc2* gene had previously been identified as required in late G_2 or early mitosis (Nurse *et al.*, 1976). Fine-structure genetic mapping of *wee2-1* showed it to map within the region spanned by the various *cdc2* mutant alleles, strongly suggesting that *wee2-1* was in fact a missense mutation within the *cdc2* coding region, and *wee2-1* was renamed *cdc2-1w*. Detailed examination of a number of mutant *cdc2* alleles showed that cell division size during growth at the permissive temperature was altered in many (Nurse and Thuriaux, 1980). Two showed partial *wee* phenotype at 25°C and a straightforward mitotic defect at 35°C, confirming that a single mutation within the protein coding region could cause *wee* phenotype. The dominance relationships of the various *cdc2* alleles showed that loss of function led to *cdc* phenotype, while *cdc2-1w* was likely due to an alteration or gain in activity. According to these observations, the wild-type *cdc2* product would be required for entry into mitosis and would in some way be rate-limiting for this key cycle event (Nurse and Thuriaux, 1980).

The involvement of a third control element, *cdc25*, was proposed on the basis of genetic interaction between *wee* and *cdc25* mutants. *cdc25* mutants are typical *cdc* mutants with transition points shortly before mitosis: when combined with *wee1* mutants the *wee* phenotype is epistatic and double *wee1 cdc25* mutant strains are *wee* rather than *cdc* in phenotype (Fantes, 1979). *cdc2-1w* weakly suppressed *cdc25* phenotype in contrast to the strong suppression conferred by *wee1* mutations.

The interactions between *cdc25* and *wee* mutations were used as a means of identifying new cell size mutations by obtaining and analyzing extragenic suppressors of *cdc25* that allowed growth at 35°C. The analysis of some 700 strains showed that more than one-half were *wee1* mutations with phenotypes similar to previously isolated *wee1* mutants. In addition, a few *wee1* mutants with partial *wee* phenotype and two new *cdc2w* alleles were obtained (Fantes, 1981). One of the latter, *cdc2-2w* (= *wee2-2*), was very similar to *cdc2-1w* both in size and in strength of *cdc25* suppression, and it has recently been shown to have the same amino acid substitution as *cdc2-1w* (S. MacNeill and P. Nurse, personal communication). Another new allele, *cdc2-3w* (= *wee2-3*) is different, though: the cell size it confers is similar to *cdc2-1w*, but it is a much stronger suppressor of *cdc25*, leading to the conclusion that the *cdc2-3w* product is qualitatively rather than merely quantitatively different from that of *cdc2-1w* (Fantes, 1981). Several models were proposed on the basis of these observations to explain the interactions between *cdc25* and *wee1* and *cdc2w* mutants as well as the roles the wild-type genes played in the control (Fantes, 1979, 1981). One firm conclusion was the involvement of *cdc25* in the mitotic

control, but no unambiguous interpretation of the exact role of any gene was possible. Further progress had to await the cloning of the genes.

B. Molecular Properties of Control Elements

Classical genetic analysis of the mitotic control showed that the products of *cdc2* and *wee1* were in some way rate-limiting for entry into mitosis, while the evidence for *cdc25* was less direct. A property expected of a rate-limiting element in such a control is that the final phenotype (cell size at mitosis in this case) should be sensitive to the level of functional gene product present. Classical genetics allows this question to be posed in a very limited way, by the use of diploid strains heterozygous for mutant alleles. Indeed, Nurse and Thuriaux (1980) used the dominance relations of *cdc2, cdc2w,* and *wee1* alleles to suggest that *cdc2* and *wee1* had positive and negative roles, respectively, in the mitotic control (Fig. 1).

Further analysis of the control system required the cloning and analysis of the main genetic elements, *cdc2, cdc25,* and *wee1*. The ability to clone these genes and reintroduce them into *S. pombe* has allowed far more extensive sorts of analysis to be done. The effect of overproducing the gene products on multicopy plasmids and by the use of strong promoters, and of deleting the chromosomal copy by gene transplacement, has substantiated predictions based on classical genetics, and has allowed a deeper insight into the system. In addition, determination of the DNA sequence of the genes has allowed predictions to be made about the possible functions of the gene products and has opened the way to a full biochemical analysis. In the following, the three major genes in the control are first discussed individually. Then the interactions between the genes are considered, and in Section V,D the roles of other genetic elements are introduced.

1. cdc2

The cloning and some properties of the *cdc2* gene have already been discussed in Section III. To summarize, the gene encodes a transcript of 1.6 kb and a protein of 34 kDa with protein kinase activity. The gene product is phosphorylated during exponential growth, with no evidence of changes in phosphorylation pattern during the cell cycle (Simanis and Nurse, 1986).

The observation that the *cdc2* gene is rate limiting for mitosis and a major determinant of cell size at mitosis suggested that cell size might be a

sensitive function of *cdc2* transcript level. This was tested by the introduction of multiple copies of the gene into *S. pombe;* the level of transcript was increased some 30-fold, but no effect on cell size was observed (Durkacz *et al.*, 1986). Conversely, the level of *cdc2* transcript was only slightly increased in *cdc2w* or *wee1* mutants compared with a wild-type control. It is clear from these observations that there is no direct relationship between the level of *cdc2* transcript, and cell size. It may be that the slight increase in transcript level in *cdc2w* and *wee1* is a consequence of reduced cell size; at any rate reduced size is not a consequence of high transcript level.

The *cdc2* transcript level did not change during the cell cycle in synchronous culture, nor did arresting the cell cycle at the G_2 *cdc2* block have any effect (Durkacz *et al.*, 1986). This shows that any cell cycle-related changes in *cdc2* transcription are unlikely to be involved in the mitotic control.

All the above results show that the regulatory effect of the *cdc2* product on entry into mitosis must be mediated at a posttranscriptional level. Obvious possibilities are that the level of the *cdc2* p34 protein, or the extent or type of p34 phosphorylation, might be critical, but again the available evidence is negative. Simanis and Nurse (1986) showed there to be no cycle-related changes in either p34 level or level of gross phosphorylation in synchronous culture. Two possibilities remain: one is that the level of p34 protein kinase activity might be critical, in which case changes in activity during the cell cycle or increased activity in small cells (or both) might be expected. No measurements of p34 protein kinase activity in synchronous culture have been reported, nor has the p34 kinase activity of *wee1* or *cdc2w* cells. A second possibility is that the situation is more complex, and that it is the interaction between p34 and regulatory elements or substrates which directly regulates entry into mitosis. So far one potential regulator and one potential substrate have been proposed; these are discussed in Section V,D,1.

2. cdc25

The *cdc25* gene was cloned by complementation of *cdc25-22,* as was another sequence named *nim1* that suppresses *cdc25* mutations when present in multiple copies (Russell and Nurse, 1986, 1987b). The DNA sequence of *cdc25* was determined, but its predicted translation product of 67 kDa showed no homology with any sequence in databases and so was not informative about its possible biochemical function (Russell and Nurse, 1986). A more exciting set of observations were made by increasing the expression of the $cdc25^+$ gene in *S. pombe*. Initially this was done

by using a multicopy plasmid, when many of the resulting cells showed partial *wee* phenotype. This supported the previous indirect genetic evidence that the *cdc25* product was directly involved in mitotic control. The effect was investigated systematically by examination of strains with different numbers of copies of *cdc25*$^+$ integrated in the genome. As the number of copies and level of expression increased, the cell division size decreased, showing the *cdc25* gene product to be a rate-limiting element of mitotic control. Massive overexpression of *cdc25*$^+$ from the ADH promoter led to fully *wee* phenotype, with a mean cell division size at least as small as that of the smallest *wee1* strains (Russell and Nurse, 1986).

3. wee1

The uniform phenotype and other properties of *wee1* mutants suggested that they were likely due to complete loss of gene function (Nurse and Thuriaux, 1980). The lack of lethality of *wee1* mutations made it unlikely that screening for the *wee1*$^+$ gene by complementation would be successful, since no direct growth selection was available. Two attempts were made to use the known *wee1–cdc25* interaction: the idea was that a *wee1 cdc25* strain in which the *wee1* defect was restored by a plasmid-borne copy of the wild-type gene would become *cdc*$^-$, and that such transformants might be distinguishable by growth tests alone. Ultimately this procedure was not successful, but a chromosomal mutation *win1.1* that reversed the suppression of *cdc25* by *wee1* was identified (Ogden and Fantes, 1986). In a parallel attempt, a plasmid that reversed the suppression was obtained, but this turned out not to be the authentic *wee1* gene (R. Booher and D. Beach, personal communication).

A direct route to the cloning of *wee1* followed the observation (Russell and Nurse, 1986) that the combination of a *wee1* mutation and massive overexpression of *cdc25*$^+$ was lethal, as discussed in greater detail below (Section V,C,1). A strain carrying the temperature-sensitive *wee1-50* allele and with *cdc25*$^+$ strongly overexpressed was phenotypically *wee* at 25°C but incapable of colony formation at 35°C. This strain was transformed and transformants able to grow at 35°C isolated. From these transformants were obtained two plasmids carrying overlapping inserts, which directed integration to a site very close to the *wee1* locus, confirming that the cloned sequences did indeed carry *wee1*$^+$ (Russell and Nurse, 1987a).

As expected of an inhibitor of mitosis, increased expression of the *wee1*$^+$ gene by integration of one or more extra copies delays mitosis so that cell size at division was increased. The greater the number of integrated copies of *wee1*$^+$, the larger the cells. Furthermore, very strong

expression of the temperature-sensitive allele *wee1-50* caused cell cycle arrest at 25°C, a temperature at which the *wee1* allele is functional (Russell and Nurse, 1987a). Deletion of the *wee1* gene function by gene transplacement resulted in viable cells with *wee* phenotype very similar to that of known mutants.

The predicted product of the *wee1* gene is a protein originally reported to have a molecular weight of 112,000, now amended to 96,000 (P. Nurse, personal communication). Part of its sequence is homoloous to protein kinases. In particular, there are regions which are very similar to the consensus ATP binding and phosphorylation sites of protein kinases (Russell and Nurse, 1987a). This makes it very likely that the *wee1* gene product has protein kinase activity, although no test of this prediction has yet been reported.

C. Interactions between Major Control Elements

The timing of mitosis and cell size at that cycle stage are intimately related. Mitosis in a *wee* mutant can be regarded as advanced relative to a wild-type, in the sense that the *wee* mutant undergoes mitosis at a size corresponding to a much earlier stage in the wild-type cell cycle. Conversely, a cell that undergoes mitosis and division at a larger size than normal is in some sense delayed for mitosis: ultimately an indefinite delay becomes a complete failure to divide. The observations discussed above show that the $wee1^+$ and $cdc25^+$ genes are quantitatively rate-limiting in the control over entry into mitosis, since changes in the amount of the gene products directly and substantially affect the timing of mitosis. The *cdc2* gene is rate limiting in a different way: changes in level of expression have little or no effect, but point mutants can be either advanced or delayed in entry into mitosis.

A priori it would be expected that the three genes or their products must interact in the control mechanism, and specific interactions have already been mentioned. The following describes the investigation of interactions between the genes and what this tells us about the circuitry of the control mechanism itself (Fig. 1).

1. wee1 and cdc25 Have Opposing Effects

Loss of *wee1* function results in premature mitosis and small cell size, though the cells are still viable; loss of $cdc25^+$ function results in the inability to undergo mitosis. Double mutants carrying both *wee1* and con-

ditional *cdc25* mutations are phenotypically *wee* at the restrictive temperature for *cdc25*, showing that the effect of *wee1* is in some sense stronger. Double mutants of different *cdc* alleles with *wee1* are not always fully *wee* in phenotype, however, suggesting that *wee1* defects do not fully override the effect of *cdc25* (Fantes, 1979, 1981). In these early studies it was never certain that the *cdc25* alleles were fully inactivated, and no clear interpretation of the interactions with *wee1* was possible. By manipulation of the cloned *cdc25* gene, Russell and Nurse (1986) showed that *wee1* mutations could suppress the otherwise lethal defect of a partial deletion of the *cdc25* gene. This ruled out one possible interpretation of the genetic interaction, that a defective *wee1* allele derepresses residual activity of the conditional *cdc25* alleles (Fantes, 1979). This result also showed that *cdc25*$^+$ function is not always essential for viability, but only in the presence of a functional *wee1*$^+$ gene. Rather, the function of *cdc25*$^+$ might be to counteract the otherwise indefinite delay to mitosis were the *wee1*$^+$ product allowed to act unhindered (Russell and Nurse, 1986).

The phenotype resulting from overexpression of *cdc25*$^+$ is very similar to that of loss of *wee1* function. Russell and Nurse (1986) showed that loss of *wee1* function simultaneously with overexpression of *cdc25*$^+$ led to the appearance of highly aberrant nuclear behavior at the expected time of mitosis, followed by high cell lethality. This phenomenon, termed mitotic catastrophe, was ascribed to cells attempting to undergo mitosis at too small a size or at too early a stage in the cell cycle, although the precise reason for the failure of mitosis remains uncertain. The interaction suggests strongly that the *wee1*$^+$ and *cdc25*$^+$ products act independently and in opposite directions on the same system (Fig. 1). The *wee1* product is likely to be a protein kinase, and this activity may well be the one required for inhibiting mitosis. One speculative possibility suggested by Russell and Nurse (1987a) is that *cdc25* encodes a phosphoprotein phosphatase which reverses the effect of *wee1*.

The system on which the *wee1*$^+$ and *cdc25*$^+$ products act must be one required in a rate-limiting step for entry into mitosis: this would explain their opposing and regulatory effects. If the two gene products act on the same protein substrate then an obvious candidate for this would be the p34 *cdc2* product, which is known to be a phosphoprotein, to have protein kinase activity, and to be rate limiting for mitosis. Genetic interactions between *cdc2*, and *wee1* and *cdc25*, are considered below.

2. *Two Types of cdc2w Mutation Suggest Dual Control of p34*

One clear mechanism by which the *cdc2* p34 protein might be affected by the *wee1* or *cdc25* products is at the level of p34 phosphorylation. No

changes in gross phosphorylation have been detected over a range of conditions (see Section III). However, changes in the precise pattern of phosphorylation might occur at the G_1/S or G_2/M transitions, and the effect of *wee1* and *cdc25* mutations on phosphorylation might provide many answers about the mechanism of mitotic control. There is little pertinent information, and no firm conclusions can be drawn about the biochemical basis of the control (but see below and Section V,D,1). An alternative approach which has proved informative has been to study interactions between *cdc2* mutations and various types of genetic change in the *wee1* and *cdc25* genes.

The first suggestion that there might be interaction between the *cdc25* and *cdc2* protein products came from the difference in behavior among *cdc2w* alleles. *cdc2-1w* (and the allele *cdc2-2w* which has an identical amino acid change) are both poor suppressors of *cdc25-22*, while *cdc2-3w* is a strong suppressor. A similar pattern is seen with a null as well as with a missense *cdc25* mutation, only here *cdc2-1w* is completely ineffective as a suppressor (Russell and Nurse, 1987a). *cdc2-3w* suppresses a null *cdc25* mutation, and cells of the double mutant are about twice the size of the *cdc2-3w* parent and 30% larger than wild type. Since all three *cdc2w* alleles reduce cell size by about the same amount, the different patterns of interaction with *cdc25* indicate that the function of the $cdc2^+$ p34 product which controls the time of mitosis and cell size is separate from that which is responsive to *cdc25*. The product of the *cdc2-1w* (and *cdc2-2w*) allele, like the wild type, requires $cdc25^+$ product in order to initiate mitosis. Hence this allele in combination with a *cdc25* defect confers *cdc* phenotype. The *cdc2-3w* allele can function independently of $cdc25^+$ function.

Interactions between the *cdc2w* alleles and *wee1* mutations are also allele specific for the *cdc2* allele. The combination *wee1-50 cdc2-3w* is lethal at 35°C (restrictive for *wee1-50*), and the behavior of the cells on shift to 35°C is very similar to that of a *wee1-50* mutant with $cdc25^+$ overexpressed, that is, they suffer mitotic catastrophe (Russell and Nurse, 1987a). Loss of *wee1* function causes an advancement of mitosis in *cdc2-3w* and $cdc2^+$ genetic backgrounds, but in *cdc2-3w* the advancement is lethal. This leads to the conclusion that the *cdc2-3w* product is sensitive to the *wee1* product as is the $cdc2^+$ p34. Consistent with this, overexpression of $wee1^+$ in a *cdc2-3w* mutant leads to an increase in division size, as it does in a $cdc2^+$ strain. In contrast, a double mutant of *wee1-50* with *cdc2-1w* is viable at 35°C and is phenotypically very similar to both parents (Thuriaux *et al.*, 1978). There is only a slight change in size of the *cdc2-1w* mutant when $wee1^+$ is moderately overexpressed (Russell and Nurse, 1987a). This can be interpreted as insensitivity of the product of *cdc2-1w* to inhibition by *wee1* function.

These rather complex data and interpretations are summarized in one possible model in Fig. 1. There are several comments which need to be made.

1. The function of the *cdc2* gene p34 product in promoting mitosis (presumably its protein kinase activity) is normally sensitive to inhibition by the *wee1* gene product. Since *cdc2* acts in both G_1 and in G_2, there must be differences either in the state of p34 or in the availability of its substrates during the prereplicative and premitotic periods. The G_2 activity is affected by mutations in *wee1* and *cdc25* and by other factors discussed below.

2. The relative amount of *cdc2* transcript is unaffected by *wee1* mutations (Durkacz *et al.*, 1986), and so the effect of *wee1* must be at the level of the *cdc2* protein product. The phenotypic differences observed with different *cdc2w* alleles in a *wee1* background show that the *cdc2* product is itself part of the interaction. A likely explanation is that the normal p34 has a site which is somehow affected by *wee1* function. The p34 protein encoded by the *cdc2-1w* allele has reduced sensitivity at this site, and its inability to be inhibited causes an increase in p34 activity and advanced mitosis. The p34 encoded by *cdc2-3w* retains the site, and loss of *wee1* function advances mitosis to the point of mitotic catastrophe and lethality.

3. The $cdc2^+$ product has a site which is somehow sensitive to *cdc25* gene function. For the wild-type and *cdc2-1w* alleles, activation by $cdc25^+$ is essential for function, so that a null *cdc25* allele prevents entry into mitosis. The weak suppression of missense *cdc25* mutations by *cdc2-1w* can be explained by residual *cdc25* activity. If the product of the *cdc2-3w* allele can function in the absence of $cdc25^+$ stimulation, the suppression of *cdc25* by *cdc2-3w* can be explained, but why should *cdc2-3w* confer small cell phenotype on $cdc25^+$ cells? It is likely that *cdc25* function is quantitatively limiting for *cdc2* activity under normal circumstances. Relief from the requirement for $cdc25^+$ in *cdc2-3w* would in that case allow mitosis to occur prematurely.

4. There is no evidence for physical interaction between the *cdc2* product and the products of *wee1* or *cdc25*. The genetic interactions are specific to the *cdc2* allele, but allele specificity for *wee1* and *cdc25* alleles has not been demonstrated. This and other evidence suggest that the functions of the latter two genes are quantitatively but not qualitatively important. This does not exclude direct interaction between the gene products, but the possibility that the *wee1* and *cdc25* products affect *cdc2* activity via intermediate, perhaps unidentified, molecules remains open.

5. Although an appealing possibility, there is no evidence that phosphorylation of the *cdc2* p34 protein plays any role in modulating its activity either in G_1 or G_2. Indeed, there is evidence (Potashkin and Beach, 1988) that *wee1*$^+$ function has no effect on the phosphorylation pattern of p34.

6. The three components of the mitotic control so far described are either quantitatively or qualitatively rate limiting for mitosis. There are certainly more genetic elements than this: for instance, *nim1* (discussed in Section V,D,2,a) has been identified as one. Whether there are any more is an open question, and a complete understanding of how the major elements cooperate to produce a functional control may only be possible when the identities and roles of others have been investigated.

D. Associated Elements of Mitotic Control

A considerable number of genetic elements have been identified which may interact with the mitotic control in some way. Some of them are mutants or DNA fragments which were isolated either as the result of deliberate screening for particular *cdc* or *wee* phenotypes or fortuitously in the course of screening for something else. With others the connection to the cell cycle was (and in some cases, still is) by no means obvious, while yet other genes have been known for a considerable time but were not thought to be involved in the control. My approach here is to group the genes into those whose interaction with *cdc2* is most obvious and those that appear to interact with the presumably upstream *wee1* and *cdc25* functions.

1. cdc2 Interacting Elements

a. *suc1*. The *suc1* gene was first identified as a cloned DNA fragment able to suppress a *cdc2 ts* allele when present on a multicopy plasmid (Hayles *et al.*, 1986a). The chromosomal *suc1* locus is unlinked to the *cdc2* locus, and there is no cross-hybridization between the two genes. Subsequently, two mutations that map at the *suc1* chromosomal locus were identified among a collection of revertant strains of *cdc2 ts* mutants (Hayles *et al.*, 1986b). The mutations were dominant, suggesting a specific alteration rather than loss of function. Deletion mutants of *suc1* are lethal, and the phenotype of *suc1* spores suggests a defect in cell cycle progress (Hayles *et al.*, 1986b; Hindley *et al.*, 1987) and in cell growth (Hayles *et al.*, 1986b). Surprisingly, overexpression of *suc1*$^+$ by the intro-

duction of a single extra copy in a *cdc2*$^+$ strain leads to a partial cell cycle defect in that the cells are substantially larger than normal. Strong overexpression leads to even larger cells (Hayles *et al.*, 1986b; Hindley *et al.*, 1987). The cellular growth rate is stated to be much reduced in one report (Hayles *et al.*, 1986b) but only minimally affected in another (Hindley *et al.*, 1987). The reason for this apparent discrepancy is unclear.

The *suc1* gene product itself is likely to affect G_2 more strongly than G_1, since no G_1 accumulation is observed in cells with altered *suc1* activity (Hayles *et al.*, 1986b) though stronger evidence such as the use of a temperature conditional lethal *suc1* allele would be desirable. In *cdc2* mutants suppressed by *suc1*, both the G_1 and G_2 defects must be suppressed, and *suc1* must therefore interact with *cdc2* during both cycle phases. *suc1* mutant strains, and strains in which *suc1*$^+$ is overexpressed, are defective in the second meiotic division, which in many respects resembles a mitotic division. Many of the asci formed contain only two spores (Hayles *et al.*, 1986b).

Suppression of *cdc2 ts* defects by *suc1* mutations or by overexpressing the *suc1*$^+$ gene is highly allele specific, the same *cdc2* alleles being most readily suppressed by *suc1* mutation or overexpression (Hayles *et al.*, 1986a,b). This shows that *suc1* does not suppress *cdc2* mutations by a bypass mechanism: if it did, all alleles should be suppressed. Strains carrying *suc1* mutations show the normal level of *cdc2* transcript, so that *suc1* is unlikely to interact with *cdc2* by controlling its expression (Hayles *et al.*, 1986b). The most likely conclusion is that interaction between *suc1* and *cdc2* occurs by direct physical interaction between the protein products. Experiments supporting this possibility are described below.

The *suc1* gene encodes two messenger RNAs of 0.8–1.2 kb, which are produced from the primary transcript after splicing out of two introns. The predicted translation product of 13 kDa showed no close similarity to any other by comparison with database sequences (Hindley *et al.*, 1987). Manipulation of the cloned *suc1* gene allowed high-level production in *E. coli* of the encoded protein, which was then used to raise an antiserum. The antiserum was used in immune precipitation assays to demonstrate the presence in *S. pombe* lysates of a 13-kDa protein, which was shown to be the authentic *suc1*$^+$ product (Brizuela *et al.*, 1987). The levels of *suc1* transcripts and of p13 do not vary during the cell cycle (Hayles *et al.*, 1986b; Brizuela *et al.*, 1987).

Direct association of the *suc1*$^+$ and *cdc2*$^+$ gene products *in vitro* was demonstrated by Brizuela *et al.* (1987). Immunoaffinity columns prepared with anti-p13 serum were used to separate from *S. pombe* lysates p13 and other proteins closely associated with it. In addition to p13, the *cdc2* p34

product was retained by the column. This appears not to be due to cross-reaction of the antiserum with p34, since the antiserum does not recognize p34 alone, but rather to association between the p13 and p34 species (Brizuela *et al.*, 1987). It is not certain from this analysis alone whether the complex exists *in vivo;* however, a different sort of experiment supports this idea. Cell lysates were prepared from strains carrying one of four mutant *cdc2* alleles or the *cdc2*$^+$ allele. Immune precipitates prepared using the anti-p13 antibody were analyzed for their p34 content. Control experiments in which the immune precipitation step was omitted showed the total level of p13 and p34 to be very similar in all strains.

Two of the *cdc2* alleles used are strongly suppressed by overexpression of *suc1*$^+$ (Hayles *et al.*, 1986a,b; Brizuela *et al.*, 1987). Immunoprecipitates of cell extracts from these strains contained either much reduced or undetectable levels of *cdc2* p34. Overexpression of *suc1*$^+$ weakly suppresses the other two alleles examined (Hayles *et al.*, 1986b), rather than completely failing to suppress them (Brizuela *et al.*, 1987). The p34 products of these alleles precipitated with anti-p13 antibody essentially as the wild-type protein (Brizuela *et al.*, 1987). Since the products of different *cdc2* alleles behave differently in the coprecipitation assay, it seems likely that there is direct physical interaction between the *suc1*$^+$ and *cdc2*$^+$ products, which is altered in some *cdc2* mutants. However, there may not be as close a correlation between allele suppressibility and the strength of the *in vitro* interaction as suggested by Brizuela *et al.* (1987), as all four *cdc2* alleles tested are suppressed by *suc1* (Hayles *et al.*, 1986b).

Only about 5% of the *cdc2* p34 molecules appear to be complexed with the *suc1* p13 protein after immunoaffinity purification. Again, it is not certain whether this fraction is representative of the fraction complexed in intact cells. The interaction between the two proteins is thought to be unstable, and the possibility of dissociation of the complex during the various manipulations cannot be excluded.

The protein kinase activity of the p34–p13 complex was examined by testing the phosphorylation of endogenous *S. pombe* polypeptides coprecipitated during immune precipitation with anti-p13 antiserum. After incubation with radiolabeled ATP, the *cdc2*$^+$ lysate contained a range of labeled bands, indicating the presence of protein kinase activity in the immune precipitate. This was taken to imply (Brizuela *et al.*, 1987) that the p13–p34 complex has protein kinase activity, but the evidence is not compelling. It is not certain that all the p34 in the kinase assay is still complexed with p13: given the probable lability of the interaction, dissociation might take place, and the observed kinase activity might be due to free p34. In addition, the number of phosphorylated bands observed after

incubation of the immune precipitate with ATP opens the possibility that the interaction between p13 and p34 is not direct, but rather mediated by one of the other proteins which coprecipitate.

b. *cdc13.* The *cdc13* gene was one of the first cell cycle genes to be identified (Nurse *et al.*, 1976). The archetypal mutant *cdc13-117* is unable to complete mitosis at 35°C. Its terminal phenotype is striking in two ways: the chromosomes visibly condense after arrest, and septation, which is prevented by many types of mitotic block, continues (Nasmyth and Nurse, 1981). These characteristics are different from those of the majority of mutants known at the time to be defective in mitosis. The original interpretation was that the *cdc13* arrest point was at a later stage in mitosis than the other mutants. Functional dependency analysis, using a combination of reciprocal shift experiments and double mutant phenotypes, supported the hypothesis that the *cdc13* step was dependent on earlier steps in mitosis (Fantes, 1982; see below, Section VI,A).

New insight into the role of *cdc13* came by a circuitous genetic route (Booher and Beach, 1987). A new *cdc2* allele, *cdc2-59* was isolated from *cdc2-3w* and conferred a leaky cold-sensitive *cdc* phenotype at 25°C. The *cdc2-59* allele differs from $cdc2^+$ in two ways: it contains the original *cdc2-3w* mutation and the additional mutation conferring sensitivity to cold. Among revertant strains derived from *cdc2-59* able to grow at 25°C, six showed heat-sensitive *cdc* phenotype owing to the presence of an extragenic mutation. One of these defined a new *cdc13* allele, *cdc13-c1*. The double mutant *cdc2-59 cdc13-c1* shows no cold-sensitive phenotype, indicating that the *cdc13* mutation suppresses the *cdc2-59* phenotype. The double mutant is strongly sensitive to heat, showing full arrest at 33°C. In contrast the *cdc13-c1* mutation alone is only weakly heat sensitive even at 37°C. These particular effects are restricted to the *cdc2-59* and *cdc13-c1* alleles, as expected if there were direct physical interaction between the *cdc2* and *cdc13* gene products. *cdc13-c1* does not suppress a *cdc2* null mutation, consistent with this possibility. Other allele-specific effects supporting it were also observed. The combination of *cdc13-117* with *cdc2-59* is lethal at any temperature, and surprisingly this was also true for the *cdc2-3w cdc13-117* combination, showing the *cdc2-3w* component of *cdc2-59* to be the critical factor. In contrast *cdc2-1w* did not interact with *cdc13-117* in any unexpected way: the double mutant is viable at 25°C and *cdc* at 35°C (Booher and Beach, 1987). *cdc2-3w* also enhanced the heat sensitivity of *cdc13-c1* in the same way as *cdc2-59*. Different aspects of the interaction are shown by two observations. First, multicopy expression of $cdc2^+$ suppresses both *cdc13-c1* and *cdc13-117*. Second, the mitotic catastrophe phenotype of *cdc2-3w wee1-50* is suppressed by the presence of *cdc13-c1*.

These experiments (Booher and Beach, 1987) suggest strongly that the *cdc2*$^+$ and *cdc13*$^+$ products interact physically in mitosis (Fig. 1), and they are apparently inconsistent with the previous supposition that the cell cycle step controlled by *cdc13*$^+$ was later in mitosis than that controlled by *cdc2*$^+$. It is hard to reconcile the older data with the new, particularly as the reciprocal shift data unambiguously place the *cdc2* step before, and the *cdc13* step interdependent with, the benomyl-sensitive step (Fantes, 1982; see Section VI,A). A possible explanation is discussed below (Section VI,E).

The *cdc2*$^+$ product is required in G_1 and in G_2, presumably acting on different substrates at different times. A strong possibility is that the *cdc13*$^+$ product is a substrate of the p34 *cdc2*$^+$ product during G_2 phase, and the recent cloning of the gene (R. Booher and D. Beach, personal communication, 1987) should allow this question to be addressed. A putative substrate for the *Sacch. cerevisiae CDC28* homolog of *cdc2* has recently been identified (Mendenhall *et al.*, 1987), and its relationship to *cdc13* will be of great interest. *cdc13* does not appear to be involved in G_1 (Nurse *et al.*, 1976; Booher and Beach, 1987) and may therefore be a specific G_2 substrate of p34. The new *cdc2* allele *cdc2-59* may only be defective in its G_2 function (Booher and Beach, 1987), comparable to the *CDC28.1N* mutation in the homologous *Sacch. cerevisiae* gene *CDC28* (Piggott *et al.*, 1982).

c. A Universal Control? *Schizosaccharomyces pombe* is atypical among at least the best studied eukaryotes in having its major cycle control point at the G_2/M boundary rather than the G_1/S control of mammalian cells or budding yeast. The suspicion that studying the details of the *S. pombe* cycle might be of only specialist interest has been allayed by two recent reports describing the identification of elements highly homologous to *cdc2* and *suc1* in human cells. Lee and Nurse (1987) isolated a cDNA clone from a human gene library that complements the *cdc2* defect in *S. pombe*. The DNA sequence of the human gene predicts a translation product highly homologous to the *cdc2* p34. Part of the amino acid sequence completely conserved between the *Sacch. cerevisiae CDC28* and *S. pombe cdc2*$^+$ products is also identical in the predicted translation product of the human gene. Furthermore, the human gene fully complements both the G_1 and the G_2 defects of a *cdc2* null allele in *S. pombe*.

A protein which is almost certainly the product of the complementing gene has also been identified in human cells. Antibodies specific for the totally conserved region of the two yeast proteins detect a human protein of very similar molecular weight. While the role of the protein in human cells is a matter for speculation at present, it may well be involved in the G_1 control or "R" point (Pardee *et al.*, 1978). Other evidence supports the

existence of a G_2 control in human and other vertebrate cells, mediated by maturation promotion factor (MPF) (Kirschner *et al.*, 1985), and the *cdc2* homolog may be involved here also.

The human homolog of the *cdc2* p34 protein has been independently identified by Draetta and associates (1987) using monoclonal antibodies. The presence of protein kinase activity in immune precipitates was demonstrated, and the peptide maps of the human, *Sacch. cerevisiae*, and *S. pombe* proteins were found to be very similar. A human homolog of the *suc1* p13 protein was also identified by the reaction of antibodies specific for the *S. pombe suc1* p13. Immune precipitation of p13 from human cell lysates coprecipitated human p34, behavior analogous to the *S. pombe* proteins.

2. wee1 and cdc25 Interacting Elements

a. ***nim1.*** In the course of screening a plasmid library for clones able to complement *cdc25-22*, Russell and Nurse (1987b) obtained a fragment named *nim1*. The DNA fragment showed suppression of *cdc25-22* when present on a multicopy plasmid. Independently, a fragment was isolated with similar properties, which afforded weak suppression of *cdc25-22* when a single extra copy was introduced by integration (J. E. Ogden and P. A. Fantes, unpublished observations). This fragment appears to be identical to that of *nim1* (P. Young, personal communication). The *nim1*$^+$ DNA sequence predicts that its product should have protein kinase activity, but this awaits direct confirmation. More informative are the results of genetic experiments using manipulated versions of the *nim1* gene either alone or in combination with other mutations in the mitotic control (Russell and Nurse, 1987b).

Introduction of the multicopy plasmid into a *cdc*$^+$ strain results in *wee* phenotype of some of the cells, suggesting that the *nim1*$^+$ gene is a positive dosage-dependent control element in mitotic control. This was confirmed by overexpressing *nim1*$^+$ from a strong promoter, when a fully *wee* phenotype was observed. Deletion of the *nim1* gene did not affect viability, though the resulting cells were somewhat larger than normal (Russell and Nurse, 1987b).

The overexpressed *nim1*$^+$ gene suppressed *cdc25-22* and a *cdc25* null allele, showing that strong expression of *nim1*$^+$ bypasses the *cdc25* block. Since *nim1*$^+$ is predicted to encode a protein kinase, and *cdc25*$^+$ probably acts by activating the *cdc2* protein kinase, one possibility is that overexpression of *nim1*$^+$ activity might be able to bypass the *cdc2*$^+$ requirement. However, overexpression of *nim1*$^+$ does not suppress any of four *cdc2 ts* alleles, showing that *nim1*$^+$ must work through the *cdc2*$^+$ product (Russell

and Nurse, 1987b). A strong possibility is that *nim1*$^+$ works through the *wee1* gene or its product, since manipulations resulting in either no *nim1* activity or overexpression of the gene have no effect on the size of *wee1* cells, and no overadvancement of mitosis leading to mitotic catastrophe is seen in the latter case. *nim1*$^+$ is therefore likely to be a negative regulator of *wee1*$^+$ activity and to act by preventing *wee1*$^+$ from inhibiting mitosis (Fig. 1). No effect of *nim1*$^+$ overexpression or gene disruption was observed on the level of *wee1* transcript, so that the action of *nim1*$^+$ on *wee1*$^+$ must be effected by a different, posttranscriptional, mechanism (Russell and Nurse, 1987b). Once again direct interaction between the protein products, perhaps by the *nim1*$^+$ protein kinase acting on the *wee1* product, is a possibility, albeit unsupported. If this were so then an appealing idea is that the *nim1*$^+$ protein kinase might regulate the activity of the *wee1* product by phosphorylation.

b. *win1*. A mutation defining the *win1* gene was isolated fortuitously during screening transformants of a *wee1 cdc25* strain for plasmids able to reverse the suppression of *cdc25* phenotype by the *wee1* mutation. One strain was isolated which had *cdc* phenotype at 35°C (restrictive for *cdc25*) during growth on minimal medium. This phenotype was found not to be due to the plasmid harbored by the strain but rather to a chromosomal mutation designated *win1-1* (Ogden and Fantes, 1986). The *win1* locus is not linked to any known *cdc* or *wee* genetic locus. The single *win1-1* mutation increases cell size by 40% during growth on minimal medium, and a comparable increase is observed in combinations with other *cdc* mutants. *wee1* mutants are an exception: *wee1 win1* double mutant strains are very similar to single *wee1* mutants. When combined with both a *wee1* mutation and a *cdc25* mutation, *win1-1* confers a strong *cdc* phenotype, although attempts to demonstrate first cycle arrest have so far been unsuccessful. *win1-1* is recessive for the reversal of *wee1* suppression of *cdc25*, which is specific to *wee1: wee* mutants of *cdc2* do not show any strong interaction with *win1-1* and *cdc25* (Ogden and Fantes, 1986).

A possible interpretation of the role of *win1*$^+$ is that it can bypass the normal requirement for *cdc25*$^+$ by carrying out the same function (Fig. 1). In strains carrying a functional allele of *wee1* or of *cdc25*, the effect of the major control gene(s) predominates. Thus *wee1*$^+$ *cdc25* strains are phenotypically *cdc*, while *wee1 cdc25*$^+$ strains are *wee* (Section V,C,1; Fig. 1). In double mutant *wee1 cdc25* strains, the system is essentially unregulated and operates in its default position in which mitosis is stimulated. Attainment of this position might be mediated by the *win1*$^+$ product inefficiently performing the *cdc25*$^+$ function. In the absence of *wee1*$^+$, *cdc25*$^+$,

and *win1*$^+$, the balance of activities is toward the *wee1*$^+$ *cdc25* state, and mitosis is prevented. Because *win1*$^+$ is able to carry out the *cdc25*$^+$ function inefficiently, the *win1-1* mutation has only a slight effect on cell size in most genetic backgrounds: only in the absence of both *wee1*$^+$ and *cdc25*$^+$ is a strong phenotypic effect observed. The activity of *win1*$^+$ is clearly insufficient to suppress loss of *cdc25*$^+$ function.

A striking property of *win1-1* is that its phenotypic effects are dependent on the nature of the growth medium, being most noticeable on minimal medium and less so on complex media. Indeed the *cdc* phenotype of *wee1 cdc25 win1* strains is largely suppressed during growth on complex media (Ogden and Fantes, 1986), though there is great heterogeneity of phenotype with some very long cells and some close to wild-type size (E. Warbrick and P. A. Fantes, unpublished observations). Unfortunately, no simple component of the growth medium responsible for modifying the phenotype has been identified, despite a survey of a range of supplemented and unsupplemented media (E. Warbrick and P. A. Fantes, unpublished observations). It is possible that the effect of growth medium is connected with the nutritional sensitivity of the normal mitotic control, and *win1* may play a role in mediating it.

c. *cdr* Mutants. A deliberate attempt to identify genetic elements involved in the nutritional control of mitosis was made by Young and Fantes (1987). Mutants were isolated which retained large cell size on nitrogen starvation, a condition which causes accelerated division and the production of very small cells in the wild-type (Fantes, 1984b; Nurse and Thuriaux, 1977). The mutants were all slightly larger during exponential growth than wild-type cells, though the difference was less than after nitrogen deprivation. They were tested for their division response to various nutritional shifts and to nitrogen starvation. Their cell size during nitrogen limitation in chemostat culture and after nitrogen deprivation was also examined. In some cases nitrogen starvation caused arrest in G_2 (Young and Fantes, 1987) rather than the usual G_1 (Nurse and Thuriaux, 1977; Costello *et al.*, 1986). Many of the mutants showed altered behavior compared with the wild-type, but no pattern typical of the majority of mutants was found.

Genetic analysis showed that two major loci were involved, termed *cdr1* and *cdr2*, and representative alleles were investigated further (Young and Fantes, 1987). *wee1* is epistatic to *cdr1* and *cdr2* mutants, suggesting that the *cdr*$^+$ genes exert their effect through the *wee1*$^+$ gene. Alternatively, it is possible that they work through *cdc25*$^+$ in a similar manner to that proposed for *nim1* (see above). The involvement of *cdr* genes in mitotic control is supported by the interactions of mutant alleles with *cdc2* and *cdc25* mutant alleles. Double mutants of constitution *cdr*$^-$ *cdc2* were

all substantially larger than either parent at 25°C, the permissive temperature for *cdc2*. The interaction with *cdc25-22* was even stronger: the double mutants were extremely elongated even at 20°C and had much lower restrictive temperatures (Young and Fantes, 1987). Although much remains to be discovered about the *cdr* genes, it seems likely that their main function is within the nutritional part of the mitotic control (Fig. 1).

d. Allosuppressors and Mitotic Control. An entirely unexpected connection between mitotic control and the central metabolism of the cell was made when it was discovered that certain mutants isolated for their allosuppressor activity exhibit a *cdc* phenotype. Allosuppressors are mutations that do not map within a tRNA gene but enhance the nonsense suppression ability of a weak or inactive tRNA suppressor (Nurse and Thuriaux, 1984). Prototrophic mutants were selected from a strain carrying a nonsense *ade6* allele and a mutant tRNA gene whose nonsense suppressor activity had been inactivated by a second mutation within the tRNA gene itself. From a number of strains which showed temperature-sensitive prototrophy, six were identified as also showing a degree of cell elongation. In each case the ability to confer prototrophy was dependent on the presence of the original tRNA mutation. Four of the mutants mapped to a single locus, *sal3*, while another mapped to *sal2*. The *sal2* mutation also showed temperature-sensitive *cdc* phenotype, and genetic tests showed it to be allelic to *cdc25*. Further tests on both of the preexisting *cdc25* mutant alleles showed that they also had allosuppressor activity, which was reversed by *wee1* or *cdc2-1w* mutations (Nurse and Thuriaux, 1984).

The *sal3* mutants behaved in a similar way except that the *cdc* phenotype was not as pronounced (Nurse and Thuriaux, 1984). The *cdr* mutants also show weak allosuppressor activity and in this respect are very similar to *sal3* (Young and Fantes, 1984). Neither is allelic with *sal3*, however, since they map to chromosome I whereas *sal3* is on chromosome III (Nurse and Thuriaux, 1984).

It is striking that at least four genes have been identified in which mutants have two distinct phenotypes: allosuppressor activity and a pronounced effect on the cell cycle. In the case of *sal2*/*cdc25*, the cell cycle effect is unambiguously at the mitotic control. The evidence for *cdr1* and *cdr2* and for *sal3* is less specific: in each case the single mutant phenotype is cell elongation, and no temperature-sensitive alleles have been isolated that could be tested for their time of action in the cell cycle. The indirect evidence that the latter genes influence the mitotic control rather than any other part of the cell cycle is nevertheless quite strong. In all cases the cell cycle phenotype is abolished by the presence of a *wee1* mutation, and the allosuppressor activity of *sal3* mutants is also reduced (Nurse and

Thuriaux, 1984). Mutants at both *cdr* genes interact with *cdc2* and *cdc25* mutants, almost lethally in the latter case. Furthermore, the altered division responses of *cdr* mutants are consistent with the defects lying within the mitotic control (Young and Fantes, 1987).

It is likely that allosuppressor activity is due to effects on tRNA metabolism or protein synthesis. This suggests a possible mechanism by which cells might sense their growth conditions and set their critical size for mitosis accordingly (Fig. 1). As the growth conditions vary, so does the rate of growth and of protein synthesis. Some signal might be generated which is directly related to the rate of protein synthesis, perhaps at the level of the ribosome. The level of signal is monitored by the cell, perhaps through the *wee1*$^+$ gene product which is thought to mediate nutritional effects on mitosis. The interaction between the growth rate signal and the *wee1*$^+$ product modulates the critical cell size requirement for mitosis.

e. *mcs* Mutants. The lethal interactions between *cdc2-3w* and *wee1* mutations and between *cdc25*$^+$ overexpression and *wee1* have opened new routes to obtaining mutants in novel genes involved in mitotic control. Mutants able to suppress the *cdc2-3w wee1-50* lethality have been described and are the subject of current interest (Booher and Beach, 1987).

3. Transition Point Control

In addition to the sort of interactions described so far, which are manifest at the level of double mutant phenotype, more elusive interactions have been reported (Fantes, 1983). The transition or execution point of a gene is the final time in the cell cycle at which its product is required (Nurse *et al.*, 1976; Hartwell, 1974; Pringle, 1978). As noted previously (Nurse *et al.*, 1976), the transition points of *S. pombe* cell cycle mutants are clustered in the cell cycle so that most genes required for mitosis have transition points within 0.15 cycle before mitosis itself. This is so for *cdc1*, *cdc2*, *cdc13*, *cdc25*, *cdc27*, and *cdc28* and for *ben4* (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; Fantes, 1979). Mechanisms must exist that control the times of action of these mitotic gene products. In double mutants where a *wee1* mutation is combined with *cdc1* or *cdc27*, the transition point of the latter is advanced by up to half a cell cycle. The transition points of *cdc2* mutants in a *wee1* genetic background are allele specific and can be advanced by up to 0.2 cycle (Fantes, 1983). An earlier suggestion (Fantes, 1983) that loss of *wee1*$^+$ function derepresses transcription of some *cdc* genes can be rejected at least for *cdc2* and for *cdc27* since no effect on transcript level has been observed (Durkacz *et al.*, 1986; Hughes and Fantes, 1989). The effect must be mediated post-

transcriptionally, and current knowledge would suggest posttranslationally.

In support of this are some other observations (P. A. Fantes, unpublished) on the effects of nitrogen starvation on the behavior of cells carrying any of several *cdc* mutant alleles. Nitrogen starvation normally leads to a stimulation of mitosis (Fantes, 1984b; Egel and Egel-Mitani, 1974). When certain *cdc* mutants defective in mitosis are shifted to medium lacking nitrogen at the restrictive temperature, there is considerably more residual division than when the shift is done in complete minimal medium. The mutant alleles affected in this way are almost without exception those susceptible to transition point advancement by a *wee1* mutation (P. A. Fantes, unpublished observations), and the extent of residual division after shift is very similar whether the presence of a *wee1* mutation or nitrogen starvation causes the extra divisions. This is consistent with the proposal that monitoring of the nutritional status of a cell is mediated by the $wee1^+$ gene. In further support of this is the finding that in *wee1 cdc* double mutants, no further stimulation of division occurs beyond that allowed by either a *wee1* mutation or nitrogen deprivation. Lack of $wee1^+$ function mimics the effect of nutritional deprivation and no further effect occurs when nitrogen is removed.

> There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. (Adams, 1980a.) There is another theory which states that this has already happened. (Adams, 1980b.)

VI. EVENTS OF MITOSIS

There is no morphological sign of impending mitosis in *S. pombe* until, in late G_2, the spindle pole body (SPB) duplicates and the mitotic spindle is formed between the two new SPBs. The spindle extends almost immediately and the chromosomes separate at anaphase (McCully and Robinow, 1971; King and Hyams, 1982a; Tanaka and Kanbe, 1986). The three chromosomes are briefly condensed though this is hard to visualize in normal growing cells (Robinow, 1977). Arresting certain mutants or treatment with antimicrotubule agents can block chromosomes in the condensed state, as discussed below. The *Sacch. cerevisiae* cycle differs significantly in that SPB duplication occurs early in the cycle and a short spindle is present throughout the G_2 phase until it elongates shortly before mitosis (Byers, 1981). Mitotic chromosome condensation has not been reported for *Sacch. cerevisiae*. In both yeasts the mitotic centers, the SPBs, are embedded in the nuclear envelope, which remains intact

throughout mitosis. The cytology of mitosis in *S. pombe* is discussed by Robinow and Hyams (Chapter 8, this volume).

Mitosis in any cell must satisfy the requirement of accurate segregation of the chromosomes, and it is not surprising that many features, such as replication and separation of the mitotic centers, spindle assembly, chromosome condensation, separation of the centromeres, and segregation of the daughter chromatids, are shared between yeast species and higher cells. The order in which they occur need not be so tightly regulated, however, as is shown by the difference in timing of SPB replication and spindle assembly between budding and fission yeasts. This suggests that there may be parallel developmental pathways in mitosis which can run independently and only come together at a few key points. There are at least two such pathways in *S. pombe* (Fig. 2).

A. Mutants of G_2 and Mitosis

More than 25 genes have been identified whose products are required for successful mitosis (Table I). Of these, one group typified by the mitotic control genes presumably arrest in late G_2 rather than in mitosis itself. A second type show arrest in mitosis according to several criteria, discussed below. The products of the great majority of both groups of mutants are required finally in late G_2 or mitosis, with only one gene, *cdc6* (Nurse *et al.*, 1976), having a transition point in mid-G_2. The distinction between a mutant with a late G_2 defect and one with a defect in mitosis is not in general easy to make, since there are few morphological markers

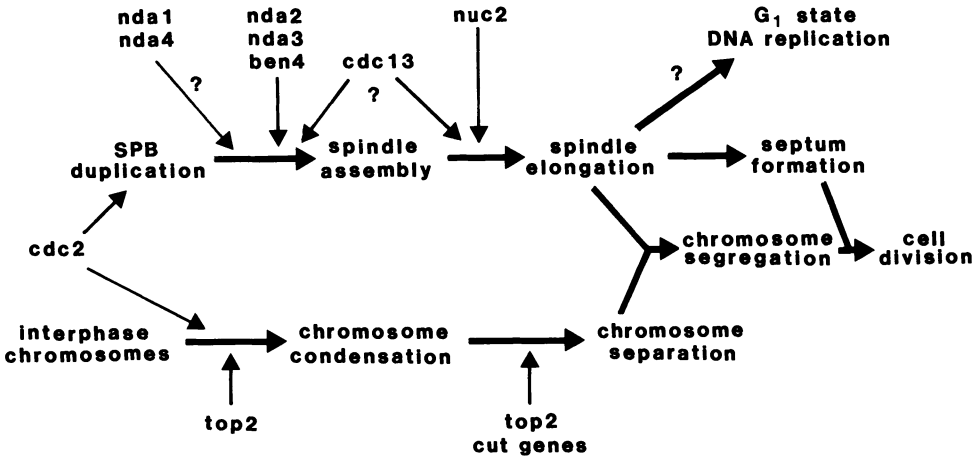


Fig. 2. Pathways in mitosis: a tentative model.

defining early mitotic events. Prophase chromosome condensation is either absent or highly elusive, although changes in the morphology of the nuclear chromatin region are observed during mitosis in normal cells (Toda *et al.*, 1981) and in mutants of mitosis (Umesono *et al.*, 1983b; also see below). The continued integrity of the nuclear envelope through mitosis precludes the use of its breakdown as a marker.

It is probable that the distinction between defects in late G_2 and early mitosis will prove to be purely semantic. The last event in G_2 is almost by definition the first in mitosis, and only our ignorance of the biochemical basis of such an event forces us to classify functions as premitotic or mitotic. Until the detailed molecular mechanisms of mitosis are understood, however, it is useful to attempt a classification of gene-controlled functions into G_2 and mitotic.

Using the limited available evidence, I have classified some mutants as defective in late G_2 rather than mitosis (Fig. 2). In addition to the genes of mitotic control, genes such as *cdc1*, *cdc27* and *cdc28* are included, largely on the basis of the resulting nuclear morphology being similar to that of normal interphase cells, though the nuclei are elongated in some cases (Nurse *et al.*, 1976; King and Hyams, 1982b). No chromosome condensation is evident and no septa are formed (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; King and Hyams, 1982a,b). The state of the SPBs and the presence of the spindle are uncertain. The only report (King and Hyams, 1982a) describes electron microscopy of cells which had been arrested for 5–7 hr. Since some of the mutants used do not maintain a tight division block for as long as this, it is possible that the small number of cells examined were atypical. Mutants whose nuclear morphology after arrest differs significantly from normal interphase will be considered as defective in the process of mitosis rather than its initiation, and are considered below.

The relationships between the functions controlled by the various genes and the events of mitosis have been investigated in several ways. Reciprocal shift experiments using various heat-sensitive mutants and the antimicrotubule agent benomyl show that the benomyl-sensitive step is functionally dependent on the steps controlled by *cdc2* and *cdc27* (Fantes, 1982). These observations fit well with the idea that the (G_2) *cdc2* function occurs early in mitosis, consistent with its regulatory role. The steps controlled by the *cdc1*, *cdc13*, and *cdc25* genes were found to be interdependent with the benomyl-sensitive step, presenting several conceptual problems. The $cdc13^+$ and $cdc25^+$ products are thought to interact directly or indirectly with the $cdc2^+$ product (Section V,C). Functional dependency analysis would therefore be expected to show the steps controlled by *cdc2*, *cdc13*, and *cdc25* to be interdependent with each other, and to share the same relationship with other steps such as that sensitive

to benomyl. A possible resolution of this paradox is discussed in Section VI,E, but its existence stresses the point that the results of reciprocal shift experiments need to be treated with extreme caution (Pringle, 1978).

B. Mitotic Spindle

The major components of the mitotic spindle are the microtubules, structures composed of polymerized α- and β-tubulin, associated with other elements. In *S. pombe* as in *Sacch. cerevisiae* two α-tubulin genes and a single β-tubulin gene have been identified (Toda *et al.*, 1984; Hiraoka *et al.*, 1984).

1. β-Tubulin

Mutants at the β-tubulin locus, *ben1*, have been identified as resistant to the benzimidazole compounds thiabendazole (TBZ) and benomyl, or its active moiety, methyl benzimidazolecarbamate (MBC) (Yamamoto, 1980). Another β-tubulin mutant, *nda3-KM311*, was first identified among a collection of mutants cold sensitive for mitosis (Toda *et al.*, 1981) and was subsequently shown to map at the *ben1* locus (Toda *et al.*, 1983). The allelism of *ben1* and *nda3* was strongly supported by the isolation of a mutant, *ben1-TB1005*, which shows resistance to benomyl and TBZ (and is actually drug dependent) at high temperature and is cold sensitive for mitosis (Umesono *et al.*, 1983a). Other mutants supersensitive to TBZ which map at *ben1/nda3* were also obtained.

A variety of indirect evidence was obtained suggesting that *ben1/nda3* encoded a tubulin protein: as well as the altered resistance of different alleles to known antimicrotubule agents, ultrastructural studies showed alterations to the spindle (Toda *et al.*, 1983). Observations on other fungi (Davidse, 1986) suggest that β-tubulin is the primary *in vivo* target of MBC. Under suitable conditions three highly condensed chromosomes were visualized in *nda3-KM311* cells. Similar condensation was also observed in wild-type cells treated with TBZ (Umesono *et al.*, 1983b). Direct proof that the *ben1/nda3* locus encoded the β-tubulin gene came from the cloning of the gene by complementation and determination of the DNA sequence. The predicted translation product was highly homologous to known β-tubulins (Hiraoka *et al.*, 1984). Southern analysis shows that *nda3* is the only gene of *S. pombe* likely to encode a β-tubulin (Hiraoka *et al.*, 1984).

Overexpression of the wild-type β-tubulin gene is lethal in *S. pombe*. This made the gene impossible to clone by complementation from a wild-

type genomic library in a conventional multicopy plasmid, since transformants carrying the gene are inviable. Instead, a library made with DNA from a drug-resistant strain was used to transform a sensitive strain to resistance (Hiraoka *et al.*, 1984).

There are several phenotypes associated with cold-sensitive *nda3* mutants. Nuclear division is blocked, sometimes showing the chromosome condensation characteristic of metaphase arrest in higher cells (Fig. 2). The chromatin is often displaced from its normal central position (Hiraoka *et al.*, 1984). It is not certain whether a spindle is present in arrested cells: spindle microtubules were observed by electron microscopy in one study (Toda *et al.*, 1983), but according to a subsequent report no spindle was present, as judged by immunofluorescence microscopy (Hiraoka *et al.*, 1984). Differences in technique may account for this discrepancy: in particular the arrest temperature in the latter report was lower, and a better arrest may have been attained (Hiraoka *et al.*, 1984). There is an interesting comparison in *Aspergillus nidulans*, where mutant alleles of the β-tubulin gene can lead to mitotic arrest either with a blocked spindle or with no spindle (Oakley and Morris, 1981). Cell morphology is aberrant in cold-sensitive *nda3* mutants: rather than the cells simply elongating like most cell cycle mutants, swelling and branching is seen (Hiraoka *et al.*, 1984; Umesono *et al.*, 1983b). This is consistent with the disruption of cytoplasmic microtubules in addition to those of the spindle.

2. α-Tubulin

The cold-sensitive mutation *nda2-KM52* is in several ways phenotypically similar to *nda3-KM311*. Both show cold sensitivity for mitosis, chromosome condensation, and eccentrically positioned chromosomes after arrest. There is no spindle in *nda2-KM52* cells, and there are anomalies in SPB behavior (Toda *et al.*, 1983). The cells are aberrant in morphology in that a septum is often formed on prolonged incubation at the restrictive temperature, frequently resulting in one half of the cell being anucleate (Toda *et al.*, 1981, 1983). Septation may only occur after an abortive mitosis, since separation of the two chromatin regions occurs after prolonged arrest, perhaps because the block is leaky.

No *nda2* mutant more resistant to TBZ or MBC than the wild type has been identified, and indeed the majority are supersensitive to these drugs (Umesono *et al.*, 1983a). The suggestion based on this (Umesono *et al.*, 1983a) that *nda2* might encode an α-tubulin was confirmed by isolation of two plasmids that complemented the cold sensitivity (Toda *et al.*, 1984). DNA sequence analysis revealed that both the insert sequences were different, and that both could encode α-tubulin proteins. Transcripts from

both genes were identified. One cloned fragment, $(NDA2)_1$, was shown by homologous integration and mapping to contain the wild-type *nda2*$^+$, and its protein was designated the α_1-tubulin. Presumably *nda2* mutants are supersensitive to microtubule inhibitors because the interaction between mutant α-tubulin and β-tubulin is abnormal, rendering the complex more susceptible to inhibition. The other cloned gene, $(NDA2)_2$, mapped elsewhere and encodes the α_2-tubulin (Toda *et al.*, 1984). There is high homology (86%) between the predicted amino acid sequences of the α_1- and α_2-tubulins.

Point mutations in the α_1-tubulin (*nda2*) gene confer drug sensitivity and/or cold sensitivity, and a disrupted *nda2* allele is lethal. In contrast, the $(NDA2)_2$ sequence encoding the α_2-tubulin is mutationally silent, and even cells carrying a null allele are viable (Adachi *et al.*, 1986). The α_2 gene is nevertheless at least potentially functional, since its expression from a plasmid complements a *nda2* mutation. This also shows that there is no crucial difference between the two translation products. The second gene might in principle be nonessential because it is not normally expressed from the chromosome during mitotic growth. This seems unlikely since (1) α_2 transcript is present, (2) the disrupted α_2 gene confers increased sensitivity to TBZ, (3) mutants with the combination of α_2 disruption and *nda2-KM52* grow extremely slowly even at a temperature fully permissive for the *nda2* mutation alone, and (4) a protein which is presumably the product of the α_2 gene is present in microtubules in normal cells but not in the α_2 disruptants (Adachi *et al.*, 1986).

The most likely explanation is based on the finding (Adachi *et al.*, 1986) that the α-tubulin genes are subject to different transcriptional controls. In normal cells the α_1 transcript predominates, while in a transformant carrying multiple copies of the α_2 gene the level of α_2 transcript is much increased. Surprisingly, the α_2 transformant has a much reduced level of α_1 transcript. In an α_1 transformant, the level of the α_1 transcript is essentially unaffected, showing that the α_1 level is autoregulated. In contrast, the level of α_2 transcript is unaffected by the presence of extra α_1 genes and increases in line with gene copy number in the α_2 transformant. These observations led Adachi *et al.* (1986) to formulate a model in which a limited pool of α-tubulin regulates the level of α_1 expression, while α_2 expression is constitutive. The cell is able to compensate for the absence of α_2-tubulin by increasing the expression of the α_1 (*nda2*) gene. Disruption of the *nda2* gene cannot be compensated by any increase in α_2 expression and this leads to lethality. This model is a useful hypothesis, and is consistent with the occurrence of autoregulation in other tubulin systems (Cleveland, 1983; Minami *et al.*, 1981). One of the two *Sacch. cerevisiae* α-tubulin genes is essential, but not the other (Schatz *et al.*, 1986). Some

discrepancies between model and data remain, however, and the difference in biological role of the two genes and their products remains to be explained.

3. nuc2

A recently isolated mutant *nuc2* is phenotypically similar to tubulin mutants in showing chromosome condensation and nuclear displacement from the center of the cell. However, a short spindle is present, of uniform length, showing that the defect is in spindle elongation rather than assembly (Yanagida *et al.*, 1986).

4. ben4

The majority of mutants resistant to antimicrotubule agents map to the *nda3* locus, consistent with β-tubulin being the major target for their binding. A separate class of mutants selected for resistance to low concentrations of benomyl and for cold sensitivity for mitosis map at a different locus, designated *ben4* (Roy and Fantes, 1982). The *ben4* gene was originally proposed as a β-tubulin gene, but it is not allelic with any known tubulin gene (D. Roy, personal communication; Adachi *et al.*, 1986). *ben4* mutants are resistant to MBC and benomyl, but not in general to TBZ, during growth at 35°C. Mutant cells arrested at 20°C show an unusual nuclear morphology: the chromatin extends along the length of the elongated nucleus, while the nucleolus lies at one end. No septum is formed, and the cells are elongated rather than aberrantly shaped. These properties suggest a defect in spindle structure but not in the arrangement of the cytoskeletal microtubules, though this remains to be directly tested. In contrast, mutations at the α- or β-tubulin loci appear to affect both spindle and cytoplasmic microtubules.

A possible role for the *ben4*$^+$ product is as a microtubule-interacting protein which is preferentially required for spindle function. The resistance of *ben4* mutants to MBC suggests that the *ben4* product might bind to β-tubulin and affect the affinity of the latter for MBC. In *ben4* mutants the altered interaction reduces the affinity for MBC, perhaps by masking the MBC binding site, and consistent with this, *ben4* mutants are dominant for MBC resistance (Roy and Fantes, 1982). *ben4* mutations show genetic interaction with the β-tubulin gene. The presence of a duplication of the *nda3* gene in a *ben4* strain reduces its level of MBC resistance (A. Schroeder-Lorenz and P. A. Fantes, unpublished observations). This is again consistent with a triple interaction between β-tubulin, the *ben4*$^+$ product, and MBC.

A more striking interaction is shown between *ben4* and the actin gene (Mertins and Gallwitz, 1987). Cells carrying an extra copy of the actin gene are viable although a substantial proportion show multiple septa and other aberrations (A. Schroeder-Lorenz, J. Marks, and J. S. Hyams, personal communication). However, the combination of an actin gene duplication and a *ben4* mutation is completely lethal (A. Schroeder-Lorenz and P. A. Fantes, unpublished observations). While the significance of this interaction is unknown, the distribution of actin filaments in *ben4* cells after mitotic arrest is strikingly different from normal, supporting the possibility of a direct interaction between the *ben4* product and actin (Marks and Hyams, 1985; Marks *et al.*, 1986).

5. cid Mutants

Colcemid is an effective agent at submicromolar concentrations against the microtubules of higher eukaryotic cells. At high (millimolar) concentrations Colcemid inhibits the cell cycle of *S. pombe* (Stetten and Lederberg, 1973), and a collection of mutants (designated *cid* mutants) resistant to the compound are described by Sackett and Lederberg (1986). The mutants are not conditional lethals but have altered cell cycles. There is no evidence that either Colcemid or the *cid* mutations affect spindle formation or function in *S. pombe*. The primary target appears to be cell separation rather than mitosis, although mitosis is delayed in the presence of Colcemid.

C. Early Stages of Mitosis

The shape of the chromatin region changes in a regular way during mitosis (Toda *et al.*, 1981; Yanagida *et al.*, 1986). The spherical interphase nucleus contains a roughly hemispherical region of chromatin and a crescent of nucleolar material (McCully and Robinow, 1971; Toda *et al.*, 1981). Two "pegs" of chromatin protrude into the nucleolus. The chromatin then compacts, sometimes into an ellipsoidal shape. A U-shaped structure is formed, the arms of the U facing opposite ends of the cell. The center of the U constricts and separates the thickened arms which become the daughter nuclei (Toda *et al.*, 1981; Fig. 2). These stages have been used to classify the arrest stages of various mutants.

The *nda1* and *nda4* genes are defined by cold-sensitive mutations in mitosis (Toda *et al.*, 1983; Yanagida *et al.*, 1986). Chromosome condensation does not occur after arrest in these mutants, but *nda1* cells arrest at the compact chromatin stage, and *nda4* cells with a U-shaped chromatin region (Yanagida *et al.*, 1986). Double mutants of *nda1* with *nda2* and

nda3 have the terminal phenotype of *nda1*, suggesting that the *nda1* function is required before those of the tubulin genes (Toda *et al.*, 1983). Similar observations were made on double mutants of *nda4* with *nda2* and *nda3* (Yanagida *et al.*, 1986). These observations suggest that *nda1* and *nda4* control early events in mitosis (Fig. 2).

D. Chromosome Movement and Condensation

Disrupting microtubule function in tubulin mutants allows chromosomes to condense while preventing chromosome separation or spindle function (Fig. 2). Chromosome condensation also occurs in *cdc13* mutants after prolonged arrest. This suggests that two separate sets of processes are involved in mitosis, and that blocking one can allow the other to continue up to a point. If that were so, then conditions might be found in which spindle function was normal but chromosome behavior was aberrant. Mutants affected in this way have been isolated, and form the subject of this section.

1. Roles of Topoisomerases

Topoisomerases are enzymes which affect the higher order configuration of double-stranded DNA molecules without altering the primary sequence of bases. The chromatin of both chromosomes and plasmids contains highly supercoiled DNA, and the density of supercoiling is related to the packaging of DNA into nucleosomes. The degree of supercoiling is controlled by topoisomerases. Type I topoisomerases create single-strand nicks in DNA molecules and rejoin the cut ends after rotation around the intact strand, whereas type II topoisomerases make a double-strand cut followed by rejoining (Wang, 1985). Type II enzymes, which require ATP and Mg^{2+} for activity, can unknot or decatenate interlinked DNA molecules by cutting and rejoining, while type I enzymes cannot. Topoisomerase II is a major component of the scaffold of mitotic chromosomes (Earnshaw *et al.*, 1985).

Since the roles of the two enzymes in *S. pombe* and the likely phenotypes of mutants lacking them were uncertain, Uemura and Yanagida (1984) screened the survivors of heavy mutagenesis directly for *in vitro* topoisomerase I activity by assaying the conversion of supercoiled plasmid to more relaxed forms. Two mutants mapping at the same (*top1*) locus were identified which showed low *in vitro* activity in the presence of EDTA and absence of ATP. One mutant had less than 1% of the wild-type activity, yet was viable over the normal temperature range, suggesting that the enzyme was dispensable. The *top1* mutants retained ATP-Mg^{2+}

DNA relaxing activity as well as unknotting activity. Starting from a *top1* mutant which was also defective in endonucleolytic activity, three strains defective in these activities were isolated among a collection of temperature-sensitive lethals (Uemura and Yanagida, 1984, 1986). All three mapped to the same (*top2*) locus: the gene was cloned by complementation, and DNA sequence analysis confirmed that the gene encoded topoisomerase II (Uemura *et al.*, 1986).

Single *top2* mutants behave as *cdc* mutants with a defect in mitosis. Septum formation is not prevented, however, and the growing septum bisects the nucleus. This makes it impossible to analyze the morphology of the arrested nucleus (Uemura and Yanagida, 1984). This difficulty was circumvented by combining the *top2* defect with a mutation (*cdc11*) preventing septation (Uemura and Yanagida, 1986). After temperature shift, nuclear division in the double mutant is still prevented. Spindles are present in the double *top2 cdc11* mutant and in those cells of the single *top2* strain in which no septum has formed. Most of the chromatin is present in an almost spherical form, with the spindle passing through the center. Small fragments of chromatin are present along the spindle, suggesting that the force which normally pulls the chromosomes apart is present, although they are unable to separate (Uemura and Yanagida, 1986).

In order to investigate the role of topoisomerase II in more detail, a situation where the spindle did not interfere with the *top2* phenotype was arranged. A cold-sensitive allele of *top2* was obtained which showed the same mutant phenotype as heat-sensitive alleles, and whose topoisomerase II activity was cold sensitive *in vitro* (Uemura *et al.*, 1987). This was combined with the cold-sensitive β-tubulin mutant *nda3.K311*. When the double *nda3 top2* mutant was shifted to the restrictive temperature, no spindle was formed, as in the *nda3* parent. However, individual condensed chromosomes were not observed: rather, long, apparently entangled chromosomes were seen. These chromosomes were not as fully condensed as those seen in *nda3*. This implicates the *top2* product in chromosome condensation (Fig. 2). Shifting the arrested double mutant back to the permissive temperature allowed the chromosomes rapidly to condense and to start to separate. Condensation slightly preceded separation, suggesting that separation involving spindle forces was not required for chromosome condensation. To confirm this, the cold-sensitive blocks were released in the presence of nocodazole to prevent spindle formation. Condensation of the chromosomes without separation was seen (Uemura *et al.*, 1987).

Temperature pulse experiments, in which the double *nda3 top2* mutant was arrested at 20°C, transferred to 36°C for a few minutes, and then returned to 20°C, showed that $top2^+$ function was required throughout the

separation of the condensed chromosomes during anaphase (Uemura *et al.*, 1987). This was confirmed by order of function experiments. A double mutant carrying a heat-sensitive *top2* allele and the cold-sensitive *nda3* allele was arrested by incubation at 20°C at the *nda3* block: no spindles were present and the chromosomes were condensed. On shifting to 36°C the spindle formed rapidly, but failed to properly separate the chromosomes. Instead, nonseparating "streaked" chromosomes were pulled along the spindle axis, showing that topoisomerase II is required both before and after spindle assembly.

The phenotype of *top1 top2* double mutants is strikingly different from that of single *top2* mutants. The nuclear morphology of double mutant cells changes rapidly after temperature shift to an aberrant ring-shaped or hollow spherical form, in all cells, irrespective of their initial cell cycle position (Uemura and Yanagida, 1984). This suggests that some activity able to change the extent of DNA supercoiling is required throughout the cell cycle, and that its complete absence results in aberrant chromatin structure. Normally topoisomerase I provides this activity, but in its absence in *top1* mutants the topoisomerase II enzyme can substitute for it, explaining the viability of *top1* mutants. *top2* mutants are viable during interphase because topoisomerase II activity is not required until mitosis, and the $top1^+$ gene supplies the topoisomerase I activity needed in interphase. Topoisomerase II activity is absolutely required for mitosis as described above, and arrested *top2* cells rapidly lose viability on reaching mitosis (Uemura and Yanagida, 1986). Observations made with *top2* mutants of *Sacch. cerevisiae* also show that topoisomerase II is essential for mitosis but not in interphase (Holm *et al.*, 1985). The lethality of *top2* mutants at mitosis is probably a consequence of the spindle attempting to separate intertwined chromosomes.

Although chromosomal behavior in mitosis is drastically affected in *top2* mutants, the ability of chromosomes to be replicated is apparently unimpaired provided the nucleus remains intact (Fig. 2) (Uemura and Yanagida, 1986). In *cdc11 top2* double mutants DNA (and RNA and protein) continues to accumulate after arrest at a rate comparable to that in the single *cdc11* mutant. No division of the nuclei takes place, and the chromatin region enlarges considerably. This unexpected finding is discussed below.

2. nuc1 and cut Mutants

The discovery that mutants of mitosis such as *top2* continue to form septa after arrest, while *top1 top2* double mutants have a "ring chromatin" phenotype, suggested that new genes might be identified by screening for phenotypically similar mutants. One mutant, *nuc1*, arrests with the

ring chromatin phenotype of *top1 top2*, independently of *top1* function. Neither topoisomerase I nor II is defective in *nuc1*, and the defect probably lies in the architecture of the nuclear chromatin.

Mutants mapping in nine previously unidentified genes, *cut1* to *cut9*, have been identified which have terminal phenotypes similar to *top2* (Hirano *et al.*, 1986). The phenotype is independent of *top1*. In all cases the primary arrest is at nuclear division; septation takes place and splits a fraction of the cells. In *cut1* and *cut2* mutants, chromosome separation appears to be defective. A short spindle is present in a proportion of arrested *cut1* and *cut2* mutant cells. In these cells, the chromatin is in the form of an elongated and slightly curved rod which follows the line of the spindle. The presence of a late G_2 mutation (as defined earlier) in a *cut1* strain prevents expression of the *cut1* phenotype. In double mutants of *cut1* with the *cdc11* septation defect, DNA replication continues despite the absence of nuclear division, just as for *top2 cdc11*. The cloned $cut1^+$ gene complements not only *cut1* but also *cut2* mutations, showing that the two genes control related functions (Hirano *et al.*, 1986).

It seems likely that many of the *cut* genes will turn out to be involved in chromosome structure and behavior. The most surprising aspect of the *cut* and *top2* mutations is the high frequency with which mutants defective in mitosis yet able to form a septum have been obtained. In terms of the number of genes, there are about the same number of mitotic mutants able to form a septum as unable to do so (Table I) (Hirano *et al.*, 1986). Hirano *et al.* (1986) suggest that the *cut* and *top* mutants act late in mitosis, beyond the time of commitment to septum formation, as originally suggested for *cdc13* (Nasmyth and Nurse, 1981). However, other mutants acting late in mitosis such as *nda3* and others defective in spindle function do not form septa after arrest. Since the $top2^+$ product is required in mitosis at least as late as the $nda3^+$ function, arrested *nda3* mutants according to this model are predicted to form septa. An alternative explanation derives from an extension of the scheme in which mitosis comprises two parallel pathways, one concerned with chromosome condensation and segregation and the other with spindle functions (Fig. 2) (Yanagida *et al.*, 1986). Septum formation would be dependent on a late stage in the spindle pathway. Blocks in the chromosome pathway, which have no effect on spindle function, therefore allow septation.

E. Coordination of Mitosis with Cell Cycle

It is clear that mitosis is not a simple linear series of events: the spindle and chromosomes can behave more or less independently in mutants

affected in the complementary pathway (Fig. 2). Successful mitosis is only possible if both pathways are functional. Septum formation seems to be dependent on only the spindle pathway and can proceed even if the chromosomes are unable to separate. This observation poses questions about the rules that govern the overall structure of the cell cycle: in particular, why do mitosis and DNA replication normally occur in strict alternation?

It is notable that in *cut* and *top2* mutants DNA replication is unimpaired, although aspects of chromosome behavior such as condensation and segregation are defective. This is highly anomalous: in most mutants arrested in late G_2 or in mitosis (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981) no further DNA replication occurs. It is as though the normal control ensuring alternation of chromosome replication during S phase and segregation during mitosis has broken down. Such a mechanism might operate by the whole cell, or of components such as the nucleus or chromosomes, switching from a G_2 to a G_1 state by passage through mitosis, and back to G_2 after passage through S phase. If this is so, then the continuation of DNA replication in an arrested *top2* mutant suggests that the G_2 state can be altered to the G_1 state without the completion of mitosis. The processes of mitosis concerned with spindle function continue in *top2* mutants, and the formation of a septum implies that the pathway leading to septation is still open (Fig. 2). One of these processes, or another as yet unidentified one, might be responsible for enabling the transition from the G_2 to the G_1 state. Recent findings (D. Broek and P. Nurse, personal communication) suggest that some property of the $cdc2^+$ protein kinase may control whether the cell is in a G_1 or G_2 state.

Mitosis is initiated by the $cdc2^+$ product attaining a state of readiness to set the two (or more) mitotic pathways into motion. The $cdc2^+$ product has protein kinase activity which may be regulated by the products of *wee1*, *cdc25*, *suc1*, and perhaps other regulator molecules. It seems likely that the *cdc2* p34 kinase can act on a set of protein substrates, some involved in the G_1/S transition and others in aspects of mitosis. The simplest hypothesis is that each of these substrates are phosphorylated at the appropriate time and in the appropriate place so that the dependent pathways can proceed. This suggests that there may be two substrates which must be phosphorylated to initiate mitosis: one to initiate the processes of spindle formation, the other to bring about chromosome condensation (Fig. 2). It is likely that the *cdc13* product is a substrate of the *cdc2* product, and it is pertinent to ask which pathway it might be part of. The terminal phenotype of *cdc13* mutants suggests that their defect is in spindle formation, since chromosome condensation occurs and the nucleus is not elongated (Nasmyth and Nurse, 1981). The *cdc13* step in

mitosis is also interdependent with the benomyl-sensitive step(s) in mitosis (Fantes, 1982). DNA replication is arrested (Nurse *et al.*, 1976), similar to other mutants of the spindle pathway. It is true that septation occurs in arrested cells, in which respect *cdc13* differs from other spindle mutants apart from *nda2*. In both *nda2* and *cdc13* septa form only after prolonged arrest, however, and this could conceivably be due to leakiness of the blocks.

Whether this largely speculative model is correct remains to be seen: a strong prediction is that mutants should be found which interact with *cdc2* in some way and are specifically defective in the chromosome condensation pathway. In any case the *cdc2* product has a central role in cell cycle control, and elucidation of its natural substrates will be an important next step in investigating these fundamental processes.

F. Chromosome Maintenance

Schizosaccharomyces pombe has three chromosomes whose genetic lengths are in the ratio 3 : 2 : 1 for chromosomes I, II, and III, respectively (Munz *et al.*, Chapter 1, this volume). Consistent with this, three chromatin bodies with similar length ratios can be visualized by light and electron microscopy (Robinow, 1977; Umesono *et al.*, 1983b; Erard and Barker, 1985). Recently the techniques of pulsed-field gel electrophoresis have been extended so that molecules of several megabase pairs can be resolved. The absolute sizes of the *S. pombe* chromosomes estimated at 3, 6, and 9 Mbp (Smith *et al.*, 1987) are now estimated as 3.5, 4.7, and 5.7 Mbp (C. Smith and M. Yanagida, personal communication).

Chromosomes require three functional elements for maintenance. The chromosomal DNA needs to be replicated during the S phase under the control of replication origins. Centromeres are required to attach the chromosomes to the microtubules at mitosis. Telomeres may be involved in attaching the chromosomes to the nuclear envelope, and they protect the ends of the chromosomes from degradation (Blackburn and Szostak, 1984). In *Sacch. cerevisiae* DNA sequences able to confer each of the three functions have been identified and cloned (Blackburn, 1985). The question of *ars* sequences and their relationship to replication origins and *ars* sequences in *S. pombe* is discussed in Section IV,C. Much less is known about the centromeres and telomeres of *S. pombe* than of the *Sacch. cerevisiae* elements since the field has only recently been opened up for investigation. Much of the available information pertaining to *S. pombe* is summarized below; details of the DNA sequences involved are omitted since no clear picture has yet emerged.

1. Centromeres

Saccharomyces cerevisiae centromeres can be cloned directly since they confer stability of mitotic transmission on circular or linear plasmids, and genetic systems designed to facilitate the identification of stably maintained plasmids have been used (Hsiao and Carbon, 1981). In *S. pombe* similar selection systems have so far failed to identify sequences with centromeric activity. There are several possible reasons for this: the selection systems may for some reason not be appropriate; the *S. pombe* centromeric sequences may be refractory to cloning in *E. coli*; or the entire system which attaches centromeres to spindle microtubules may be fundamentally different from that in *Sacch. cerevisiae*. At present the most likely reason is that the length of the DNA sequence required for centromere function in *S. pombe* may be much larger than in *Sacch. cerevisiae*, precluding its cloning using current vector systems. Sequences as short as 300 bp have centromere activity in *Sacch. cerevisiae* (Clarke and Carbon, 1985). No directly comparable figure is available for *S. pombe*, since no functional centromere has been isolated, but meiotic recombination is greatly suppressed over at least 50 kb of centromeric DNA of chromosome II (Nakaseko *et al.*, 1986; Clarke *et al.*, 1986), a characteristic of centromeric regions (Charlesworth *et al.*, 1986). While not all of the 50-kb region may be required for spindle attachment, it is reasonable to suppose that much of it may be involved somehow. There are sequence elements that are repeated in the centromeric regions of all three chromosomes and do not occur elsewhere in the genome (Nakaseko *et al.*, 1986, 1987; Clarke *et al.*, 1986), again characteristic of mammalian centromeric regions (Singer, 1982). It is possible that these repeated domains are involved in attachment of microtubules or binding of kinetochore proteins. The complexity of *S. pombe* centromeric regions compared to those of *Sacch. cerevisiae* may reflect the larger size of *S. pombe* chromosomes or their greater degree of condensation at mitosis (Umesono *et al.*, 1983b).

A DNA sequence has been fortuitously isolated from *S. pombe* that improves the normal mitotic instability of circular plasmids in *S. pombe* (Heyer *et al.*, 1986). This sequence, named *stb*, does not reduce the copy number as do centromeric (*CEN*) sequences in *Sacch. cerevisiae* (Clarke and Carbon, 1985). *stb* seems to work by improving the partitioning of plasmid molecules at mitosis, but the mechanism is unknown. Only one copy of the *stb* sequence is present in the *S. pombe* genome, unlinked to the centromere of chromosome III (Heyer *et al.*, 1986). It is therefore unlikely that the *stb* element has any centromeric function in the genome.

2. *Telomeres*

Linear DNA molecules in eukaryotic cells suffer one of two fates, namely, degradation or integration into a chromosome by recombination, unless the ends are protected in some way (Blackburn and Szostak, 1984). In the case of chromosomes, telomeres perform the protective function, and they may also interact with the nuclear envelope (Agard and Sedat, 1983). In certain lower eukaryotes such as *Tetrahymena* and *Physarum*, the ribosomal cistrons are organized extrachromosomally in relatively short molecules. Such rDNA molecules can be introduced into yeasts by transformation, and are stably maintained as linear molecules. The ends of the molecules are modified during the generation of transformants by addition of telomeric sequences from the host (Shampay *et al.*, 1984). This has enabled the telomeric sequences from *Sacch. cerevisiae* and *S. pombe* to be cloned (Shampay *et al.*, 1984; Matsumoto *et al.*, 1987b). The existence of a telomere-specific terminal transferase in *Tetrahymena* has been demonstrated (Greider and Blackburn, 1985); similar activities are presumably responsible for telomere addition in yeasts.

rDNA molecules from *Physarum* (Kuenzler, 1985) or *Tetrahymena* (Suguwara and Szostak, cited in Matsumoto *et al.*, 1987b) acquire *S. pombe* telomeric sequences on transformation. A plasmid originally containing *Tetrahymena* rDNA termini which had acquired telomeres from *Sacch. cerevisiae* by its propagation in that yeast is also maintained in *S. pombe*. Little or no change in telomeric structure was observed during transfer from *Sacch. cerevisiae* to *S. pombe* (Guerrini *et al.*, 1985). This suggests that telomeres from the two yeasts are functionally interchangeable, though this has not yet been shown by direct transfer of authentic chromosomes. Minichromosomes derived from chromosome III by γ-ray irradiation are stably maintained in *S. pombe* (Niwa *et al.*, 1986; Matsumoto *et al.*, 1987b). They have termini "healed" by the addition of endogenous telomeric sequences (Matsumoto *et al.*, 1987b).

A *S. pombe* chromosome has recently been transferred to mouse cells by fusion to yeast protoplasts (Allshire *et al.*, 1987). It is maintained in at least one fusion line as an apparently intact autonomous element, although the possibility that it has acquired mouse DNA sequences cannot be excluded. The nature of the telomeric sequences in particular is yet to be determined, a strong possibility being that mouse telomeres have been added on. The introduced chromosome is not stably maintained, suggesting either that the *S. pombe* centromere functions poorly in higher cells or that it is devoid of function and mitotic transmission of the chromosome is random.

VII. CONCLUDING REMARKS

Study of the cell cycle in *S. pombe* and other organisms has now expanded to a point where it extends into many areas not traditionally associated with the cycle. Investigations into the mechanisms of mitosis have led to the genetic and cytological analysis of the cytoskeleton on the one hand and into the field of chromosome structure and function on the other. The basis of almost universal rules such as the alternation of the S and mitotic phases may only be understood by investigating situations where the rule is broken, and these have recently emerged in *S. pombe*.

Detailed investigation into the cell cycle itself has required the approaches of genetics, molecular biology, and protein chemistry to be applied to what were originally questions posed purely at the physiological level. Recent advances in molecular biological techniques have fortunately proceeded hand in hand with the sophistication of the questions posed, to the extent that the first *S. pombe* gene to be cloned was the major cell cycle control gene *cdc2*. Without the ability to transform, replace, delete, and overexpress genes in *S. pombe*, our understanding of the major cell cycle control over mitosis might not have progressed significantly since 1980. As it is, the available range of molecular techniques has allowed deep insight into this system, mainly in the laboratories of Paul Nurse and David Beach. Perhaps even more important, use of these techniques has shown that at least some elements of the system exist in mammalian cells.

Molecular biological and related techniques are indeed powerful aids to investigation, but workers studying the *S. pombe* cell cycle have remained aware that they are purely tools to be used alongside more traditional methods. The investigation of the complex mitotic and S phase control systems of *S. pombe* was initiated by detailed analysis of conditional lethal *cdc* and cell size mutants. A purely molecular biological approach would not have led to the correct biological questions being asked of the organism, and it is improbable that our present level of understanding would have been reached. The detailed investigations into the mechanisms of mitosis by Mitsuhiro Yanagida's group were initiated by the analysis of classical mutants, and their use, combined with sophisticated cytological techniques, remains a central part of the study. The suggestion that elements required for chromosome maintenance in *S. pombe* may function in higher cells arose from simple concepts and relatively unsophisticated experiments. The combination of the ability to carry out simple experiments to address new problems and the availability of sophisticated tools where they are needed will ensure that *S. pombe*

will remain the organism of choice for many studies in the foreseeable future.

VIII. ADDENDUM

The growing interest in various aspects of the *S. pombe* cell cycle has resulted in a "burst" of new publications. Some of the major new developments are discussed in this addendum.

A. Maturation Promoting Factor and Mitotic Control in *Schizosaccharomyces pombe*

The most exciting discoveries have been in the analysis of maturation promoting factor (MPF, mentioned in Section V,D,1,c), which drives unfertilized *Xenopus* eggs into meiosis and fertilized eggs into mitosis (Ford, 1985). Much of what follows has been reviewed recently (Fantes, 1988; Murray, 1988; Lee and Nurse, 1988). MPF has been biochemically intractable, but was recently purified by Lohka *et al.* (1988). The active factor contains proteins of approximately 45 and 32 kDa. The latter protein is recognized by an antiserum (Gautier *et al.*, 1988) specific for a 16 amino acid sequence of the *S. pombe cdc2* protein kinase (p34) which is identical in the *Sacch. cerevisiae* and human p34 homologs (Lee and Nurse, 1987). This antiserum immunoprecipitates both components of MPF (Gautier *et al.*, 1988). It appears that one component of MPF is the *Xenopus* p34 homolog: furthermore, the *S. pombe suc1* gene product (p13), which binds to p34 *in vitro* (Section V,D,1,a), strongly inhibits MPF function (Dunphy *et al.*, 1988). *Schizosaccharomyces pombe* p13 affinity columns deplete crude MPF extracts of MPF activity, and *Xenopus* proteins of approximately 33 and 45 kDa are specifically bound (Dunphy *et al.*, 1988). The nature of the 45-kDa component of MPF is unknown; it is phosphorylated in the presence of the p32 component and ATP, and this may be important for function (Lohka *et al.*, 1988). Proteins have been identified in human cells and in *Sacch. cerevisiae* which associate with the respective p34 homologs but are of rather different sizes than the *Xenopus* protein (Draetta and Beach, 1988; Mendenhall *et al.*, 1987). They are phosphorylated in a cell cycle-specific manner (Draetta and Beach, 1988; Mendenhall *et al.*, 1987). In the human system, the maximum occurs just before mitosis, coincident with a maximum in the level of protein kinase activity against an exogenous substrate (Draetta and Beach, 1988).

The physiological substrate(s) of *S. pombe* p34 are still unknown. One

clue is provided by the finding that a mitosis-specific protein kinase purified from starfish oocytes appears to be another p34 homolog (Labbe *et al.*, 1988; Arion *et al.*, 1988). This enzyme shows activity against histone H1, whose phosphorylation has been suggested as the trigger for mitosis, notably in *Physarum polycephalum* (Inglis *et al.*, 1986). MPF is also active against this substrate, among others (Lohka *et al.*, 1988).

The *cdc13* gene was implicated in mitotic control by genetic interactions with *cdc2* (Section V,D,1,b). The *cdc13* gene has been cloned: the predicted amino acid sequence of its product (Booher and Beach, 1988; Hagan *et al.*, 1988) shows striking homology to cyclins (Hagan *et al.*, 1988; Solomon *et al.*, 1988; Goebl and Byers, 1988), proteins thought to control the early embryonic divisions of clams and sea urchins (Murray, 1987; Pines and Hunt, 1987). The terminal phenotype of *cdc13-117* is different from that of a null allele (Booher and Beach, 1988), suggesting that the mutant gene product is not completely defective at the restrictive temperature, but whether it is qualitatively or only quantitatively different from normal remains to be seen. The *cdc13-117* mutant allele leads to hypersensitivity to thiabendazole, suggesting a connection with microtubules, perhaps with a role in spindle assembly (Booher and Beach, 1988). These findings may help reconcile the paradox discussed in Section V,D,1,b.

B. Other Topics

1. Phosphorylation of the cdc2 p34 Protein Kinase

There is evidence for differential phosphorylation of the p34 homologs of *Xenopus* (Draetta and Beach, 1988) and human cells (Gautier *et al.*, 1988); no comparable findings for *S. pombe* have been reported. The phosphorylation pattern of the *Sacch. cerevisiae CDC28* product appears to be invariant under a range of conditions (Hadwiger and Reed, 1988).

2. Entry into Meiosis

Direct interaction between the *ran1* protein kinase and the product of the *mei3* gene has been demonstrated. Transcriptional activation of the *mei3* gene initiates meiosis by binding of the *mei3* product to the *ran1* protein kinase and inactivating it (McLeod and Beach, 1988). The role of the *mei2* gene has been investigated by Watanabe *et al.* (1988). *mei2* transcription is stimulated by nitrogen starvation, a signal for the initiation of meiosis, and inhibited by cAMP. These events are independent of the *mei3–ran1* pathway, but both pathways are required for meiotic initiation.

3. *ars Sequences*

The complete DNA sequences of eight cloned *S. pombe ars* fragments have been determined (Maundrell *et al.*, 1988). None is smaller than 0.8 kb, and each contains an AT-rich 11-bp consensus within the functional *ars* domain. Removal of the consensus region, however, does not abolish *ars* activity, although the mitotic stability of transformants is reduced. This is similar to previous observations by Johnston and Barker (1987) and suggests that the short consensus is part of a larger *ars* domain and has some function in its natural chromosomal position.

4. *Mitosis*

Two new and interesting types of mutants have been described by Yanagida's group (Okhura *et al.*, 1988; Hirano *et al.*, 1988). Mutants in three *dis* genes are defective in chromosome disjunction at mitosis (Okhura *et al.*, 1988). The spindle elongates, and unseparated chromosomes are distributed to the poles of the cell in an irregular way. All the mutants are hypersensitive to caffeine, suggesting a role for cAMP in regulating mitosis.

The *nuc2* gene encodes a highly insoluble protein that is located within the nucleus (Hirano *et al.*, 1988). The *nuc2* mutant arrests with a short spindle, but nuclear elongation is blocked. The chromosomes condense and are arranged centrally in the cell, around the spindle, resembling metaphase in higher eukaryotes. The *nuc2* gene product is analogous to a nuclear scaffold protein, and it may connect the nucleus with the cytoskeleton.

5. *Microtubule Array*

Hagan and Hyams (1988) describe the cytoplasmic array of microtubules in wild-type and *cdc* mutant cells. The microtubules are involved in positioning the cell nucleus. The spindle pole bodies of elongated spindles have astral arrays of microtubules extending into the cytoplasm until the end of anaphase.

ACKNOWLEDGMENTS

I am indebted to many friends and colleagues too numerous to mention individually for their help in the preparation of this chapter. Special thanks are extended to Paul Nurse, the past and present members of my laboratory, and Murdoch Mitchison for helpful discussions

at various stages. I am grateful to Paul Nurse, David Beach, Mitsuhiro Yanagida, Paul Young, Judy Potashkin, Bob Booher, and Masayuki Yamamoto for access to preprints and other unpublished communications. Finally I thank Paul Young and Judy Fantes for their forbearance during my preparation of this chapter. Research in my laboratory is supported by the Cancer Research Campaign and the Science and Engineering Research Council.

REFERENCES

Adachi, Y., Toda, T., Niwa, O., and Yanagida, M. (1986). Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **6,** 2168–2178.

Adams, D. (1980a). "The Restaurant at the End of the Universe," p. 7. Pan Books, London.

Adams, D. (1980b). "The Restaurant at the End of the Universe," p. 8. Pan Books, London.

Adams, R. P., Knowler, J. T., and Leader, D. P. (1986). "The Biochemistry of the Nucleic Acids," 10th Ed., Ch. 6. Cambridge Univ. Press, London and New York.

Agard, D. A., and Sedat, J. W. (1983). Three dimensional architecture of a polytene nucleus. *Nature (London)* **302,** 676–681.

Allshire, R. C., Cranston, G., Gosden, J. R., Maule, J. C., Hastie, N. D., and Fantes, P. A. (1987). A fission yeast chromosome can replicate autonomously in mouse cells. *Cell* **50,** 391–403.

Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988). *cdc2* is a component of the M phase-specific histone H1 kinase: Evidence for identity with MPF. *Cell* **55,** 371–378.

Aves, S. J., Durkacz, B. W., Carr, A., and Nurse, P. (1985). Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc10* "start" gene. *EMBO J.* **4** 457–463.

Barker, D. G., and Johnston, L. H. (1983). *Saccharomyces cerevisiae cdc9,* a structural gene for DNA ligase which complements *Schizosaccharomyces pombe cdc17*. *Eur. J. Biochem.* **134,** 315–319.

Barker, D. G., White, J. M., and Johnston, L. H. (1987). Molecular characterisation of the DNA ligase gene, *cdc17,* from the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **162,** 659–667.

Beach, D. H., and Nurse, P. (1981). High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature (London)* **290,** 140–142.

Beach, D., Durkacz, B., and Nurse, P. (1982a). Functionally homologous cell cycle control genes in budding and fission yeast. *Nature (London)* **300,** 706–709.

Beach, D., Piper, M., and Nurse, P. (1982b). Construction of a *Schizosaccharomyces pombe* gene bank and its use to isolate genes by complementation. *Mol. Gen. Genet.* **187,** 326–329.

Beach, D., Rodgers, L., and Gould, J. (1985). *ran1*$^+$ controls the transition from mitotic division to meiosis in fission yeast. *Curr. Genet.* **10,** 297–311.

Bishop, J. M. (1983). Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52,** 301–354.

Blackburn, E. H. (1985). Artificial chromosomes in yeast. *Trends Genet.* **1,** 8–12.

Blackburn, E. H., and Szostak, J. W. (1984). The molecular structure of centromeres and telomeres. *Annu. Rev. Biochem.* **53,** 163–194.

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of *cdc2*$^+$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **6,** 3523–3530.

Booher, R., and Beach, D. (1987). Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast; dissociation of the G_1 and G_2 roles of the *cdc2*$^+$ protein kinase. *EMBO J.* **6,** 3441–3447.

Booher, R., and Beach, D. (1988). Involvement of *cdc13*$^+$ in mitotic control in *Schizosaccharomyces pombe:* Possible interaction of the gene product with microtubules. *EMBO J.* **7,** 2321–2327.

Bostock, C. J. (1970). DNA synthesis in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **60,** 16–26.

Breeden, L., and Nasmyth, K. (1987). Similarity between cell-cycle genes of budding yeast and fission yeast and the *Notch* gene of *Drosophila*. *Nature (London)* **329,** 651–654.

Brizuela, L., Draetta, G., and Beach, D. (1987). $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. *EMBO J.* **6,** 3507–3514.

Byers, B. (1981). Cytology of the yeast life cycle. *In* "The Molecular Biology of the Yeast *Saccharomyces*" (J. N. Strathern, E. W. Jones, and J. R. Broach, eds.), pp. 59–96. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Carter, B. L. A. (1981). The control of cell division in *Saccharomyces cerevisiae*. *In* "The Cell Cycle" (P. C. L. John, ed.), pp. 99–117. Cambridge Univ. Press, London and New York.

Certa, U., Colavito-Shepanski, M., and Grunstein, M. (1984). Yeast may not contain histone H1: The only known "histone H1-like protein" in *Saccharomyces cerevisiae* is a mitochondrial protein. *Nucleic Acids Res.* **12,** 7975–7985.

Charlesworth, B., Langley, C. H., and Stephan, W. (1986). The evolution of restricted recombination and the accumulation of repeated DNA sequences. *Genetics* **112,** 947–962.

Choe, J., Schuster, T., and Grunstein, M. (1985). Organization, primary structure, and evolution of histone H2A and H2B genes of the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **5,** 3261–3269.

Clarke, L., and Carbon, J. (1985). The structure and function of yeast centromeres. *Annu. Rev. Genet.* **19,** 29–56.

Clarke, L., Amstutz, H., Fishel, B., and Carbon, J. (1986). Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8253–8257.

Cleveland, D. W. (1983). The tubulins: From DNA to RNA to protein and back again. *Cell* **34,** 330–332.

Cooper, S. (1979). A unifying model for the G_1 period in prokaryotes and eukaryotes. *Nature (London)* **280,** 17–19.

Costello, G., Rodgers, L., and Beach, D. (1986). Fission yeast enters the stationary phase G_0 state from either mitotic G_1 or G_2. *Curr. Genet.* **11,** 119–125.

Davidse, L. (1986). Benzimidazole fungicides: Mechanism of action and biological impact. *Annu. Rev. Phytopathol.* **24,** 43–65.

Dickinson, D. P. (1981). Polypeptide synthesis in cell cycle mutants of fission yeast. *J. Cell Sci.* **51,** 203–217.

Dickinson, J. R. (1981). The *cdc22* mutation in *Schizosaccharomyces pombe* is a temperature-sensitive defect in nucleoside diphosphokinase. *Eur. J. Biochem.* **119,** 341–345.

Draetta, G., and Beach, D. (1988). Activation of *cdc2* protein kinase during mitosis in human cells: Cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54,** 17–26.

Draetta, G., Brizuela, L., Potashkin, J., and Beach, D. (1987). Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by *cdc2*$^+$ and *suc1*$^+$. *Cell* **50,** 319–325.

Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988). The *Xenopus* homolog *cdc2* is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54,** 423–431.

Durkacz, B., Beach, D., Hayles, J., and Nurse, P. (1985). The fission yeast cell cycle control gene *cdc2*: Structure of the *cdc2* region. *Mol. Gen. Genet.* **201,** 543–545.

Durkacz, B., Carr, A., and Nurse, P. (1986). Transcription of the *cdc2* cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *EMBO J.* **5,** 369–373.

Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. S., and Liu, L. F. (1985). Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* **100,** 1706–1715.

Egel, R. (1973). Commitment to meiosis in fission yeast. *Mol. Gen. Genet.* **121,** 277–284.

Egel, R., and Egel-Mitani, M. (1974). Premeiotic DNA synthesis in fission yeast. *Exp. Cell Res.* **88,** 127–134.

Erard, M., and Barker, D. G. (1985). Electron microscopic studies of condensed mitotic chromosomes in the fission yeast *Schizosaccharomyces pombe*. *Biol. Cell.* **55,** 27–34.

Fantes, P. A. (1977). Control of cell size and cycle time in *Schizosaccharomyces pombe*. *J. Cell Sci.* **24,** 51–67.

Fantes, P. (1979). Epistatic gene interactions in the control of division in fission yeast. *Nature (London)* **279,** 428–430.

Fantes, P. A. (1981). Isolation of cell size mutants of a fission yeast by a new selective method: Characterization of mutants and implications for division control mechanisms. *J. Bacteriol.* **146,** 746–754.

Fantes, P. A. (1982). Dependency relations between events in mitosis in *Schizosaccharomyces pombe*. *J. Cell Sci.* **55,** 383–402.

Fantes, P. A. (1983). Control of timing of cell cycle events in fission yeast by the *wee1*$^+$ gene. *Nature (London)* **302,** 153–155.

Fantes, P. A. (1984a). Cell cycle control in *Schizosaccharomyces pombe*. *In* "The Microbial Cell Cycle" (P. Nurse and E. Streiblova, eds.), pp. 109–125. CRC Press, Boca Raton, Florida.

Fantes, P. A. (1984b). Temporal control of the *Schizosaccharomyces pombe* cell cycle. *In* "Cell Cycle Clocks" (L. N. Edmunds, ed.), pp. 233–252. Dekker, New York.

Fantes, P. A. (1988). Intersecting cell cycles. *Trends Genet.* **4,** 275–276.

Fantes, P., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth modulated size control over nuclear division. *Exp. Cell Res.* **107,** 377–386.

Fantes, P. A., and Nurse, P. (1978). Control of the timing of cell division in fission yeast: Cell size mutants reveal a second control pathway. *Exp. Cell Res.* **115,** 317–329.

Fantes, P. A., Grant, W. D., Pritchard, R. H., Sudbery, P. E., and Wheals, A. E. (1975). The regulation of cell size and the control of mitosis. *J. Theor. Biol.* **50,** 213–244.

Ford, C. C. (1985). Maturation promoting factor and cell cycle regulation. *J. Embryol. Exp. Morphol.* **89** (Suppl.), 271–284.

Fukui, Y., Kaziro, Y., and Yamamoto, M. (1986). Mating pheromone-like diffusible factor released by *Schizosaccharomyces pombe*. *EMBO J.* **5,** 1991–1993.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^+$. *Cell* **54,** 433–439.

Goebl, M., and Byers, B. (1988). Cyclin in fission yeast. *Cell* **54,** 739–740.

Gordon, C. B., and Fantes, P. A. (1986). The *cdc22* gene of *Schizosaccharomyces pombe* encodes a cell cycle-regulated transcript. *EMBO J.* **5,** 2981–2985.

Greider, C. W., and Blackburn, E. H. (1985). Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* **43,** 405–413.

Guerrini, A. M., Ascenzioni, F., Tribioli, C., and Donini, P. (1985). Transformation of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with linear plasmids containing 2μ sequences. *EMBO J.* **4,** 1569–1573.

Gygax, A., and Thuriaux, P. (1984). A revised chromosome map of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **8,** 85–92.

Hadwiger, J. A., and Reed, S. I. (1988). Invariant phosphorylation of the *Saccharomyces cerevisiae CDC28* protein kinase. *Mol. Cell. Biol.* **8,** 2976–2979.

Hagan, I. M., and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **89,** 343–357.

Hagan, I., Hayles, J., and Nurse, P. (1988). Cloning and sequencing of the cyclin related *cdc13*$^+$ gene and a cytological study of its role in fission yeast mitosis. *J. Cell Sci.* **91,** 587–595.

Harland, R. M., and Laskey, R. A. (1980). Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. *Cell* **21,** 761–771.

Hartwell, L. H. (1974). *Saccharomyces cerevisiae* cell cycle. *Bacteriol. Rev.* **38,** 164–198.

Hartwell, L. H., Culotti, J., Pringle, J. R., and Reid, B. J. (1974). Genetic control of the cell division cycle in yeast: A model. *Science* **183,** 46–51.

Hayles, J., Beach, D., Durkacz, B., and Nurse, P. (1986a). The fission yeast cell cycle control gene *cdc2*: Isolation of a sequence *suc1* that suppresses *cdc2* mutant function. *Mol. Gen. Genet.* **202,** 291–293.

Hayles, J., Aves, S., and Nurse, P. (1986b). *suc1* is an essential gene involved in both the cell cycle and growth in fission yeast. *EMBO J.* **5,** 3373–3379.

Hereford, L. M., and Hartwell, L. H. (1974). Sequential gene function in the initiation of *S. cerevisiae* DNA synthesis. *J. Mol. Biol.* **84,** 445–461.

Hereford, L. M., Osley, M. A., Ludwig, J. R., and McLaughlin, C. (1981). Cell-cycle regulation of yeast histone mRNA. *Cell* **24,** 367–375.

Heyer, W. D., Sipiczki, M., and Kohli, J. (1986). Replicating plasmids in *Schizosaccharomyces pombe*: Improvement of symmetric segregation by a new genetic element. *Mol. Cell. Biol.* **6,** 80–89.

Hindley, J., and Phear, G. (1984). Sequence of the cell division gene *cdc2* from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinasese. *Gene* **31,** 129–134.

Hindley, J., Phear, G., Stein, M., and Beach, D. (1987). *Suc1*$^+$ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **7,** 504–511.

Hinnen, A., Hicks, J. B., and Fink, G. R. (1978). Transformation of yeast. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 1929–1933.

Hirano, T., Funahashi, S., Uemura, T., and Yanagida, M. (1986). Isolation and characterization of *Schizosaccharomyces pombe cut* mutants that block in nuclear division but not cytokinesis. *EMBO J.* **5,** 2973–2979.

Hirano, T., Hiraoka, Y., and Yanagida, M. (1988). A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene *nuc2*$^+$ that encodes a nuclear scaffold-like protein blocks spindle formation in mitotic anaphase. *J. Cell Biol.* **106,** 1171–1183.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *nda3* gene of fission yeast encodes β-tubulin: A cold-sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39,** 349–358.

Hirschberg, J., and Simchen, G. (1977). Commitment to the mitotic cell cycle in yeast in relation to meiosis. *Exp. Cell Res.* **106,** 245–252.

Holm, C., Goto, T., Wang, J. C., and Botstein, D. (1985). DNA topoisomerase II is required at the time of mitosis in yeast. *Cell* **41,** 553–563.

Howard, A., and Pelc, S. R. (1953). Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity (Suppl.)* **6,** 261–273.

Hsiao, C. L., and Carbon, J. (1981). Direct selection procedure for the isolation of functional centromeric DNA. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3760–3764.

Hughes, D. A., and Fantes, P. A. (1989). In preparation.

Iino, Y., and Yamamoto, M. (1985a). Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. *Mol. Gen. Genet.* **198,** 416–421.

Iino, Y., and Yamamoto, M. (1985b). Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2447–2451.

Inglis, R. J., Langan, T. A., Matthews, H. R., Hardie, D. G., and Bradbury, E. M. (1976). Advance of mitosis by histone phosphokinase. *Exp. Cell Res.* **97,** 418–425.

Johnston, G. C., Pringle, J. R., and Hartwell, L. H. (1977). Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* **105,** 79–98.

Johnston, L. H., and Barker, D. G. (1987). Characterisation of an autonomously replicating sequence from the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **207,** 161–164.

Johnston, L. H., Barker, D. G., and Nurse, P. (1986). Cloning and characterisation of the *Schizosaccharomyces pombe* DNA ligase gene *CDC17*. *Gene* **41,** 321–325.

Kaufer, N., Simanis, V., and Nurse, P. (1985). Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. *Nature (London)* **318,** 78–80.

Kearsey, S. E. (1983). Analysis of sequences conferring autonomous replication in baker's yeast. *EMBO J.* **2,** 1571–1575.

Kearsey, S. (1984). Structural requirements for the function of a yeast chromosomal replicator. *Cell* **37,** 299.

King, S. M., and Hyams, J. S. (1982a). Interdependence of cell cycle events in *Schizosaccharomyces pombe*. Terminal phenotypes of cell division cycle mutants arrested during DNA synthesis and nuclear division. *Protoplasma* **110,** 54–62.

King, S. M., and Hyams, J. S. (1982b). Synchronisation of mitosis in a cell division cycle mutant of *Schizosaccharomyces pombe* released from temperature arrest. *Can. J. Microbiol.* **28,** 261–264.

Kirschner, M., Newport, J., and Gerhart, J. (1985). The timing of early developmental events in *Xenopus*. *Trends Genet.* **1,** 41–47.

Kohli, J. (1987). Genetic nomenclature and gene list of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **11,** 575–589.

Kohli, J., Hottinger, H., Munz, P., Strauss, A., and Thuriaux, P. (1977). Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidization. *Genetics* **87,** 471–489.

Kuenzler, P. (1985). The linear extrachromosomal DNA of *Physarum polycephalum* replicates and is maintained under non-selective conditions in two different lower eukaryotes. *Nucleic Acids Res.* **13,** 1855.

Labbe, J. C., Lee, M. G., Nurse, P., Picard, A., and Doree, M. (1988). Activiation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene *cdc2*$^+$. *Nature (London)* **335,** 251–254.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature (London)* **327,** 31–35.

Lee, M., and Nurse, P. (1988). Cell cycle control genes in fission yeast and mammalian cells. *Trends Genet.* **4,** 287–290.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterothallie bei *Schizosaccharomyces pombe*. *C. R. Lab. Carlsberg Ser. Physiol.* **24,** 381.

Leupold, U. (1987). Sex appeal in fission yeast. *Curr. Genet.* **12,** 543–545.

Lohka, M. J., Hayes, M. K., and Maller, J. L. (1988). Purification of maturation-promoting factor, and intracellular regulator of early mitotic events. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 3009–3013.

Lorincz, A., and Reed, S. (1984). Primary structure homology between the product of yeast cell division cycle gene *cdc28* and vertebrate oncogenes. *Nature (London)* **307,** 183–185.

Lycan, D. E., Osley, M. A., and Hereford, L. M. (1987). Role of transcriptional and posttranscriptional regulation in expression of histone genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7,** 614–621.

McCully, E. K., and Robinow, C. F. (1971). Mitosis in the fission yeast *Schizosaccharomyces pombe*: A comparative study with light and electron microscopy. *J. Cell Sci.* **9,** 475–507.

McLeod, M., and Beach, D. (1986). Homology between the *ran1*$^+$ gene of fission yeast and protein kinases. *EMBO J.* **5,** 3665–3672.

McLeod, M., and Beach, D. (1988). A specific inhibitor of the *ran1*$^+$ protein kinase regulates entry into meiosis in *Schizosaccharomyces pombe*. *Nature (London)* **332,** 509–514.

McLeod, M., Stein, M., and Beach, D. (1987). The product of the *mei3*$^+$ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J.* **6,** 729–736.

Marks, J., and Hyams, J. S. (1985). Localization of F-actin through the cell cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* **39,** 27–32.

Marks, J., Hagan, I. M., and Hyams, J. S. (1986). Growth polarity and cytokinesis in fission yeast: The role of the cytoskeleton. *J. Cell Sci. Suppl.* **5,** 229–241.

Matsumoto, S., and Yanagida, M. (1985). Histone gene organisation of fission yeast: A common upstream sequence. *EMBO J.* **4,** 3531–3538.

Matsumoto, S., Yanagida, M., and Nurse, P. (1987a). Histone transcription in cell cycle mutants of fission yeast. *EMBO J.* **6,** 1093–1097.

Matsumoto, T., Fukui, K., Niwa, O., Suguwara, N., Szostak, J. W., and Yanagida, M. (1987b). Identification of healed terminal DNA fragments in linear minichromosomes of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **7,** 4424–4430.

Maundrell, K., Wright, A. P. H., Piper, M., and Shall, S. (1985). Evaluation of heterologous *ARS* activity in *Saccharomyces cerevisiae* using cloned DNA from *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **13,** 3711–3722.

Maundrell, K., Hutchison, A., and Shall, S. (1986). Sequence analysis of *ars* elements from fission yeast. *Yeast* **2,** S233.

Maundrell, K., Hutchison, A., and Shall, S. (1988). Sequence analysis of *ARS* elements in fission yeast. *EMBO J.* **7,** 2203–2209.

Mechali, M., and Kearsey, S. (1984). Lack of specific sequence requirement for DNA replication in *Xenopus* eggs compared with high sequence specificity in yeast. *Cell* **38,** 55–56.

Mendenhall, M. D., Jones, C. A., and Reed, S. I. (1987). Dual regulation of the yeast *CDC28*–p40 protein kinase complex: Cell cycle, pheromone, and nutrient limitation effects. *Cell* **50,** 927–935.

Mertins, P., and Gallwitz, D. (1987). A single intronless actin gene in the fission yeast *Schizosaccharomyces pombe*: Nucleotide sequence and transcripts formed in homologous and heterologous yeast. *Nucleic Acids Res.* **15,** 7369–7379.

Minami, S., Collins, P. S., Young, E. E., and Weeks, D. P. (1981). Tubulin induction in *Chlamydomonas reinhardii*: Requirement for tubulin mRNA synthesis. *Cell* **24,** 89–95.

Minet, M., Nurse, P., Thuriaux, P., and Mitchison, J. M. (1979). Uncontrolled septation in a cell division cycle mutant of the fission yeast *Schizosaccharomyces pombe*. *J. Bacteriol.* **137,** 440–446.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exp. Cell Res.* **13,** 244–262.

Mitchison, J. M. (1970). Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* **4,** 131–165.

Mitchison, J. M. (1974). Sequences, pathways and timers in the cell cycle. *In* "Cell Cycle Controls" (G. M. Padilla, I. L. Cameron, and A. Zimmermann, eds.), pp. 125–142. Academic Press, New York.

Mitchison, T. J., Evans, L., Schulze, E. S., and Kirschner, M. W. (1986). Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell* **45,** 515–527.

Murray, A. W. (1987). Cyclins in meiosis and mitosis. *Nature (London)* **326,** 542–543.

Murray, A. W. (1988). A mitotic inducer matures. *Nature (London)* **335,** 207–208.

Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O., and Yanagida, M. (1986). Chromosome walking shows a highly homologous repetitive sequence present in all the centromeric regions of fission yeast. *EMBO J.* **5,** 1011–1021.

Nakaseko, Y., Kinoshita, N., and Yanagida, M. (1987). A novel sequence common to the centromere regions of *Schizosaccharomyces pombe* chromosomes. *Nucleic Acids Res.* **15,** 4705–4715.

Nasmyth, K. A. (1977). Temperature-sensitive lethal mutants in the structural gene for DNA ligase in the yeast *Schizosaccharomyces pombe*. *Cell* **12,** 101–106.

Nasmyth, K. (1979a). Genetic and enzymatic characterization of conditional lethal mutants of the yeast *Schizosaccharomyces pombe* with a temperature-sensitive DNA ligase. *J. Mol. Biol.* **130,** 275–284.

Nasmyth, K. A. (1979b). A control acting over the initiation of DNA replication in the yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **36,** 155–168.

Nasmyth, K., and Nurse, P. (1981). Cell division cycle mutants altered in DNA replication and mitosis in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **182,** 119–124.

Nasmyth, K., Nurse, P., and Fraser, R. S. S. (1979). The effect of cell mass on the cell cycle timing and duration of S-phase in fission yeast. *J. Cell Sci.* **39,** 215–233.

Niwa, O., Matsumoto, T., and Yanagida, M. (1986). Construction of a mini-chromosome by deletion and its mitotic and meiotic behaviour in fission yeast. *Mol. Gen. Genet.* **203,** 397–405.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. *Nature (London)* **256,** 547–551.

Nurse, P. (1981). Genetic control of the yeast cell cycle: A reappraisal of "start." *In* "The Fungal Nucleus" (K. Gull and S. Oliver, eds.), pp. 331–345. Cambridge Univ. Press, London and New York.

Nurse, P. (1985a). Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. *Mol. Gen. Genet.* **198,** 497–502.

Nurse, P. (1985b). Cell cycle controls in yeast. *Trends Genet.* **1,** 51–55.

Nurse, P., and Bissett, Y. (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. *Nature (London)* **292,** 558–560.

Nurse, P., and Fantes, P. A. (1981). Cell cycle controls in fission yeast: A genetic analysis. *In* "The Cell Cycle" (P. C. L. John, ed.), pp. 85–98. Cambridge Univ. Press, London and New York.

Nurse, P., and Thuriaux, P. (1977). Controls over the timing of DNA replication during the cell cycle of fission yeast. *Exp. Cell Res.* **107,** 365–375.

Nurse, P., and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics* **96,** 627–637.

Nurse, P., and Thuriaux, P. (1984). Temperature sensitive allosuppressor mutants of the fission yeast *Schizosaccharomyces pombe* influence cell cycle control over mitosis. *Mol. Gen. Genet.* **196,** 332–338.

Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **146,** 167–178.

Oakley, B. R., and Morris, N. R. (1981). A β-tubulin mutation in *Aspergillus nidulans* that blocks microtubule function without blocking assembly. *Cell* **24,** 837–845.

Ogden, J. E., and Fantes, P. A. (1986). Isolation of a novel type of mutation in the mitotic control of *Schizosaccharomyces pombe* whose phenotypic expression is dependent on the genetic background and nutritional environment. *Curr. Genet.* **10,** 509–514.

Okhura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T., and Yanagida, M. (1988). Cold-sensitive and caffeine-supersensitive mutants of the *Schizosaccharomyces pombe dis* genes implicated in sister chromatid separation during mitosis. *EMBO J.* **7,** 1465–1473.

Pardee, A. B., Dubrow, R., Hamlin, J. L., and Kletzien, R. F. (1978). Animal cell cycle. *Annu. Rev. Biochem.* **47,** 715–750.

Piggott, J., Rai, R., and Carter, B. (1982). A bifunctional gene product involved in two phases of the yeast cell cycle. *Nature (London)* **298,** 391–393.

Pines, J., and Hunt, T. (1987). Molecular cloning and characterisation of the mRNA for cyclin from sea urchin eggs. *EMBO J.* **6,** 2987–2995.

Potashkin, J. A., and Beach, D. (1988). Multiple forms of the product of the fission yeast cell division cycle gene *cdc2*$^+$. *Curr. Genet.* **14,** 235–240.

Pringle, J. R. (1978). The use of conditional lethal cell cycle mutants for temporal and functional mapping of cell cycle events. *J. Cell. Physiol.* **95,** 393–405.

Pringle, J. R., and Hartwell, L. H. (1981). The *Saccharomyces cerevisiae* cell cycle. *In* "The Molecular Biology of the Yeast *Saccharomyces*" (J. N. Strathern, E. W. Jones, and J. R. Broach, eds.), pp. 97–142. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Quastler, H., and Sherman, F. G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. *Exp. Cell Res.* **17,** 420–438.

Reed, S. I., Hadwiger, J. A., and Lorincz, A. T. (1985). Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4055–4059.

Reid, B. J., and Hartwell, L. H. (1977). Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. *J. Cell Biol.* **75,** 355–365.

Robinow, C. F. (1977). The number of chromosomes in *Schizosaccharomyces pombe*: Light microscopy of stained preparations. *Genetics* **87,** 491–497.

Rothstein, R. J. (1983). One-step gene disruption in yeast. *In* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, pp. 202–211. Academic Press, New York.

Roy, D., and Fantes, P. A. (1982). Benomyl resistant mutants of *Schizosaccharomyces pombe* cold-sensitive for mitosis. *Curr. Genet.* **6,** 195–201.

Russell, P., and Nurse, P. (1986). *cdc25*$^+$ functions as an inducer in the mitotic control of fission yeast. *Cell* **45,** 145–153.

Russell, P., and Nurse, P. (1987a). Negative regulation of mitosis by *wee1*$^+$, a gene encoding a protein kinase homolog. *Cell* **49,** 559–567.

Russell, P., and Nurse, P. (1987b). The mitotic inducer *nim1*$^+$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49,** 569–576.

Sachsenmaier, W. (1981). The mitotic cycle in *Physarum*. *In* "The Cell Cycle" (P. C. L. John, ed.), pp. 139–160. Cambridge Univ. Press, London and New York.

Sackett, D. L., and Lederberg, S. (1986). Colcemid-resistant mutants of fission yeast have an altered cell cycle. *Exp. Cell Res.* **163,** 467–476.

Schatz, P. J., Solomon, F., and Botstein, D. (1986). Genetically essential and nonessential α-tubulin genes specify functionally interchangeable proteins. *Mol. Cell. Biol.* **6,** 3722–3733.

Schild, D., and Byers, B. (1978). Meiotic effects of DNA-defective cell division cycle mutations of *Saccharomyces cerevisiae*. *Chromosoma* **70,** 109–130.

Schumperli, D. (1986). Cell-cycle regulation of histone gene expression. *Cell* **45,** 471–472.

Shampay, J., Szostak, J. W., and Blackburn, E. H. (1984). DNA sequences of telomeres maintained in yeast. *Nature (London)* **310,** 154–157.

Shortle, D., Novick, P., and Botstein, D. (1984). Construction and genetic characterization of temperature-sensitive alleles of the yeast actin gene. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4889–4893.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene *cdc2*$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45,** 261–268.

Singer, M. F. (1982). Highly repeated sequences in mammalian genomes. *Int. Rev. Cytol.* **76,** 67–111.

Singer, R. A., and Johnston, G. C. (1981). Nature of the G_1 phase of the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3030–3033.

Singer, R. A., and Johnston, G. C. (1985). Indirect suppression of the *wee1* mutant phenotype in *Schizosaccharomyces pombe*. *Exp. Cell Res.* **158,** 533–543.

Smith, C. L., Matsumoto, T., Niwa, O., Klco, S., Fan, J.-B., Yanagida, M., and Cantor, C. R. (1987). An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. *Nucleic Acids Res.* **15,** 4481–4489.

Solomon, M., Booher, R., Kirschner, M., and Beach, D. (1988). Cyclin in fission yeast. *Cell* **54,** 738–739.

Stetten, G., and Lederberg, S. (1973). Colcemid sensitivity of fission yeast. II. Sensitivity of stages of the cell cycle. *J. Cell Biol.* **56,** 259–262.

Streiblova, E., Hasek, J., and Jelke, E. (1984). Septum pattern in *ts* mutants of *Schizosaccharomyces pombe* defective in genes *cdc3, cdc4, cdc8* and *cdc12*. *J. Cell Sci.* **69,** 47–65.

Struhl, K., Stinchcomb, D. T., Sherer, S., and Davis, R. W. (1979). High frequency transformation of yeast: Autonomous replication of hybrid DNA molecules. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1035–1039.

Takeda, T., and Yamamoto, M. (1987). Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3580–3584.

Tanaka, K., and Kanbe, T. (1986). Mitosis in the fission yeast *Schizosaccharomyces pombe* as revealed by freeze-substitution electron microscopy. *J. Cell Sci.* **80,** 253–268.

Taylor, J. H. (1963). The replication and organization of DNA in chromosomes. *In* "Molecular Genetics" (J. H. Taylor, ed.), Part 1, pp. 65–111. Academic Press, New York.

Thuriaux, P., Nurse, P., and Carter, B. (1978). Mutants altered in the control co-ordinating cell division with cell growth in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **161,** 215–220.

Toda, T., Yamamoto, M., and Yanagida, M. (1981). Sequential alterations in the nuclear chromatin region during mitosis of the fission yeast *Schizosaccharomyces pombe*: Video fluorescence microscopy of synchronously growing wild-type and cold-sensitive *cdc* mutants by using a DNA-binding fluorescent probe. *J. Cell Sci.* **52,** 271–287.

Toda, T., Umesono, K., Hirata, A., and Yanagida, M. (1983). Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. *J. Mol. Biol.* **168,** 251–270.

Toda, T., Adachi, Y., Hiraoka, Y., and Yanagida, M. (1984). Identification of the pleiotropic cell cycle gene *nda2* as one of two different α-tubulin genes in *Schizosaccharomyces pombe*. *Cell* **37,** 233–242.

Tyson, J. J. (1984). The control of nuclear division in *Physarum polycephalum*. *In* "The Microbial Cell Cycle" (P. Nurse and E. Streiblova, eds.), pp. 175–190. CRC Press, Boca Raton, Florida.

Uemura, T., and Yanagida, M. (1984). Isolation of type I and type II DNA topoisomerase mutants from fission yeast: Single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO J.* **3,** 1737–1744.

Uemura, T., and Yanagida, M. (1986). Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: Uncoordinated mitosis. *EMBO J.* **5,** 1003–1010.

Uemura, T., Morikawa, K., and Yanagida, M. (1986). The nucleotide sequence of the fission yeast DNA topoisomerase II structural gene: Structural and functional relationships to other DNA topoisomerases. *EMBO J.* **5,** 2355–2362.

Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., and Yanagida, M. (1987). DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *Schizosaccharomyces pombe*. *Cell* **50,** 917–925.

Umesono, K., Toda, T., Hayashi, S., and Yanagida, M. (1983a). Two cell division cycle genes *NDA2* and *NDA3* of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to antimitotic benzimidazole compounds. *J. Mol. Biol.* **168,** 271–284.

Umesono, K., Hiraoka, Y., Toda, T., and Yanagida, M. (1983b). Visualisation of chromosomes in mitotically arrested cells of the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **7,** 123–128.

Wang, J. C. (1985). DNA topoisomerases. *Annu. Rev. Biochem.* **54,** 665–697.

Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C., and Yamamoto, M. (1988). The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *EMBO J.* **7,** 761–767.

White, J. H. M., Barker, D. G., Nurse, P., and Johnston, L. H. (1986). Periodic transcription as a means of regulating gene expression during the cell cycle: Contrasting modes of expression of DNA ligase genes in budding and fission yeast. *EMBO J.* **5,** 1705–1709.

Williamson, D. H. (1985). The yeast ARS element, six years on: A progress report. *Yeast* **1,** 1–14.

Wright, A. P. H., Maundrell, K., and Shall, S. (1986). Transformation of *Schizosaccharomyces pombe* by non-homologous unstable integration of plasmids in the genome. *Curr. Genet.* **10,** 503–508.

Yamamoto, M. (1980). Genetic analysis of resistant mutants to antimitotic benzimidazole compounds in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **180,** 231–234.

Yanagida, M., Hiraoka, Y., Uemura, T., Miyake, S., and Hirano, T. (1986). Control mechanisms of chromosome movement in mitosis of fission yeast. *In* "Yeast Cell Biology" (J. Hicks, ed.), pp. 279–297. Liss, New York.

Young, P. G., and Fantes, P. A. (1984). Changed division response mutants function as allosuppressors. *In* "Growth, Cancer, and the Cell Cycle" (P. Skehan and S. J. Friedman, eds.), pp. 221–228. Humana Press, Clifton, New Jersey.

Young, P. G., and Fantes, P. A. (1987). *Schizosaccharomyces pombe* mutants affected in their division response to starvation. *J. Cell Sci.* **88,** 295–304.

6

Cell Cycle Growth and Periodicities

J. M. MITCHISON

Department of Zoology
University of Edinburgh
Edinburgh EH9 3JT, Scotland

I. INTRODUCTION

An important question about the cell cycle is how a cell grows between one division and the next. Given that the strategy of a cell in balanced growth is to double all its components during a cell cycle, the tactics by which it does so can and do vary. DNA, for instance, is synthesized periodically, whereas a popular image is that many proteins follow a

MOLECULAR BIOLOGY OF THE
FISSION YEAST

smooth exponential pattern of synthesis. The aim of this chapter is to describe the patterns of cell cycle growth in *Schizosaccharomyces pombe* in which they have been better defined than in most other cellular systems.

Without exception, cell cycle growth in *S. pombe* is not a smooth exponential process but instead shows periodicities when the measurements have been made with sufficiently sensitive techniques. Periodicity is used here in a broad sense to include rate changes in a continuous pattern of synthesis as well as discontinuous synthesis. Three main patterns of growth have been found in the components that have been measured, and they are illustrated in a simplified form in Fig. 1. In the step pattern, there is a sharp doubling in amount at one stage of the cycle—a sharply periodic pattern. Figure 1A is what would happen in a single cell or a perfectly synchronous culture. Figure 1B is what happens in a normal synchronous culture where there is a spread of division times. The time for the step to be completed would be about the same as the time for completion of the cell number step. Figure 1C shows the peak that would

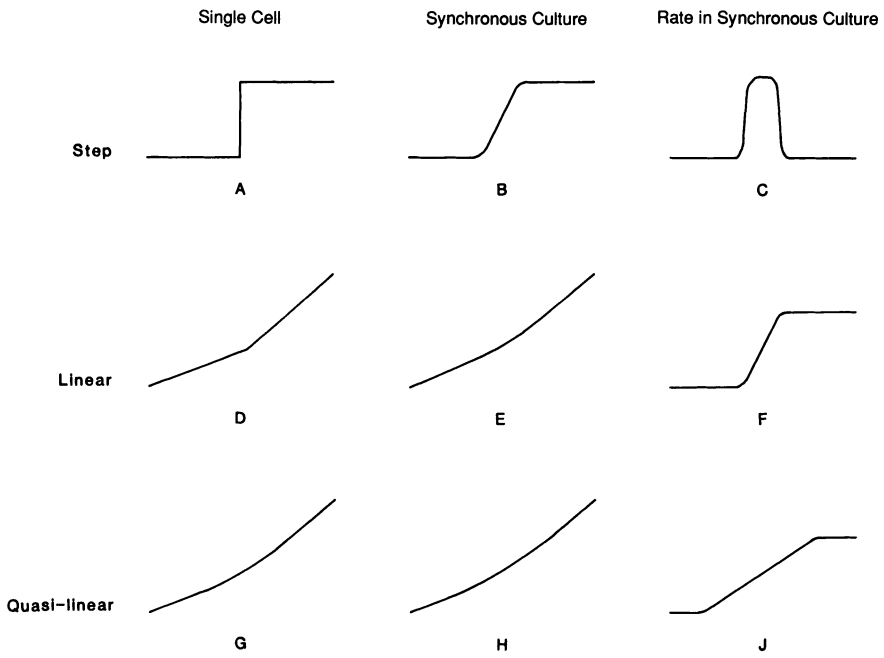


Fig. 1. (A–J) Patterns of growth during the cell cycle.

occur in a rate measurement, since it is the first differential of Fig. 1B. A common pattern in *S. pombe* is the linear one. A cell component is made at a constant rate until a particular point in the cycle when the rate doubles. This gives a pattern of two lines and a rate change point (Fig. 1D). Synthesis is continuous, but there is a periodic change in rate. In a normal synchronous culture, the rate change will not be instantaneous but will be spread out over a period of time equal to the time of completion of the cell number step (Fig. 1E). This pattern can be detected if the measurements are frequent and accurate, but it is better to use a rate measurement, if this is possible, since the linear pattern then produces a step (Fig. 1F). A variant of the linear pattern is the quasi-linear one. Here the change in rate takes an appreciable time (Fig. 1G) and is therefore even longer in a synchronous culture (Fig. 1H). It is difficult to detect this pattern in a synchronous culture, and a rate measurement is usually needed. The step in rate (Fig. 1J) takes a longer time than the step in rate for the ordinary linear pattern (Fig. 1F) or the step in cell number.

Some of the results come from single cell methods, as discussed later. But most of the work has been done with synchronous cultures, and it is worth making two important points about them here. The first is that the most reliable method of following growth patterns is to use a selection method where a fraction of cells at the same stage of the cycle are separated off from an asynchronous culture and grown up as a synchronous one. It is usually misleading to use an induction method in which an asynchronous culture is induced to divide synchronously by environmental shocks or chemical treatment (Mitchison, 1988). The best method of selection is to use an elutriating rotor (Creanor and Mitchison, 1979) or, if such a rotor is not available, sedimentation in a sucrose gradient (Mitchison and Carter, 1975; Mitchison, 1988). The second important point is the need to run asynchronous controls which have been through most of the selection procedure so as to ensure that the effects seen in the synchronous cultures are genuine cell cycle effects and not perturbations caused by the selection procedure which can, in some cases, be long lasting (Mitchison, 1977; Creanor and Mitchison, 1979). Another method of bulk analysis is age fractionation in which cells from an asynchronous culture are spread out on a gradient (usually in a zonal rotor). Samples from the gradient give cells at different stages of the cell cycle. This technique, which gives a high yield, has been used extensively for budding yeast but only to a limited extent for *S. pombe*. There is a considerable problem in determining the exact cycle stage of each sample (Creanor *et al.*, 1983; Mitchison, 1988). In addition, it is difficult to have a control against artifacts produced by separation on the gradient.

II. CELL CYCLE GROWTH

A. Length and Volume

Growth in cell length is relatively easy to measure on living cells under the microscope either directly or from photographs. Except in one case which is discussed later, there is general agreement that length increases for the first three-quarters of the cycle after which there is no further increase while the septum is being formed (Mitchison, 1957; May, 1962; Streiblova and Wolf, 1972; James *et al.*, 1975; Miyata *et al.*, 1978; Mitchison and Nurse, 1985).

This fairly simple pattern of "go–stop" conceals some complexities in the arrangements of the growing points of the cell wall. A septated cell toward the end of the cycle shows wall growth at the septum. When the cell divides, each daughter has a "new end" formed from splitting the septum and an "old end" which was present in the mother. Somewhat surprisingly, the growing point moves from the new end (where it had been forming the septum) to the old end. This has been called "Mitchison's rule" by Calleja *et al.* (1977), but it does not apply in all situations (Johnson *et al.*, 1982; Miyata *et al.*, 1986). Tip growth occurs at the old end for the first part of the cycle, and there is no wall growth elsewhere in the cell. Later in the cycle growth starts at the new end at a time called NETO, an acronym for "new end take-off" first observed by Streiblova and Wolf (1972). Growth stops at both ends at about 0.75 of cycle, and the septum becomes visible at about 0.85. The septum is roughly medial, but a careful study by Johnson *et al.* (1979) shows that although there are asymmetries in its length position, the volumes of the daughters are equal. The location of the growth points has been determined either by normal microscopy using division scars as markers or by fluorescence microscopy using Calcofluor or primulin. In addition, the changing position of the growth points is neatly paralleled by changes in the location of F-actin revealed within the cell by fluorescent microscopy and the use of phalloidin coupled to a fluorochrome (Marks and Hyams, 1985; Marks *et al.*, 1986).

In wild-type cells (strain 972), NETO takes place at 0.34 of the cycle with a cell length of 9.5 μm (Mitchison and Nurse, 1985). In the small *wee* mutants, however, the timing of NETO is size dependent. For example, in *wee1-1* NETO happens at the same length as in the wild type, but this length is not reached until later in the cycle (0.85). The position with large-size mutants (larger than wild-type at septation) is different. Here NETO occurs at the same stage of the cycle as wild type (about 0.32) but at a

larger cell size. The control in this case is probably a requirement to have completed an event in early G_2 since most *cdc* mutant cells blocked before this point do not show NETO whereas most of those blocked in late G_2 do show it. The general conclusion is that NETO only happens if two criteria are satisfied: (1) that the cell length is greater than a critical value of 9.0–9.5 μm and (2) that the cell has traversed the first 0.3–0.35 of the cycle and passed early G_2.

Although NETO is a well-defined event in the cycle, there is an appreciable variation in its timing (coefficient of variation of 19%) in wild-type cells. The mean timing varies not only in *wee* mutants but also in some other situations. It is delayed in poor media, in which cell size is also reduced. It is also delayed transiently after a temperature shift. In another wild-type strain, 132, it is 0.2 of a cycle later than in 972.

The observations above were made with cells stained with Calcofluor. This is quick and efficient but lacks a direct time dimension. Supplementary observations were therefore made of the rate of tip growth in time-lapse films (Mitchison and Nurse, 1985). These showed that the growth in length during the first three-quarters of the cycle follows a linear pattern with a rate change point (RCP) at which the rate of overall growth increases by 35%. Earlier observers had missed this pattern and had regarded growth as exponential, but it is not easy to demonstrate a linear pattern when the rate increase is markedly less than a doubling. It is also difficult to demonstrate if the RCP is near one end of the growth curve, as it may be in strain 132. The RCP is coincident with NETO in strain 972, and the simplest explanation would be that the commencement of growth at the end added an extra increment of growth rate onto a constant rate of growth at the old end. But this is not so since in some cells growth at the old end slows down after the RCP. It seems rather that there is an increase in length growth after the RCP but the partitioning of this growth between the two ends can vary considerably.

Films of blocked *cdc* mutants brought out three interesting points. *cdc11-123* continues growth and nuclear division at the restrictive temperature but septation is blocked. The result is long, multinucleate cells. Tip growth, however, stops at intervals, producing constant length phases which occur at normal cell cycle timings and which last for the same length of time as in the normal cycle. This suggests that the signal for the cessation of tip growth comes from mitosis and is not dependent on septum formation and the diversion of the cell's capacity for cell wall synthesis from the tips to the septum. *cdc2-33* continues to grow at the restrictive temperature but both nuclear division and septation are blocked, producing long, uninucleate cells. In some but not all blocked cells, there is a linear pattern of length growth with a RCP. This is an example, which

is discussed later, of a periodic cell cycle event (the RCP) which persists after a block to the DNA–division cycle. In both blocked mutants there are cells in which the normally tight coupling between NETO and the RCP is broken: a RCP can occur without NETO. This is another example of the dissociation of cell cycle events that can occur in blocked mutants.

The rates of tip growth in blocked cells of *cdc2-33* were measured by May and Mitchison (1986) using novel techniques equivalent to pulse labeling. Cells were coated with *Bandeiraea* lectin, plain or conjugated to a fluorochrome. They were then left to grow for a period after which the newly formed wall at the tip was coated again with the same lectin plus a different fluorochrome. The bicolored cells were analyzed in a fluorescence-activated cell sorter. A simpler and perhaps more striking technique, however, is to continue the alternating sequence of tip labeling for several hours. The result is a cell with a series of stripes (a "tiger-tail") which defines the rates of tip growth throughout the period. The tiger tails confirm the results from the films, namely, that some but not all of the blocked cells show a RCP during the period at the restrictive temperature. They also show, as did the films, that there is considerable variability among individual cells. This is more marked in this mutant but also occurs in wild-type cells, and it emphasises that analyses of whole populations can conceal what individual cells do.

The only analysis of the growth of a chemical component of the cell wall was made by Johnson (1965) using autoradiographs of alkali-resistant ghosts which had been pulse labeled with [^{3}H]glucose. The rate of labeling of the old end (primary end) increases with cell length. About 20% of the cells show incorporation at the new end (secondary end), but this is independent of length. There is also some incorporation in nonextensile regions of the wall which may represent thickening or turnover. This is consistent with the patterns of length growth described above except for one point. The old end shows an increasing rate of incorporation as the cells increase in length whereas its rate of elongation remains on average very nearly constant (Mitchison and Nurse, 1985). Two possible reasons for this are that different *S. pombe* strains (and different media) were used and also that the rate of glucose incorporation into the ghosts may not parallel the rate of tip growth.

Since *S. pombe* is a cylindrical cell which grows only at the tips and seems to maintain a constant diameter, it has sometimes been assumed that volume can be directly calculated from length. This was done in an early study (Mitchison, 1957) where the length of growing cells was measured frequently but the diameter only at the beginning of a run. This is clearly invalid if the diameter changes. Many people who have observed living cells or used films have not noticed changes in diameter, but they

may not have been looking carefully enough. Be that as it may, there is very little published information about diameter changes through the cell cycle. Kubitschek and Clay (1986), in a paper discussed below, find that cell diameter remains constant after minor adjustments early in the cycle providing the cells are growing with a "biphasic pattern" of length growth. Johnson and Lu (1975), however, find from population studies that there is an increase of about 10% in the maximum diameter toward the end of the cycle. The increase is associated with the number of division scars so the (rare) cells with five scars have a maximum diameter of 4.8 μm, 40% greater than the diameter of 3.4 μm in single-scar cells. But it is not easy to translate these changes into volumes since the multiscarred cells vary in diameter in different regions of the wall. This is shown in the diagram in Johnson and Lu (1975) where the regions between scars have a larger diameter than the ends of the cell. It would be interesting to know from electron microscopy whether the interscar regions had a thicker cell wall though it seems most unlikely that this alone could account for the increase in diameter.

A paper by Kubitschek and Clay (1986) throws an interesting new light on growth since they find two distinct patterns. The first one, called "biphasic," is very similar to the standard pattern described above. The length increases for the first three-quarters of the cycle, and there is a final plateau. The rate of length growth increases during the first part of the cycle, but it is not clear whether there is a smooth exponential rise or two linear segments. The diameter remains constant over nearly all the cycle. All the cells show this pattern when grown on an agar medium of high osmolality, for example, yeast extract–peptone–dextrose (YEPD) 4% agar. But if the osmolality is reduced, for example, YEPD plus 1.6% agar, the majority of the cells show another "linear" pattern (not the same as the linear pattern in Fig. 1). The constant length plateau appears still to be present but is markedly reduced, and, for most of the cycle, length growth is at a constant rate (linear). Another difference from the "biphasic" pattern is that the diameter increases at a steady rate through nearly all the cycle. As the authors say "... the difference between these two growth forms involves fundamental differences in their mechanisms for regulating increase in cell length and width." This is a very interesting situation and needs further investigation especially in liquid cultures, as the authors suggest. It should not be too difficult to do this since *S. pombe* cells with small amounts of polylysine in the medium stick very firmly to glass. But it should also be noted that the results with high and low agar concentrations are not altogether consistent with earlier work. The uniformly biphasic patterns with wild-type cells analyzed by Mitchison and Nurse (1985) came from films made by Fantes (1977) using a low agar concentration of 0.5%.

B. Dry Mass and Concentration

One of the earliest set of measurements of cell cycle growth in *S. pombe* was made by Mitchison (1957) using an integrating interference microscope to measure the total dry mass and "volume" (see above) of single growing cells. The dry mass follows a linear pattern with an RCP at the end of the cycle. The position of the RCP was not given in the paper, but I have examined the original data and find a mean position of the RCP as 0.91 of the cycle (SD = 0.15, n = 7). Later (Mitchison *et al.*, 1963) similar measurements were made at different growth temperatures over a range from 17 to 32°C. The volume growth curve remains the same over the temperature range but at the lower temperatures the dry mass curve shows a marked fall off in the rate of increase during the last one-quarter of the cycle, so coming to resemble the volume curve. This is one of the few occasions when the effect of temperature on cell cycle patterns has been followed, and the differences that emerge are interesting.

Dividing cell dry mass by volume gives a measure of (mass) concentration, and the derived curve for this fluctuates with a minimum at the start of the constant length plateau and a maximum at the end of the cycle. It is likely that this would cause fluctuations in the buoyant density. Kubitschek and Ward (1985), however, have measured buoyant density by gradient centrifugation and do not find any changes through the cycle. This is in accord with the "linear" growth pattern described by Kubitschek and Clay (1986) and discussed above, provided the cells in these experiments were growing with this pattern (and not "biphasically") and that mass growth was also linear. It is fair to say that we have not been able to separate cell cycle stages in *S. pombe* at all reliably using isopycnic centrifugation, which is consistent with unchanging buoyant density (J. M. Mitchison, unpublished). On the other hand, it is possible to do this in *Saccharomyces cerevisiae* (Hartwell, 1971), in which there are fluctuations in concentration (Mitchison, 1958) and in buoyant density (Baldwin and Kubitschek, 1984).

C. Protein

The synthesis of total protein is continuous, but there are changes in rate during the cell cycle. These are shown in Fig. 2, which illustrates three experiments (two synchronous cultures and one control) in which the rate is measured by the amount of labeled tryptophan incorporated in a 12-min pulse (Creanor and Mitchison, 1982). The rate follows a periodic pattern which is similar to the quasilinear pattern in Fig. 1J but is less symmetrical. There is a sharp increase in the rate of the rate at about 0.9

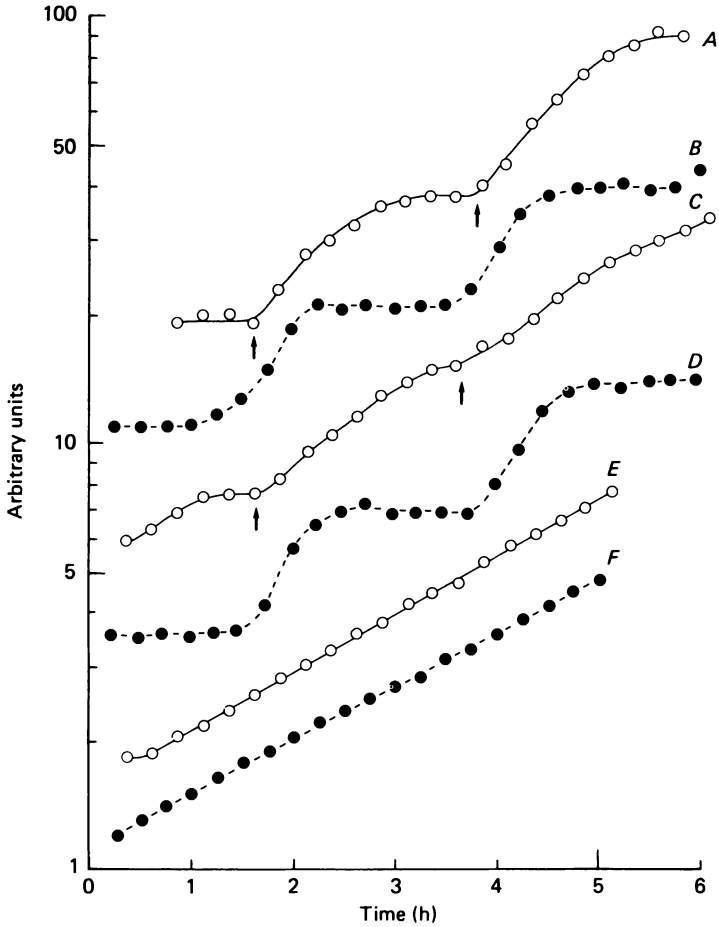


Fig. 2. Rate of tryptophan incorporation in *S. pombe* strain 972 at 35°C in Edinburgh minimal medium (EMM3) plus 10 μg/ml tryptophan. Each point in *A* and *C* is the mean of two samples. *(A)* Synchronous culture. Samples (0.25 ml) labeled for 12 min with 307 kBq [^{3}H]tryptophan. Arrows indicate acceleration points; 10 arbitrary units (au) equal 1560 cpm. *(B)* Cell numbers in synchronous culture *A*; 10 au equal 0.92 × 10^6 cells/ml. *(C)* Synchronous culture. Labeling as for *A*; 1 au equals 682 cpm. *(D)* Cell numbers in synchronous culture *C*; 1 au equals 0.35 × 10^6 cells/ml. *(E)* Asynchronous control cultures. Samples labeled for 14 min with 370 kBq [^{3}H]tryptophan; 1 au equals 4170 cpm. *(F)* Cell numbers in control culture *E*; 1 au equals 1.85 × 10^6 cells/ml. [Reproduced from Creanor and Mitchison (1982).]

of the cycle, called the "acceleration point," followed by a period of declining rate of increase of rate until there is short plateau before the next acceleration point in the next cycle. The acceleration point was used as a marker of the pattern because the slight asymmetry of the pattern

makes this point conspicuous. But if the midpoint of the rate step had been used as the marker (as is more usual with cell cycle steps) this would have been at 0.36 of the cycle.

Growth patterns that occur at the level of the single cell are smoothed out by the normal imperfect synchrony that occurs in all types of synchronous culture. It is possible to correct for these imperfections by modeling, though assumptions have to be made about the model that is applied. When applied to these experiments, this procedure with one model suggests that the average single cell has an increasing rate of synthesis for the first 60% of the cycle and a constant rate for the remaining 40%.

The same cell cycle pattern occurs with two other labeled amino acids, leucine and phenylalanine. It also occurs with a range of size mutants, which indicates that the pattern is not size related, in contrast to the situation with growth in length. But there is one exception in the large mutant (*cdc2-M35r20*), where the acceleration point is slightly but significantly earlier.

Certain assumptions are made when the rate of total protein synthesis is taken to be proportional to the rate of incorporation of an exogenous amino acid. These assumptions, discussed by Creanor and Mitchison (1982) in the light of further experiments with a tryptophan auxotroph, have been largely justified.

It is important to know whether the periodic fluctuations in the rate of synthesis are associated with any of the periodic events of the DNA–division cycle. They do not appear to be associated with the S period since the acceleration points have the same cell cycle position in *wee* mutants as in wild-type cells. Yet the S period is delayed in such mutants by about one-third of the cycle compared to wild type (Table I). Nor are the patterns associated with cleavage since they persist in synchronous cultures of *cdc11* growing at the restrictive temperature where nuclear division continues but septation and cleavage are blocked (Creanor and Mitchison, 1984). There is a definite association with mitosis, however, since the fluctuations vanish after DNA–division blocks imposed by a shift-up in temperature of *cdc2* and *cdc10* in synchronous cultures. The rate of synthesis continues to rise after the block at the normal rate, but there are no cell cycle periodicities. It seems from these results with *cdc* mutants and from the earlier ones with wild-type cells that mitosis affects the rate of synthesis and that this effect is inhibitory since the rate falls off toward the end of the cycle prior to the acceleration point. One model, mentioned above, suggests that the inhibitory effect starts at 0.6 of cycle, which is 0.15 of the cycle before mitosis. If true, this would be a very early event in the preparations for mitosis. But such deductions are critically dependent on the model used, and another model in which there is a

sharp reduction in the rate of protein synthesis for a short time at mitosis would give quite a good fit to the experimental results (P. A. Fantes, unpublished). There is evidence against this model described in Section II,E, but the only safe conclusion at present is that the exact timing and extent of the mitotic inhibition are uncertain.

An interesting aspect of the results after a *cdc* mutant block is that the rate of synthesis continues to rise but only for a time. At the end of this period, the rate reaches a plateau and becomes constant. The length of the period varies with cell size, and cells which are initially small (e.g., *wee* mutants) take longer to reach the plateau. From limited information with three size mutants of *cdc2* and with *cdc10,* it seems that the plateau starts when cells reach a critical protein/DNA ratio. As mentioned later, there are similar effects with other rate measurements such as RNA synthesis, CO_2 production, and enzyme potential.

Protein synthesis in *S. pombe* has been examined in some earlier papers. In the days before synchronous cultures, Mitchison and Wilbur (1962) measured the rate of incorporation of labeled amino acids by single cells using grain counting on autoradiographs and positioning the cells in the cycle by their length. This method is less sensitive than synchronous cultures both because cells have to be grouped into large length classes to reduce the variation in grain numbers and because there is some uncertainty about the limits of the cycle (an inherent problem in age fractionation). The results are consistent with the tryptophan pulses in synchronous cultures described above. There is a rising rate of incorporation with leucine, glycine, and methionine at 25°C and an acceleration point toward the end of the cycle.

Stebbing (1971) found an approximately exponential rise in total protein in synchronous cultures. These, however, were measurements of absolute amounts rather than rates and were too insensitive to detect the quasi-linear pattern in rate. Wain (1971) measured the rate of incorporation of leucine into soluble protein in synchronous cultures and showed that the rate increased during the cycle. There is no indication of an acceleration point in these results, but the scatter is considerable, owing primarily to the method of sample preparation. Wain also followed the apparent rate of synthesis of protein or groups of proteins separated by one-dimensional gel electrophoresis and did not find any evidence of periodic synthesis at this level of resolution. There is no equivalent in *S. pombe* of the careful two-dimensional gel analysis of the cell cycle of *Sacch. cerevisiae* by Lorincz *et al.* (1982) and by Elliott and McLauchlin (1978). Two-dimensional gels of *S. pombe* have, however, shown that there are no qualitative changes in the labeling of over 700 polypeptides after the expression of *wee* and *cdc* phenotypes (Dickinson, 1981a).

TABLE I

Timing and Duration of the S. Phase in *Schizosaccharomyces pombe*[a]

Strain	Temperature (°C)	Midpoint of S phase	Length of G_1 (from mitosis to initiation of S phase)		Duration of S phase	References
			From synchronous cultures	From autoradiographs		
WT 132	32	0.86				Bostock (1970a)
WT 132	32	0.01				Mitchison and Creanor (1971)
WT 972	32	0.97				Mitchison and Creanor (1971)
WT 972 diploid	32	0.00				Mitchison and Creanor (1971)
WT 972	35	0.00				Nurse and Thuriaux (1977)
WT 972	25	0.98	0.12	0.08	0.09	Nasmyth *et al.* (1979)
wee1-1	25	0.31	0.51		0.11	Nasmyth *et al.* (1979)
wee1-6	25	0.38				Dickinson (1983)
wee1-50	35	0.29				Nurse and Thuriaux (1977)
wee1-50	25	0.05	0.23	0.17	0.12	Nasmyth *et al.* (1979)
wee1-302	35	0.98				Novak and Mitchison (1986)
wee2-1	35	0.23				Nurse and Thuriaux (1977)
wee2-1	25	0.15	0.36		0.07	Nasmyth *et al.* (1979)
cdc2-M35	25	0.99	0.13	0.09	0.07	Nasmyth *et al.* (1979)

[a]All results are from cells growing in minimal medium and are expressed in fractions of the cycle. Some other results on wild-type cells have been omitted since they are essentially confirmatory (Bostock *et al.*, 1966; Bostock, 1970b; Fraser and Moreno, 1976).

D. DNA

It has been known since the early 1970s that DNA synthesis in *S. pombe* is sharply periodic in wild-type cells and takes place at about the time of final cell cleavage right at the end of the cycle. As with budding yeast, it is impossible to label DNA specifically with radioactive thymidine because the cells lack thymidine kinase. Most of the earlier experiments therefore used a bulk DNA assay (e.g., the diphenylamine reaction) on synchronous cultures. In later experiments, Nasmyth *et al.* (1979) developed methods of pulse labeling DNA either for bulk counting or for autoradiographs in which a general nucleic acid label was used ([^{3}H]uracil or [^{3}H]guanosine) and the RNA removed by treatment with alkali and RNase.

Table I shows collected results for wild-type and mutant cells which have approximately the same generation times. Wild-type cells of two strains (132 and 972) have the midpoint of the S period at the end of the cycle. This period is preceded by a short G_1 and followed by a long G_2. The approximate values for the length of these periods are given in Table II, but it must be remembered that it is difficult to tell the beginning and the end of mitosis. Although there are no gross differences between the different wild-type strains and among different temperatures in the range 25–35C, there may perhaps be fine differences. The same pattern was also found in the large cells of both 972 diploids and the mutant *cdc2-M35* which is 70% bigger than 972 at the permissive temperature of 25°C.

The *wee* mutants in Table I (apart from *wee1-302*) all show a delayed S period when they are expressing the *wee* phenotype of small size at divi-

TABLE II

Nuclear and DNA Cycle[a,b]

Phase	Start	Finish	Duration
Mitosis	0.76	0.81	0.05
G_1	0.81	0.95	0.14
S	0.95	0.05	0.10
G_2	0.05	0.76	0.71

[a]Approximate values for WT strains 972 and 132, in fractions of the cycle.

[b]The mitotic timing comes from a film of strain 132 at 29.5°C in complex medium with the refractive index raised with gelatin (J. M. Mitchison, unpublished). The start was the first sign of nuclear elongation, and the finish was when the nuclei reached the ends of the cell. Other timings are from Table I.

sion. *wee1-50* is temperature sensitive and only expresses the full phenotype at 35°C. This elongated G_1 and delayed S can be explained by assuming two types of control over the initiation of DNA synthesis (Nurse and Thuriaux, 1977). In the first type, there is a "size control" such that the cell has to reach a minimum size (6–7.5 pg protein/cell) before DNA synthesis can be initiated. *wee* cells are below this size so they have to grow during G_1 to achieve it. Larger *wee* cells such as *wee2-1* reach it sooner than small ones such as *wee1-1*, and so there is less delay of the S period. A minimum size for initiation is also found outside the normal cell cycle in spore germination and in recovery after nitrogen starvation. In the second type of control, shown in growing wild-type cells, the DNA size control is cryptic since the cells are always above the minimum size and a mitotic size control is in operation (Fantes, Chapter 5, this volume). DNA initiation is subject to the normal dependency relation which insists that it has to follow mitosis though it is not clear why there is a G_1 even though it is short. A final point about Table I is that it shows no S period delay in *wee1-302*. This mutant is a "partial" *wee* with a size intermediate between full *wee* mutants and wild type. The protein content after division is 6.2 pg/cell (Creanor and Mitchison, 1982) so it just escapes an S period delay.

The question arises as to what happens to the pattern of DNA synthesis if the cycle time is extended. Altering cycle time by altering temperature does not change the pattern, at any rate within the range of 25–35°C. However, extending the cycle time by other means does change the pattern by increasing the relative length of G_1 with S plus G_2 being constant, as is true for many other cells. This has been shown in *S. pombe* with nitrogen-limited growth in a chemostat (Nasmyth, 1979), with phosphate-limited growth in a chemostat (Bostock, 1970b), with the presence of phenylethanol (Bostock, 1970a) and with 2 *M* glucose in the medium (Duffus and Mitchell, 1970). Some of these techniques produce small cells, and G_1 could be extended for the same reason as in *wee* cells, though Nasmyth (1979) finds a smaller minimum size for DNA initiation than Nurse and Thuriaux (1977).

There are no reliable measurements of cell cycle changes in mitochondrial DNA. This type of DNA makes up 6% of the total DNA in exponential growth and 14% in stationary phase (Bostock, 1969).

E. RNA

Earlier experiments, before the advent of synchronous cultures, showed a continuous and approximately exponential increase through the

cycle in total RNA, largely ribosomal (Mitchison and Walker, 1959; Mitchison and Lark, 1962; Mitchison *et al.*, 1969). Later work with the more precise technique of pulse labeling synchronous cultures suggested that the pattern of increase was a linear one with a rate-doubling point at the end of the cycle, that is, a step in rate (Wain and Staatz, 1973; Fraser and Moreno, 1976). The most thorough and extensive measurements on total RNA (very largely rRNA) have been made by Elliott (1983a,b), using a range of mutants and diploids as well as wild-type cells. He also investigated the precursor pools in order to justify the use of [^{3}H]uridine pulses as a measure of the rate of RNA synthesis. The picture that emerges is similar to that for total protein. The pattern in synchronous cultures is a quasi-linear one with an acceleration point marginally earlier than that for protein. In wild-type cells of strain 972 in minimal medium, the acceleration point is at 0.84 of the cycle, and the midpoint of the rate-doubling step is at 0.10. The timing of this step is not size related since it does not change significantly in mutants which vary in size by nearly 3 times. Nor is it related to the S period since it does not change in *wee* mutants, where the S period is delayed. As with protein, the steps appear to be related to mitosis since they vanish after imposing a block in synchronous cultures of *wee1-6 cdc2-33* and *wee-2-1 cdc10-129*. There is an initial perturbation on shifting up the temperature, but thereafter the rate of RNA synthesis increases exponentially until it reaches a "transcription maximum," a rate plateau similar to that with protein.

Although there are substantial similarities between the patterns for rRNA and total protein, there are differences in the timing of the start of the rate plateau, which is earlier for RNA than for protein (discussed in Creanor and Mitchison, 1984). In addition, the results from a wide range of mutants do not support the hypothesis that the RNA plateau starts at a critical protein/DNA ratio (Elliott, 1983b). It remains to be seen whether the maximal rates of synthesis of protein and rRNA in blocked mutants are controlled by different mechanisms.

One possible explanation for the falloff in rate increase before the acceleration point in synchronous cultures is that there is a short period during mitosis when RNA synthesis stops completely. This happens in most eukaryotes but has not been reported in yeast. Most of the methods that have been used, however, are not sufficiently sensitive to detect a gap in synthesis of only a few minutes. Creanor and Mitchison (1982) therefore reexamined the data of Mitchison and Lark (1962) on grain counting in autoradiographs of cells labeled with [^{3}H]adenine for 1 min. A short gap in synthesis at mitosis should cause an increase in variation in grain counts of cells at the end of the cycle. But there was no such increase either with the [^{3}H]adenine label or with an [^{3}H]leucine label for 1 min that could

detect an equivalent gap in protein synthesis (V. Zachleder, unpublished). A complete stoppage of RNA synthesis at mitosis is therefore unlikely, but, as with protein, the exact timing and extent of the mitotic inhibition are uncertain.

Fraser, Nurse, and colleagues examined the pattern of synthesis of polyadenylated messenger RNA and described their results in a series of papers (Fraser and Moreno, 1976; Fraser and Nurse, 1978; Barnes *et al.*, 1979; Fraser and Nurse, 1979). In wild-type cells, the pattern was very similar to that of rRNA with a step in rate, but the step was marginally later, at 0.12 of the cycle (Fraser and Moreno, 1976; Fraser and Nurse, 1978). In a series of size mutants and diploids, however, the step could be delayed—in the case of *wee1-50* until 0.81 of the cycle. This lead to the suggestion that the timing of the step was size related and that small cells could adjust their growth rate to lower overall values by delaying the step. The most sensitive test for this kind of hypothesis is to examine the situation of a step in midcycle since a step at the end of the cycle can be confused with one at the beginning of the next cycle. Here there is a conflict of evidence. The partial *wee* mutant *1-302* was not examined by Fraser and Nurse (1978), but from their Fig. 4 the prediction is that the step should be at midcycle (~0.5). Elliot (1983a), however, found that poly(A)$^+$ mRNA in *wee1-302* followed the pattern of rRNA with a step at 0.17, only slightly later than wild type. Another conflict, though it is strictly speaking about rRNA, is that Fraser and Nurse (1979) found a midcycle step in both rRNA and poly(A)$^+$ RNA in the heterozygous diploid *wee1-50/972* whereas Elliott (1983a) found the step in rRNA in this diploid at the beginning of the cycle at 0.16. The reason for these discrepancies with the mutants and the diploids is not obvious, and some of the experiments need repeating; however, it is worth pointing out that the technique used by Fraser and Nurse (1979) for preparing synchronous cultures from a zonal rotor is much more likely to produce perturbations than the elutriation method used by Elliott (1983a). Until these matters are clarified, the question of whether there is size-related timing of the RNA steps must remain in suspense.

Recent advances in recombinant DNA techniques and the development of efficient methods for transformation in yeast have enabled a number of genes to be isolated and cloned. The use of these clones as hybridization probes on RNA samples from synchronous cultures makes it possible to follow the cell cycle pattern of specific mRNA transcripts. In two cases, there is a single peak of transcript level during the cycle. One is for the H2A1 histone gene where the peak is at approximately the end of the cycle during the S period, as would be expected from the experiments on histone synthesis in other organisms (Aves *et al.*, 1985; Durkacz *et al.*,

1986). The other is for *cdc22* where the peak is at about 0.9 of the cycle (Gordon and Fantes, 1986). The *cdc22*$^+$ gene product is required for the initiation of DNA synthesis, but it is probably not nucleoside-diphosphate kinase as was suggested by Dickinson (1981b). As Gordon and Fantes (1986) point out, the periodicity in the transcript level might be due either to periodic transcription or to cell cycle-specific changes in the transcript stability. With four other genes concerned with DNA synthesis and mitosis, there are no conspicuous changes in transcript levels through the cycle. These are *cdc2* (Durkacz *et al.*, 1986), *cdc10* (Aves *et al.*, 1985), *cdc17* (White *et al.*, 1986), and the 1.5-kb (kilobase) transcript of *suc22* (Gordon and Fantes, 1986). It is clear that there are no peaks in transcript levels, but the problem is that subtler changes would be difficult to detect. A linear pattern of transcript level with a rate change once per cycle certainly could not be resolved on a standard Northern blot. Even if the transcript level had a stepwise doubling during the cycle, it is unlikely that this could be determined with certainty at the present time. It is therefore premature to say that the four genes above are not cell cycle regulated. Improved techniques may well reveal cell cycle patterns which are not apparent at present.

F. Carbon Dioxide Production

Carbon dioxide production is a measure of the glycolytic flux, which in *S. pombe* is responsible for about one-half the total ATP production (Hamburger *et al.*, 1977). It has been measured by manometry in synchronized cultures (Creanor, 1978a; Hamburger *et al.*, 1977; Novak and Mitchison, 1986). Hamburger *et al.* (1977) also made an impressive set of measurements on single cells and their resulting clones in diver respirometers. Apart from one small point of divergence (discussed in Novak and Mitchison, 1986), there is general agreement about the cell cycle pattern and the fact that there is a cell cycle periodicity albeit in the fine detail of CO_2 production. The rate of production follows a linear pattern and increases steadily through the cycle, with the acceleration (rate of rate increase) staying constant. At the end of the cycle there is a rate change point at which the acceleration doubles. This is equivalent to a stepwise increase in the second differential of CO_2 production. The effect is best shown as an oscillating curve of difference between acceleration in a synchronous culture and acceleration in an asynchronous control culture run at the same time (Fig. 3).

There is a tight association between the time of the rate change point and the end of the cycle, both when growth is speeded up in rich medium

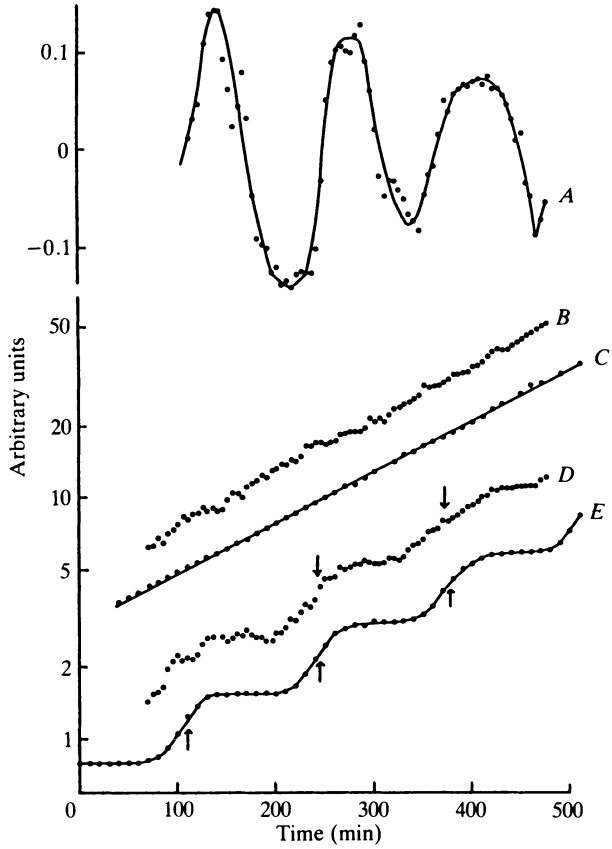


Fig. 3. Acceleration of CO_2 production in a synchronous and an asynchronous culture of *S. pombe* strain 972 cells in EMM3 at 35°C. *(A)* Difference between acceleration curves *D* and *B*. Ordinate, ln *D* − ln *B*. *(B)* Acceleration in asynchronous culture; 1 au (arbitrary unit) equals 6×10^{-4} μl CO_2/min^2. *(C)* Cell number in asynchronous culture; 1 au equals 2.1×10^5 cells/ml. *(D)* Acceleration in synchronous culture; 1 au equals 2×10^{-3} μl CO_2/min^2. *(E)* Cell number in synchronous culture; 1 au equals 10^6 cells/ml. Upper arrows show the midpoint of the acceleration steps, and the lower arrows show the midpoint of the cell number steps. [Reproduced from Novak and Mitchison (1986).]

and when it is slowed down in poor medium and at lower temperatures (Novak and Mitchison, 1986). It is also maintained after a shift-up in temperature. There is some evidence, though not conclusive, that the association is with the S period rather than division itself. This comes from results with a *wee* mutant in which the S period is shifted away from its normal coincidence with division and so is the rate change point.

Creanor (1978a) found that the periodicities continued for one to two cycle times after the DNA–division cycle had been blocked in a synchro-

nous culture by chemical inhibitors (deoxyadenosine and mitomycin C). Novak and Mitchison (1986) found the same result using a temperature-sensitive mutant (*cdc2*) to impose the block. The acceleration comes to a plateau value some time after the block has been imposed, as also happens with the rate of protein and RNA synthesis and with enzyme potential. This limits the period over which the free-running oscillations can be observed, but it can be increased by starting with the small cells of *wee* mutants. With synchronized cells of the double mutant *cdc2-33 wee1-6* there are two clear oscillations before the plateau sets in. The period of the oscillations is about 85% of the normal cell cycle time. Carbon dioxide oscillations also persist in synchronized cultures where protein synthesis has been blocked by cycloheximide (Novak and Mitchison, 1987). This is a dying system where the rate of CO_2 production is falling away, but oscillations continue for at least three cycles. Their period is about 60% of the normal cycle time.

These changes in CO_2 production are good examples of periodicities which appear when cell cycle growth is examined in detail and with accurate techniques. The generation of CO_2 is due to a series of enzyme reactions, and it is very much a bulk property of the cell. How then could there be these steps in the second differential? A very simple mechanism would be that a rate-limiting component of the glycolytic pathway, perhaps an enzyme, doubled its rate of synthesis (or activity increase) at the time of the S period. If it followed a linear pattern like the enzymes described later, and the rate of CO_2 production was proportional to the amount of the enzyme, then this rate would show the linear pattern observed and the steps in acceleration. It is possible this could be a gene dosage effect where doubling the number of gene copies would double the rate of production of the enzyme. This suggestion, however, could only apply to the normal cell cycle since the rate changes persist after a block to DNA synthesis. This persistence is a most interesting point and is discussed later in this chapter. The equally interesting persistence in the presence of cycloheximide shows that under these conditions there is a metabolic oscillation which is independent of translation, though it should be remembered that its period is appreciably shorter than the cycle time and the controls may be different from those that operate normally.

G. Oxygen Uptake

The situation concerning oxygen uptake is somewhat confused, as it is with *Sacch. cerevisiae* (reviewed briefly by Creanor, 1978b). Earlier work using unsatisfactory starvation techniques for synchronization showed one or two steps per cycle in the rate of oxygen uptake (Osumi and Sando,

1969; Osumi *et al.*, 1968; Marchant, 1971). Poole *et al.* (1973) found two peaks per cycle in the rate of uptake in glucose-grown cells but two steps per cycle in glycerol-grown cells (Poole and Lloyd, 1974). These two papers describe careful work, but there is a general problem about the lack of controls, which is discussed later in Section II,I. In addition there is some evidence of unbalanced growth in the glycerol-grown cells since the increase in rate over two steps is considerably more than a doubling.

Creanor (1978b) used selection from tube gradients and ran separate asynchronous controls. In glucose-grown cells, there were two steps in rate, one in midcycle and the other at the end of the cycle. This is similar to the results of Poole and Lloyd (1974) on glycerol-grown cells through growth was more balanced. Creanor also showed that the normal pattern of rate changes persisted after a block to DNA synthesis by deoxyadenosine. B. Novak (personal communication) has done preliminary experiments using a synchronous culture selected by an elutriating rotor and, in parallel, an asynchronous culture made from the residual cells in the rotor. This technique, first used for measuring CO_2 production by Novak and Mitchison (1986), gives the best control for eliminating synchronization artifacts. It shows that there are stepwise increases in the rate of oxygen uptake but only one per cycle, namely, in midcycle.

A safe conclusion is that there are definite periodicities in the rate of oxygen uptake and what evidence there is suggests that these periodicities persist after a DNA block. But whether there are one or two steps is not yet certain. Novak's method is probably the best, but it needs to be extended to some of the strains and media used in the earlier experiments.

H. Acid-Soluble Pool

Changes in the total dry mass of the acid-soluble pool were measured by Mitchison and Cummins (1964) using interferometry on single cells, living and acid-extracted, which had been grown on a rich medium and positioned in the cell cycle by their length. This method showed a maximum pool size in midcycle, but it can be criticized because the pool, as a percentage of the total cell dry mass did not return, as it should, to the same value at the end of the cycle as it was at the beginning. This may be because of the difficulty mentioned earlier of determining where the cycle starts and stops in age fractionation results.

Total pool dry mass together with pool "nucleotides" and amino acids (a total of 13) were measured in synchronous cultures by Stebbing (1971, 1972). All measurements show an approximately exponential rise through the cycle. The data are not sufficiently precise to establish whether there is a quasi-linear pattern, but a statistical analysis shows that there is a

better fit to an exponential pattern than to a linear one. The results from these synchronous cultures are clearly different from those from interferometry, but it should be remembered that the former were on cells growing in minimal medium and the latter on cells in a rich medium. There is an expandable metabolic pool of considerable size (Stebbing, 1971) which is much larger during growth in a rich medium. Changes in the tryptophan and leucine pool were also measured by Creanor and Mitchison (1982) using an indirect and not very accurate method. There were no signs of cell cycle periodicities.

Considering that both total protein and rRNA follow a quasi-linear pattern and that the acid-soluble pool contains many of their precursors, it would be valuable to know whether the pool and its components also show this pattern. But it would not be a trivial task to measure pool sizes with sufficient accuracy to establish this.

I. Enzyme Activity

Patterns of enzyme activity through the cell cycle have been studied for many years, but the results have not always been reliable. Some of the difficulties have arisen because the assay methods were not sufficiently reproducible for a clear definition of the pattern. But the main problem is that the methods of producing synchronous cultures can undoubtedly produce perturbations which distort the real cell cycle pattern. Using selection from tube gradients, Mitchison (1977) found that some enzymes show no perturbations, others show perturbations only for the first hour, while a third group [alcohol dehydrogenase, homoserine dehydrogenase, and maltase (α-glucosidase)] have prolonged perturbations which may persist with a frequency near to that of the cycle. Glutamine synthetase (glutamate–ammonia ligase) has a striking perturbation after continuous-flow centrifugation (Walker *et al.*, 1980). This means that in cases where there is an apparent cell cycle pattern in synchronous cultures, it is essential to run asynchronous control cultures, as outlined in Section I.

The earliest measurements on enzyme activity patterns through the *S. pombe* cycle were by Bostock *et al.* (1966). Both aspartate carbamoyltransferase and ornithine carbamoyltransferase showed a step pattern in synchronous cultures of strain 132, but an asynchronous control was assayed only for aspartate carbamoyltransferase where there was no such step. Alkaline phosphatase, sucrase (sucrose α-glucosidase), and maltase (α-glucosidase) showed a continuous rise though there was a considerable perturbation of maltase activity both in synchronous cultures and in controls. More detailed analysis of alkaline phosphatase, acid phosphatase, and sucrase showed that the continuous rise could be resolved into linear

patterns with rate change points in the first third of the cycle (Mitchison and Creanor, 1969). This linear pattern for acid phosphatase was confirmed in later experiments with strain 972 and a different method (elutriation) of generating synchronous cultures (Creanor *et al.*, 1983).

Acid phosphatase was also measured in strain IFO 0365 by Miyata and Miyata (1978). The pattern they found is a little difficult to interpret. There are fluctuations that are not complete steps since at no point does the activity stop rising. If the data in Fig. 2 of their paper are plotted on Cartesian coordinates, the pattern is not far off a linear one during the first two cycles. There is a somewhat similar situation with derepressed sucrase (sucrose α-glucosidase) (Miyata *et al.*, 1980). They also examined the effect of blocking the DNA–division cycle by treating selection synchronized cultures with hydroxyurea. Acid phosphatase activity continues to rise perhaps with a linear pattern and a rate change point 2.5 hr after the block. This is not pointed out in the paper (Miyata and Miyata, 1978) but is apparent when the total enzyme activity in Fig. 3A of their paper is plotted on Cartesian coordinates. In the case of derepressed sucrase, any possible persistent cell cycle pattern is masked by a plateau in activity for the first 2 hr after the block.

Nucleoside-diphosphate kinase has a sharp step pattern with a doubling in activity at the start of the cycle (Dickinson, 1983; Creanor and Mitchison, 1986). The step is not related to the S period since it occurs at the same point in the cycle both in strain 972 and in the mutant *wee1-6* where the S period is substantially delayed. A particular point of interest is that this pattern of steps persists for up to three cycle times after a block to the DNA–division cycle imposed by the mutant *cdc2-33*. The enzyme protein has been purified by Henley (1988) and an antibody raised against it. Radioimmunoassay shows that the amount of enzyme protein parallels the activity changes during the normal cycle and after a block.

DNA ligase (polydeoxyribonucleotide synthase), coded for by the *cdc17* gene, shows no conspicuous periodicity in activity through the cycle, as also does the level of the mRNA transcript (White *et al.*, 1986). The data, however, are not sufficiently precise to establish whether this activity increase follows a linear pattern. In contrast, there is a marked periodicity in *Sacch. cerevisiae* both in the activity of this enzyme and in the transcript levels from the structural gene (*cdc9*) in this yeast.

There is a substantial body of information from Poole and Lloyd in the 1970s using selection synchrony and age fractionation. They found one or two activity peaks in ten enzymes in glucose-grown cells (Poole and Lloyd, 1973). In glycerol-grown cells, there were four enzymes with peaks and one with steps (Poole and Lloyd, 1974). In addition to these enzyme assays, measurements of cytochromes (Poole and Lloyd, 1974;

Poole *et al.*, 1974) and oxygen uptake and heat evolution (Poole *et al.*, 1973; Poole and Lloyd, 1974) were performed, and it is convenient to consider them here. The primary problem is that this work was done before it became apparent that controls are essential to uncover the possible perturbing effects of selection methods, particularly when using large zonal rotors. A second problem is that the peak pattern found in nearly all the enzymes has not been found in any other *S. pombe* enzyme by other workers. Indeed, there is one case where there is a direct conflict of evidence. Poole and Lloyd (1973) found two peaks per cycle in acid phosphatase, which is quite different from the patterns found in the papers quoted above (Mitchison and Creanor, 1969; Miyata and Miyata, 1978; Creanor *et al.*, 1983). A third problem is that the data are not always convincing. One example is in Fig. 2(b) of Poole and Lloyd (1973) which shows assays of five enzymes over three cycles of a synchronous culture. The curve for cytochrome oxidase is persuasive, with several points defining each peak and a repeating pattern in each cycle. But this is not so with the other four enzymes, where there are differences between successive cycles and some single point peaks. In general, the conclusions from this body of work may be correct, but further work is needed before they can be regarded as definitive.

In a search for "step" patterns, seven enzymes were assayed in selection synchronized cultures (Mitchison, 1977). These were alkaline phosphodiesterase, acid phosphodiesterase, arginase, glutamine synthetase (glutamate ammonia-ligase), hexokinase, leucine aminopeptidase (cytosol aminopeptidase), and ornithine aminotransferase. These showed continuous activity rises without steps or peaks, but the results did not discriminate between linear and exponential patterns. This was survey work and the results can only be regarded as indicative especially since the relevant curves were presented for only four of the seven enzymes.

It is difficult to summarize the position but the only clear-cut evidence for sharply periodic patterns of enzyme activity is for the steps in nucleoside-diphosphate kinase and, to a lesser extent, for those in aspartate carbamoyltransferase. With many other enzymes, activity rises continuously, but, in the cases where it has been analyzed carefully, this continuous increase follows a linear pattern with a rate change.

J. Enzyme Potential

Enzyme potential is defined as the maximum rate of increase of enzyme activity under conditions of induction or derepression. In cell cycle studies, samples are taken at intervals from a synchronous culture and the

increasing activity of an enzyme is measured after transfer to an inducing medium. It is important to remember, first, that it is a rate measurement and, second, that although it is a property of the cell which can and does change through the cycle, it has a much less direct connection with growth than the other cell properties that have been surveyed here.

There is general agreement that potential for the three enzymes studied increases in a step pattern with one doubling step per cycle in the first half of the cycle. The three enzymes are sucrase (sucrose α-glucosidase) (Mitchison and Creanor, 1969, 1971; Benitez *et al.*, 1980), maltase (α-glucosidase) (Mitchison and Creanor, 1971), and arginase (Benitez *et al.*, 1980). There are some detailed arguments about the exact timing of the potential steps which are discussed by Mitchison and Creanor (1969) and by Sissons *et al.* (1973) where potential changes after induction synchrony are described.

The most detailed investigation of potential changes are those by Benitez *et al.* (1980) on sucrase (sucrose α-glucosidase) and arginase potential. They show that potential changes cannot be due to a gene dosage effect since potential continues to increase after a mutant block to DNA synthesis. It is an attractive hypothesis that potential increases as gene copies increase, and there is evidence for this in prokaryotes (reviewed in Mitchison, 1971); however, this does not seem to apply to yeast. A further demonstration of the independence of the potential steps from DNA synthesis (and mitosis) is that they continue for at least 1.5–2 cycle times after mutant blocks to the DNA–division cycle imposed on selection synchronized cultures. Potential also comes to a plateau after a time in large cells produced by mutant blocks (as with CO_2 production and protein and rRNA synthesis). There is a critical protein/genome ratio at which potential reaches a plateau, but this ratio is different for sucrase and arginase.

There is no question of the existence of the potential steps, but how they are controlled and what other events in the cycle they are associated with remain obscure. They are not dependent on gene dosage, on mitosis, nor on attainment of a critical size (Benitez *et al.*, 1980). Perhaps there is an independent "clock," a concept which is considered later.

K. Magnesium

Cellular magnesium levels have been measured by Walker and Duffus (1980) (see also a short review by Walker and Duffus, 1983). There is a pattern with a marked peak in Mg^{2+}/cell once per cycle in selection synchronized culture. The peak occurs at about 0.75 of the cycle at the time

of mitosis and reaches values of 7 times the interphase level. This is certainly a periodic pattern, but there is a problem in exactly what is being measured. The authors refer to "total cellular magnesium" but they also say that appreciable quantities of magnesium are lost in the first washing of the samples. It is of course very difficult to measure intracellular concentrations of ions that have a high flux rate and that are present in significant amounts in the medium (13 m*M* for Mg^{2+}). Part at any rate of the peaks may therefore represent an increase in bound magnesium rather than an influx into the cell.

III. CELL CYCLE PERIODICITIES

Table III gives a summary for wild-type cells of the cell cycle periodicities that have been described in more detail earlier. Three points should be made. First, periodicity is being used in a wider sense to include not only step and peak patterns of synthesis but also cases where synthesis is continuous but has a periodic rate change. Second, some cases have been omitted because the data cannot discriminate between alternative patterns of continuous increase (e.g., the acid-soluble pool and specific mRNAs). Third, in a few cases where there are differences in the data, the most credible results are presented in Table III. This obviously involves a degree of subjective judgment.

Table III groups together a wide range of cellular components and properties from cell length to enzyme potential. It also covers a wide range of patterns from conspicuous steps (as in DNA) to subtle changes in rate (as in total protein). Nevertheless, there is one striking fact, that all the patterns are periodic and there is no definitive case of smooth exponential increase. A second point is that the times of the periodic changes (steps, peaks, and rate change points) are not scattered randomly through the cycle. None of these times occurs between 0.46 and 0.75 of the cycle. This "empty quarter" lasts from about mid-G_2 until mitosis.

Very little is known as yet about the details of the controls that underlie these periodic changes. Most of the work has involved an analysis of dependency between these changes and the well-known periodic events of the DNA–division cycle (DNA synthesis, mitosis, and division). This analysis, which has been carried out on only some of the cell components in Table III, involves delaying or blocking the DNA–division cycle and observing the effect on other periodic changes. Here *cdc* and *wee* mutants have proved powerful tools. In some cases, the periodic changes continue after a block, and these are discussed in the next section. But in two

TABLE III

Patterns of Growth and Synthesis in Cell Cycle of *Schizosaccharomyces pombe* (WT)

Component or property	Pattern[a]	Rate change point or midpoint of step (fraction of cycle)	Strain	Temperature (°C)	Reference
Length	L (plus plateau)	0.34	972	25	Mitchison and Nurse (1985)
Total dry mass	L	0.91	132	25	Mitchison (1957)
Total protein	QL in rate	0.36 Acceleration pt. = 0.9	972, 132	32, 35	Creanor and Mitchison (1982)
DNA	S	0	972, 132	25, 35	See Table I
rRNA	QL in rate	0.10 Acceleration pt. = 0.84	972	35	Elliott (1983a)
mRNA [total poly(A)$^+$]	S in rate (= L)	0.11	972	35	Fraser and Nurse (1978)

mRNA (H2A1 histone)	P	~ 0	972	25	Aves *et al.* (1985), Durkacz *et al.* (1986)
mRNA *(cdc22)*	P	~ 0.9	972	35	Gordon and Fantes (1986)
Enzyme activity					
Aspartate carbamoyltransferase	S	0.40	132	32	Bostock *et al.* (1966)
Nucleosidediphosphate kinase	S	0.11	972	35	Creanor and Mitchison (1986)
Alkaline phosphatase	L	0.36	132	32	Mitchison and Creanor (1969)
Sucrase (sucrose α-glucosidase)	L	0.28	132	32	Mitchison and Creanor (1969)
Enzyme potential (rate)					
Sucrase (sucrose α-glucosidase)	S	0.36	132, 972	32, 35	Mitchison and Creanor (1969), Benitez *et al.* (1980)
Arginase	S	0.20	972	35	Benitez *et al.* (1980)
Maltase (α-glucosidase)	S	0.46	132	32	Mitchison and Creanor (1971)
Mg^{2+}	P	~ 0.75	132	32	Walker and Duffus (1980)

[a]L, Linear; QL, quasilinear; S, step; P, peak.

important cases, total protein and total rRNA, the curves of increase continue after a mitotic block but without the quasi-linear patterns that appear to be caused by an inhibitory effect at or near mitosis. This effect is not associated with DNA synthesis or cell division. Enzyme potential is also not associated with DNA synthesis. The only case where there is an association with the S period is CO_2 production.

Another dependency relation is with cell size. This is an important relationship in the mitotic control (Fantes, Chapter 5, this volume) and in the initiation of DNA synthesis. It is also a part of the control of length growth, and it may play a part in mRNA synthesis. It does not appear to be involved in the control of total protein, total rRNA, and enzyme potential.

Cell biologists may be somewhat surprised at the universal periodicity in Table III. "Step" and "peak" enzymes were popular concepts in the 1960s (Mitchison, 1971) but they have fallen out of fashion (though not out of existence) in 1970s and 1980s. The reasons are twofold. The first is technical in that enzyme activities can be distorted by synchronization techniques and that age fractionation is difficult to analyze (Creanor *et al.*, 1983; Mitchison, 1988). The second is the development of two-dimensional gels as a method of following the rate of synthesis of the abundant cellular proteins by pulse labeling in synchronous cultures or before age fractionation. These have been used over a range of cells from bacteria to mammalian cells, but the best comparator for *S. pombe* is probably the budding yeast *Sacch. cerevisiae*. Elliott and McLaughlin (1978) found an exponentially increasing rate of synthesis in the 111 proteins examined, and Lorincz *et al.* (1982) showed that only 20 proteins (including 3 histones) out of 700 examined had patterns which indicated cell cycle modulation or regulation.

There is no doubt that these results show that the vast majority of the abundant proteins do not have the step or peak patterns which would be easily detectable on the gels [as with tubulin in *Physarum* (Laffler *et al.*, 1981)]. What is much less certain is whether the methods were sufficiently sensitive to detect the linear or quasi-linear patterns that are common in many of the *S. pombe* components. In addition, the method of normalizing used by Lorincz *et al.* (1982) would not have revealed a linear pattern if it was the common one in the proteins. Elliott and McLaughlin (1978) also examined the rate of total protein synthesis after age fractionation in an elutriator and found a better fit to exponential synthesis than to a linear pattern. It may be that the two yeasts behave differently (as indeed they do in the case of DNA ligase, see Section II,I), but it is doubtful whether this technique could discriminate between an exponential and a quasi-linear pattern, particularly since the main difference between them is

toward the end of the cycle where age fractionation is least efficient. It would be necessary to use synchronous cultures of budding yeast, and it is interesting that such cultures show indications of a linear pattern in acid phosphatase activity which is very similar to that in *S. pombe* (Creanor *et al.*, 1983). A final point is that enzyme activity patterns through the cycle do not necessarily parallel enzyme protein patterns (Petzelt and Auel, 1977).

The presence of the periodicities in *S. pombe* means that growth during the cycle is not a smooth exponential process either for the bulk properties or for the individual components that have been measured. Instead, there are changes in the growth patterns that occur during three-quarters of the cycle. Many of these are rate doublings, either sharp or somewhat spread out. The mechanisms and the reasons for these changes are largely unknown, but their presence is interesting and important. It is far easier to investigate the controls of a growth pattern which shows periodicities (e.g., DNA synthesis) than one which does not. This is an exciting challenge for the molecular biologist and the cell biologist now and in the future.

Periodicities after a DNA–Division Block

There are at present two contrasting views of the control of cell cycle events. The first is one that is popular in growing systems such as yeast or mammalian cells where the dominant themes are dependent pathways (Fig. 4) and where any periodic events in growth are thought to be related to or controlled by the main periodic events of the DNA–division cycle. There will, of course, be a number of such pathways operating during the cycle. The second view is one of independent pathways. (Fig. 4) in which

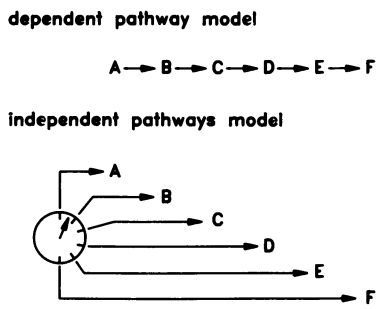


Fig. 4. Alternative models for the control of cell cycle events. [Reproduced, with permission, from Hartwell *et al.* (1974). Copyright 1974 by the AAAS.]

control is by a timer or oscillator rather than by dependent sequences. The main evidence for this model is the presence of periodic events in the cycle that persist after a block to the DNA–division cycle. Some of the recent evidence comes from eggs and early embryos where there are such persistent rhythms in surface contractions (in sea urchins, Yoneda *et al.*, 1978; in amphibia, Yoneda *et al.*, 1982; Hara *et al.*, 1980), in maturation promoting factor in amphibia (Gerhart *et al.*, 1984), and in the condensation of exogenous chromosomes in enucleate sea urchin eggs (Krystal and Poccia, 1979). Since these rhythms persist even in enucleate egg fragments, the view has been taken that there is a "cytoplasmic clock" (Hara *et al.*, 1980) and that "the cell cycle [is] driven by an endogenous oscillator that ... does not depend on the 'dispensable' elements of the cycle, such as DNA synthesis, chromosome condensation, spindle formation and cleavage" (Gerhart *et al.*, 1984).

It may be that early embryos have different controls from most other cells since they are a nongrowing system with cell cycles which are both short and invariant. But there is scattered evidence in growing systems of periodicities that persist after a DNA–division block. These occur in budding yeast with the posttranscriptional regulation of histone gene expression (Lycan *et al.*, 1987) and with the persistent budding of *cdc4* at the restrictive temperature which underlaid the models in Fig. 4 (Hartwell, 1971). The earlier evidence for cells apart from yeast is given by Mitchison (1971). Recently, persistent periodicities have been found in the cytoplasmic pH of *Dictyostelium* (Aerts *et al.*, 1985) and in oxygen uptake in *Alcaligenes* (Edwards and McCann, 1983).

In *S. pombe,* there is more evidence of persistent periodicities than in any other growing cell. It is clear that these occur in nucleoside-diphosphate kinase activity, CO_2 production, sucrase (sucrose α-glucosidase) potential, and arginase potential. They may also occur in O_2 uptake, length growth, and perhaps in acid phosphatase activity, but the evidence is less certain. The only two cases in which periodicities do not persist after a DNA–division block is with total protein and rRNA, but it is important to remember that only a limited number of periodicities have been examined for persistence after a block. Two factors limit this examination. The first is the need for a block which lasts for several cycle times and allows growth to continue more or less unaffected. *cdc2-33* is a mutant which blocks mitosis at the restrictive temperature and satisfies these criteria. It has been used for most but not all of the experiments. Most other *cdc* mutants and chemical inhibitors are less satisfactory so there has been little exploration of different kinds of block. The second is that periodicities in rate stop at some point after the block when the rate

reaches a plateau. This applies to all the cases except the steps in nucleoside-diphosphate kinase activity, and it limits the number of periods that can be observed to about two.

What can be said about the controls? The periodicities cannot be controlled by dependent sequences that include the main elements of the DNA–division cycle because they continue to "free run" at approximately cell cycle timing when the cycle is blocked. Yet there must be some connection or entrainment with events of the cycle since the periodicities have a constant position in the normal cycle. One hypothesis is that there is a single master timer or oscillator which controls all the events of the cycle and continues to run even though the DNA–division cycle has been blocked. The events of the DNA–division cycle would be a dependent sequence started from the master timer, and the periodicities in protein and rRNA would be dependent on mitosis. This would be analogous to the oscillator suggested for early embryos and is illustrated in a simple form in Fig. 4. There are two objections to this hypothesis. First, the master timer cannot be a simple fixed period oscillator since cycle time varies with size at division (Fantes and Nurse, 1981). Second, the free-running periodicities show different frequencies. The time intervals between the CO_2 rate changes are 15–20% shorter than the normal cycle (Novak and Mitchison, 1986) whereas those between the sucrase potential steps are 5–10% shorter and there is no shortening in the case of the nucleoside-diphosphate kinase steps. It is difficult to see how these different frequencies can be controlled by a single master timer, though more complex models can be devised.

A second hypothesis, which seems more plausible at the moment, is that there is a separate control for each periodicity which might involve one or more dependent sequences. One type of periodic control is "oscillatory repression" caused by end-product repression of enzyme synthesis (Mitchison, 1971), and it is interesting that Tyson (1983) has given reasons why such a system should have a period close to the cell cycle time. In the free-running situation, where the cell is freed from the constraints of the DNA–division cycle, there will be a series of oscillators or timing controls. It will be an intriguing problem to find out how many clocks there are in the "clockshop" (Winfree, 1980), whether they are connected, and, ultimately, what are their molecular bases.

Whichever hypothesis is true, there is no doubt of the importance of the free-running periodicities. They occur, of course, in an abnormal situation, but it may well be easier to study them there before tackling the more complicated situation in the normal cycle where there is the extra problem of entrainment by the DNA–division cycle. Understanding oscillatory

controls of this kind has to play an essential part in the analysis of regulatory networks which is becoming increasingly the task of molecular biologists.

IV. SUMMARY

In all the 18 components and properties that have been carefully studied in *S. pombe* there are periodic patterns during the cell cycle. "Periodic" is being used here in a wide sense to cover "linear" patterns of synthesis which show a rate change at one point in the cycle. There is no definitive evidence of any pattern of continuous exponential increase. Rate change points, steps, and peaks are scattered fairly widely through the cycle, but in wild-type cells they do not occur in the quarter of the cycle that runs from the middle of G_2 to mitosis. How the periodicities are controlled is largely unknown, but there is evidence in some cases that they are dependent on cell size and in others that they are dependent on mitosis. In the majority of cases that have been examined, periodicities continue at approximately cell cycle timing after a block to the DNA–division cycle. The implications of this surprising result are discussed.

V. A NOTE OF REMINISCENCE

Readers may be interested in the origin in the Edinburgh Zoology Department of the use of *S. pombe* for the study of the cell cycle. Early in 1955, I was talking to Michael Swann who was then Professor of Zoology. He and I had worked closely together on the mechanism of cleavage in sea urchin eggs, but we had reached the stage after nearly 10 years of work in which we were running short of testable ideas. One of us then said, and I cannot remember whom, that we had got as far as we could in trying to find out how a cell divided and that we should now try and see how a cell grew between one division and the next, a field that was largely unexplored at that time. An interference microscope which we had on loan was an obvious tool since it could be used, after modification, to measure total dry mass on single living cells. What cells could I use which would grow easily under the microscope? I thought yeasts would be good since they grew fast and easily. I rejected bacteria because they were small and not because they were prokaryotes since the distinction between them and eukaryotes was not drawn until the next decade (Stanier, 1961). I thumbed through the classic book on yeast taxonomy (Lodder

and Kreger-van Rij, 1952) and found *S. pombe* almost by accident. It was regular in shape (more so in practice than in the illustration in the book), it grew in one direction so volume calculations would be easy, and it divided in two with a medial septum (wrongly called a cell plate by me in early papers). It also did not have the unusual method of cell cycle growth which distinguishes budding yeast from nearly all other cells.

So I started looking at the patterns of growth during the *S. pombe* cell cycle and I have continued to do this off and on for more than 30 years. Many people have had their first experience of *S. pombe* in this department. These include two out of the three editors of this volume (Byron Johnson was my first postdoctoral fellow). Paul Nurse and Pierre Thuriaux made the vital link with the genetics developed by Urs Leopold, the "altmeister" of *S. pombe* genetics. Important contributions were made by Peter Fantes, Ronnie Fraser, and Kim Nasmyth, and a vital role has been played by Jim Creanor, my research associate for more than 20 years.

ACKNOWLEDGMENTS

I am grateful to Drs. J. Creanor and P. A. Fantes for helpful discussions.

REFERENCES

Aerts, R. J., Durston, A. J., and Moolenaar, W. H. (1985). Cytoplasmic pH and the regulation of the *Dictyostelium* cell cycle. *Cell* **43,** 653–657.

Aves, S. J., Durkacz, B. W., Carr, A., and Nurse, P. (1985). Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc 10* "start" gene. *EMBO J.* **4,** 457–463.

Baldwin, W. W., and Kubitschek, H. E. (1984). Buoyant density variation during the cell cycle of *Saccharomyces cerevisiae*. *J. Bacteriol.* **158,** 701–704.

Barnes, A., Nurse, P., and Fraser, R. S. S. (1979). Analysis of the significance of a periodic, cell size-controlled doubling in the rate of macromolecular synthesis for the control of balanced exponential growth of fission yeast cells. *J. Cell Sci.* **35,** 41–51.

Benitez, T., Nurse, P., and Mitchison, J. M. (1980). Arginase and sucrase potential in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **46,** 399–431.

Bostock, C. J. (1969). Mitochrondrial DNA in the fission yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **195,** 579–581.

Bostock, C. J. (1970a). The effect of 2-phenylethanol on the DNA synthesis cycle of *Schizosaccharomyces pombe*. *J. Cell Sci.* **7,** 523–530.

Bostock, C. J. (1970b). DNA synthesis in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **60,** 16–26.

Bostock, C. J., Donachie, W. D., Masters, M., and Mitchison, J. M. (1966). Synthesis of

enzymes and DNA in synchronous cultures of *Schizosaccharomyces pombe*. *Nature (London)* **210,** 808–810.

Calleja, G. B., Yoo, B. Y., and Johnson, B. F. (1977). Fusion and erosion of cell walls during conjugation in the fission yeast *(Schizosaccharomyces pombe)*. *J. Cell Sci.* **25,** 139–155.

Creanor, J. (1978a). Carbon dioxide evolution during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **33,** 385–397.

Creanor, J. (1978b). Oxygen uptake during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **33,** 399–411.

Creanor, J., and Mitchison, J. M. (1979). Reduction of perturbations in leucine incorporation in synchronous cultures of *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **112,** 385–388.

Creanor, J., and Mitchison, J. M. (1982). Patterns of protein synthesis during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **58,** 263–285.

Creanor, J., and Mitchison, J. M. (1984). Protein synthesis and its relation to the DNA-division cycle in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **69,** 199–210.

Creanor, J., and Mitchison, J. M. (1986). Nucleoside diphosphokinase, an enzyme with step changes in activity during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. I. Persistence of steps after a block to the DNA–division cycle. *J. Cell Sci.* **86,** 207–215.

Creanor, J., Elliott, S. G., Bisset, Y. C., and Mitchison, J. M. (1983). Absence of step changes in activity of certain enzymes during the cell cycle of budding and fission yeasts in synchronous cultures. *J. Cell Sci.* **61,** 339–349.

Dickinson, D. P. (1981a). Polypeptide synthesis in cell cycle mutants of fission yeast. *J. Cell Sci.* **51,** 203–217.

Dickinson, J. R. (1981b). The *cdc22* mutation in *Schizosaccharomyces pombe* is a temperature-sensitive defect in nucleoside diphosphokinase. *Eur. J. Biochem.* **119,** 341–345.

Dickinson, J. R. (1983). Nucleoside diphosphokinase and cell cycle control in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **60,** 355–365.

Duffus, J. H., and Mitchell, C. J. (1970). Effect of osmotic pressure on DNA synthesis. *Exp. Cell Res.* **61,** 213–216.

Durkacz, B., Carr, A., and Nurse, P. (1986). Transcription of the *cdc2* cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *EMBO J.* **5,** 369–373.

Edwards, C., and McCann, R. J. (1983). The effects of nalidixic acid on respiratory activity of asynchronous and synchronous cultures of *Alcaligenes eutrophus*. *J. Gen. Microbiol.* **129,** 1–5.

Elliott, S. G. (1983a). Coordination of growth with cell division: Regulation of synthesis of RNA during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **192,** 204–211.

Elliott, S. G. (1983b). Regulation of the maximal rate of RNA synthesis in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **192,** 212–217.

Elliott, S. G., and McLaughlin, C. S. (1978). Rate of macromolecular synthesis through the cell cycle of the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4384–4388.

Fantes, P. A. (1977). Control of cell size and cycle time in *Schizosaccharomyces pombe*. *J. Cell Sci.* **24,** 51-67.

Fantes, P., and Nurse, P. (1981). Division timing; controls, models and mechanisms. *In* "The Cell Cycle" (P. C. L. John, ed.), pp. 11–34. Cambridge Univ. Press, London and New York.

Fraser, R. S. S., and Moreno, F. (1976). Rates of synthesis of polyadenylated messenger RNA and ribosomal RNA during the cell cycle of *Schizosaccharomyces pombe*. *J. Cell Sci.* **21,** 497–521.

Fraser, R. S. S., and Nurse, P. (1978). Novel cell cycle control of RNA synthesis in yeast. *Nature (London)* **271,** 726–730.

Fraser, R. S. S., and Nurse, P. (1979). Altered patterns of ribonucleic acid synthesis during the cell cycle: A mechanism compensating for variation in gene concentration. *J. Cell Sci.* **35,** 25–40.

Gerhart, J., Wu, M., and Kirschner, M. (1984). Cell cycle dynamics of an M-phase-specific factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **98,** 1247–1255.

Gordon, C. B., and Fantes, P. A. (1986). The *cdc22* gene of *Schizosaccharomyces pombe* encodes a cell cycle-regulated transcript. *EMBO J.* **5,** 2981–2985.

Hamburger, K., Kramhoft, B., Nissen, S. B., and Zeuthen, E. (1977). Linear increase in glycolytic activity through the cell cycle of *Schizosaccharomyces pombe*. *J. Cell Sci.* **24,** 69–79.

Hara, K., Tydeman, P., and Kirschner, M. (1980). A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 462–466.

Hartwell, L. H. (1971). Genetic control of the cell cycle division in yeast. II. Genes controlling DNA replication and its initiation. *J. Mol. Biol.* **59,** 183–194.

Hartwell, L. H., Culotti, J., Pringle, J. R., and Reid, B. J. (1974). Genetic control of the cell division cycle in yeast. *Science* **183,** 46–51.

Henley, L. (1989). In preparation.

James, T. W., Hemond, P., Czer, G., and Bohman, R. (1975). Parametric analysis of volume distributions of *Schizosaccharomyces pombe* and other cells. *Exp. Cell Res.* **94,** 267–276.

Johnson, B. F. (1965). Autoradiographic analysis of regional wall growth of yeasts. *Exp. Cell Res.* **39,** 613–624.

Johnson, B. F., and Lu, C. (1975). Morphometric analysis of yeast cells. IV. Increase of the cylindrical diameter of *Schizosaccharomyces pombe* during the cell cycle. *Exp. Cell Res.* **95,** 154–158.

Johnson, B. F., Calleja, G. B., Boisclair, I., and Yoo, B. Y. (1979). Cell division in yeasts. III. The biased asymmetric location of the septum in the fission yeast cell *Schizosaccharomyces pombe*. *Exp. Cell Res.* **123,** 253–259.

Johnson, B. F., Calleja, G. B., Yoo, B. Y., Zukler, M., and McDonald, I. J. (1982). Cell division: Key to cellular morphogenesis in the fission yeast, *Schizosaccharomyces*. *Int. Rev. Cytol.* **75,** 167–208.

Krystal, G. W., and Poccia, D. (1979). Control of chromosome condensation in the sea urchin egg. *Exp. Cell Res.* **123,** 207–219.

Kubitschek, H. E., and Clay, K. B. (1986). A second growth state for *Schizosaccharomyces pombe*. *Exp. Cell Res.* **165,** 243–254.

Kubitschek, H. E., and Ward, A. (1985). Buoyant density constancy of cells of *Schizosaccharomyces pombe*. *J. Bacteriol.* **162,** 902–904.

Laffler, T. G., Chang, M. T., and Dove, W. F. (1981). Periodic synthesis of microtubular proteins in the cell cycle of *Physarum*. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5000–5004.

Lodder, J., and Kreger-van Rij, N. J. W. (1952). "The Yeasts, a Taxonomic Study." North Holland, Amsterdam.

Lorincz, A. T., Miller, M. J., Xuong, N.-H., and Geiduschek, E. P. (1982). Identification of proteins whose synthesis is modulated during the cell cycle of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **2,** 1532–1549.

Lycan, D. E., Osley, M. A., and Hereford, L. M. (1987). Role of transcriptional and posttranscriptional regulation in expression of histone genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7,** 614–621.

Marchant, R. (1971). The initiation of cell wall synthesis in para-synchronous cultures of *Schizosaccharomyces pombe*. *Arch. Mikrobiol.* **78,** 205–213.

Marks, J., and Hyams, J. S. (1985). Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur J. Cell Biol.* **39,** 27–32.

Marks, J., Hagan, I. M., and Hyams, J. S. (1986). Growth polarity and cytokinesis in fission yeast: The role of the cytoskeleton. *J. Cell Sci. Suppl.* **5,** 229–241.

May, J. W. (1962). Sites of cell-wall extension demonstrated by the use of fluorescent antibody. *Exp. Cell Res.* **27,** 170–172.

May, J. W., and Mitchison, J. M. (1986). Length growth in fission yeast cells measured by two novel techniques. *Nature (London)* **322,** 752–754.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exp. Cell Res.* **13,** 244–262.

Mitchison, J. M. (1958). The growth of single cells. II. *Saccharomyces cerevisiae*. *Exp. Cell Res.* **15,** 214–221.

Mitchison, J. M. (1971). "The Biology of the Cell Cycle." Cambridge Univ. Press, London and New York.

Mitchison, J. M. (1977). Enzyme synthesis during the cell cycle. *In* "Cell Differentiation in Microorganisms, Plants and Animals" (L. Nover and K. Mothes, eds.), pp. 377–401. Fischer, Jena.

Mitchison, J. M. (1988). Synchronous cultures and age fractionation. *In* "Yeast: A Practical Approach" (J. H. Duffus and I. Campbell, eds.), pp. 51–63. I. R. L. Press, Oxford.

Mitchison, J. M., and Carter, B. L. A. (1975). Cell cycle analysis. *Methods Cell Biol.* **12,** 201–219.

Mitchison, J. M., and Creanor, J. (1969). Linear synthesis of sucrase and phosphatases during the cell cycle of *Schizosaccharomyces pombe*. *J. Cell Sci.* **5,** 373–391.

Mitchison, J. M., and Creanor, J. (1971). Further measurements of DNA synthesis and enzyme potential during cell cycle of fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **69,** 244–247.

Mitchison, J. M., and Cummins, J. E. (1964). Changes in the acid-soluble pool during the cell cycle of *Schizosaccharomyces pombe*. *Exp. Cell. Res.* **35,** 394–401.

Mitchison, J. M., and Lark, K. G. (1962). Incorporation of ^{3}H-adenine into RNA during the cell cycle of *Schizosaccharomyces pombe*. *Exp. Cell Res.* **28,** 452–455.

Mitchison, J. M., and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **75,** 357–376.

Mitchison, J. M., and Walker, P. M. B. (1959). RNA synthesis during the cell life cycle of a fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **16,** 49–58.

Mitchison, J. M., and Wilbur, K. M. (1962). The incorporation of protein and carbohydrate precursors during the cell cycle of a fission yeast. *Exp. Cell Res.* **26,** 144–157.

Mitchison, J. M., Kinghorn, M. L., and Hawkins, C. (1963). The growth of single cells. IV. *Schizosaccharomyces pombe* at different temperatures. *Exp. Cell Res.* **30,** 521–527.

Mitchison, J. M., Cummins, J. E., Gross, P. R., and Creanor, J. (1969). The uptake of bases and their incorporation into RNA during the cell cycle of *Schizosaccharomyces pombe* in normal growth and after a step-down. *Exp. Cell Res.* **57,** 411–422.

Miyata, H., Miyata, M., and Ito, M. (1978). The cell cycle in the fission yeast *Schizosaccharomyces pombe*. I. Relationship between cell size and cycle time. *Cell Struct. Funct.* **3,** 39–46.

Miyata, H., Miyata, M., and Johnson, B. F. (1986). Patterns of extension growth of the fission yeast *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **32,** 528–530.

Miyata, M., and Miyata, H. (1978). Relationship between extracellular enzymes and cell growth during the cell cycle of the fission yeast *Schizosaccharomyces pombe:* Acid phosphatase. *J. Bacteriol.* **136,** 558–564.

Miyata, M., Imura, K., and Miyata, H. (1980). Relationship between extracellular enzymes and cell growth during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. II. Sucrase. *J. Gen. Appl. Microbiol.* **26,** 109–118.

Nasmyth, K. A. (1979). A control acting over the initiation of DNA replication in the yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **36,** 155–168.

Nasmyth, K., Nurse, P., and Fraser, R. S. S. (1979). The effect of cell mass on the cell cycle timing and duration of the S-phase in fission yeast. *J. Cell Sci.* **39,** 215–233.

Novak, B., and Mitchison, J. M. (1986). Change in the rate of CO_2 production in synchronous cultures of the fission yeast *Schizosaccharomyces pombe:* A periodic cell cycle even that persists after the DNA–division cycle has been blocked. *J. Cell Sci.* **86,** 191–206.

Novak, B., and Mitchison, J. M. (1987). Periodic cell cycle changes in the rate of CO_2 production in the fission yeast *Schizosaccharomyces pombe* persist after a block to protein synthesis. *J. Cell Sci.* **87,** 323–325.

Nurse, P., and Thuriaux, P. (1977). Controls over the timing of DNA replication during the cell cycle of fission yeast. *Exp. Cell Res.* **107,** 365–375.

Osumi, M., and Sando, N. (1969). Division of yeast mitochrondria in synchronous culture. *J. Electron Microsc.* **18,** 47–56.

Osumi, M., Masuzawa, E., and Sando, N. (1968). Mitochondrial formation in synchronous culture of *Schizosaccharomyces pombe*. *Jpn. Women's Univ. J.* **15,** 33–41.

Petzelt, C., and Auel, D. (1977). Synthesis and activation of mitotic Ca^{++}-adenosinetriphosphatase during the cell cycle of mouse mastocytoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 1610–1613.

Poole, R. K., and Lloyd, D. (1973). Oscillations of enzyme activities during the cell-cycle of a glucose-repressed fission-yeast *Schizosaccharomyces pombe* 972h$^-$. *Biochem. J.* **136,** 195–207.

Poole, R. K., and Lloyd, D. (1974). Changes in respiratory activities during the cell-cycle of the fission yeast *Schizosaccharomyces pombe* 972h$^-$ growing in the presence of glycerol. *Biochem. J.* **144,** 141–148.

Poole, R. K., Lloyd, D., and Kemp, R. B. (1973). Respiratory oscillations and heat evolution in synchronously dividing cultures of the fission yeast *Schizosaccharomyces pombe* 972h$^-$. *J. Gen. Microbiol.* **77,** 209–220.

Poole, R. K., Lloyd, D., and Chance, B. (1974). The development of cytochromes during the cell cycle of a glucose-repressed fission yeast, *Schizosaccharomyces pombe* 972h$^-$. *Biochem. J.* **138,** 201–210.

Stanier, R. Y. (1961). La place des bactéries dans le monde vivant. *Ann. Inst. Pasteur Paris* **101,** 297–312.

Stebbing, N. (1971). Growth and changes in pool and macromolecular components of *Schizosaccharomyces pombe* during the cell cycle. *J. Cell Sci.* **9,** 701–717.

Stebbing, N. (1972). Amino-acid pool components as regulators of protein synthesis in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **70,** 381–389.

Sissons, C. H., Mitchison, J. M., and Creanor, J. (1973). Enzyme synthesis and potential during induction synchrony in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **82,** 63–72.

Streiblova, E., and Wolf, A. (1972). Cell wall growth during the cell cycle of *Schizosaccharomyces pombe*. *Z. Allg. Microbiol.* **12,** 673–684.

Tyson, J. J. (1983). Periodic enzyme synthesis and oscillatory repression: Why is the period of oscillation close to the cell cycle time? *J. Theor. Biol.* **103,** 313–328.

Wain, W. H. (1971). Synthesis of soluble protein during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **69,** 49–56.

Wain, W. H., and Staatz, W. D. (1973). Rates of synthesis of ribosomal protein and total ribonucleic acid through the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **81,** 269–278.

Walker, G. M., and Duffus, J. H. (1980). Magnesium ions and the control of the cell cycle in yeast. *J. Cell Sci.* **42,** 329–356.

Walker, G. M., and Duffus, J. H. (1983). Magnesium as the fundamental regulator of the cell cycle. *Magnesium* **2,** 1–16.

Walker, G. M., Thompson, J. C., Slaughter, J. C., and Duffus, J. H. (1980). Perturbation of enzyme activity in cells of the fission yeast *Schizosaccharomyces pombe* subjected to continuous-flow centrifugation. *J. Gen. Microbiol.* **119,** 543–546.

White, J. H. M., Barker, D. G., Nurse, P., and Johnston, L. H. (1986). Periodic transcription as a means of regulating gene expression during the cell cycle: Contrasting mode of expression of DNA ligase genes in budding and fission yeast. *EMBO J.* **5,** 1705–1709.

Winfree, A. T. (1980). "The Geometry of Biological Time." Springer-Verlag, Berlin and New York.

Yoneda, M., Ikeda, M., and Washitani, S. (1978). Periodic change in the tension at the surface of activated non-nucleate fragments of sea-urchin eggs. *Dev. Growth Differ.* **20,** 329–336.

Yoneda, M., Kobayakawa, Y., Kubota, H. Y., and Sakai, M. (1982). Surface contraction waves in amphibian eggs. *J. Cell Sci.* **54,** 35–46.

7

Gene Cloning and Expression in Fission Yeast

PAUL RUSSELL

Department of Molecular Biology
Research Institute of Scripps Clinic
La Jolla, California 92037

MOLECULAR BIOLOGY OF THE
FISSION YEAST

I. INTRODUCTION

The development of gene cloning methods in the fission yeast *Schizosaccharomyces pombe* has progressed to a highly advanced state. This, together with the recent widespread recognition that fission yeast offers much to molecular biologists that cannot be accomplished with budding yeast alone, has encouraged many people to begin studying new problems in fission yeast. This chapter provides a compilation of information of practical use to those interested in gene cloning in *S. pombe*. Cloning strategies are reviewed first, followed by sections on cloning vectors, transformation, and manipulation of cloned genes. In the final section the general properties of gene structure and expression in fission yeast are reviewed.

II. GENE CLONING STRATEGIES

Schizosaccharomyces pombe genes have been cloned by a variety of methods. The first genes cloned were structural RNA genes, including tRNA (Mao *et al.*, 1980) and rRNA genes (Tabata, 1981). They were cloned by nucleic acid hybridization methods using purified RNA as a probe. Later the *S. pombe ura1*$^+$ and *ura3*$^+$ genes were identified by complementation of *Escherichia coli* mutants (Yamamoto *et al.*, 1981; Nakanishi and Yamamoto, 1984). These techniques have specialized or limited application; gene isolation methods of more general utility are (1) cloning using DNA probes from other species, (2) cloning *S. pombe* genes by complementation of *Saccharomyces cerevisiae* mutants, and (3) cloning by complementation of *S. pombe* mutants.

A. Cloning by Cross-Species DNA Hybridization

Many *S. pombe* genes have been cloned by DNA hybridization using genes from other species or olligonucleotide probes based on protein sequences from other species. They include genes encoding cytochrome *c* (Russell and Hall, 1982), *ras* p21 (Fukui and Kaziro, 1985; Nadin-Davis *et al.*, 1986), calmodulin (Takeda and Yamamoto, 1987), topoisomerases I and II (Uemura *et al.*, 1986), and the histone gene set (Choe *et al.*, 1985; Matsumoto and Yanagida, 1985). The feasibility of such an approach is, of course, dependent on the degree of evolutionary conservation between the probe and the homologous *S. pombe* gene. In most of the cases a *Sacch. cerevisiae* gene has been used as the probe, but it is important to

TABLE I

Comparison of Protein Sequence Similarities between *Schizosaccharomyces pombe* Proteins and Homologs in *Saccharomyces cerevisiae* and Mammals

Protein[a]	Sequence similarity (%)	
	Schizosaccharomyces pombe versus *Saccharomyces cerevisiae*	*Schizosaccharomyces pombe* versus mammals
Histone H2A	83	79
Histone H2B	82	68
Histone H4	91	91
β-Tubulin	73	75
α-Tubulin	76	74
ras p21	56	61
Calmodulin	63	74
cdc2	62	63
Cytochrome *c*	70	68
Alcohol dehydrogenase	51	25
Triose-phosphate isomerase	59	50

[a] The degree of sequence identity was calculated in the following references: histones (Matsumoto and Yanagida, 1985), β-tubulin (Hiraoka *et al.*, 1984), α-tubulin (Yanagida, 1987), *ras* (Fukui and Kaziro, 1985), calmodulin (Takeda and Yamamoto, 1987), *cdc2* (Lee and Nurse, 1987), cytochrome *c* (Russell and Hall, 1982), alcohol dehydrogenase (Russell and Hall, 1983), and triose-phosphate isomerase (Russell, 1985).

acknowledge that for most *S. pombe* genes the *Sacch. cerevisiae* homolog is at best only marginally more similar than higher eukaryotic homologs (Table I). For example, *S. pombe* H2A protein is 83% similar to *Sacch. cerevisiae* H2A and 79% similar to human H2A. Indeed, in many cases the mammalian homolog is slightly more similar to *S. pombe* than is the *Sacch. cerevisiae* homolog. Proteins which show this pattern include *ras*, calmodulin, β-tubulin (Hiraoka *et al.*, 1984), and α-tubulin (Toda *et al.*, 1984; Yanagida, 1987). Therefore, in practice there is little reason to prefer *Sacch. cerevisiae* probes over higher eukaryotic probes, and the best strategy is to try all probes that are available.

B. Cloning *Schizosaccharomyces pombe* Genes by Complementation in *Saccharomyces cerevisiae*

Many interesting genes have been identified in *Sacch. cerevisiae* which as yet have no counterparts in other organisms; in these cases it is very

important to determine the degree of evolutionary conservation of gene structure and function. An obvious organism for this work is *S. pombe* since in evolutionary terms it is only distantly related to *Sacch. cerevisiae*, and once the *S. pombe* gene is cloned it can be subjected to the same kind of analysis as is possible in *Sacch. cerevisiae*. Another major reason for choosing *S. pombe* is that in many cases it is possible to achieve cross-species complementation of genes between the two yeasts.

To clone *S. pombe* genes by complementation in *Sacch. cerevisiae* it is best to have *S. pombe* gene banks constructed in vectors that replicate and can be selected for in *Sacch. cerevisiae*. Fortunately, many of the *S. pombe* gene banks have been constructed using vectors containing the *Sacch. cerevisiae LEU2* gene and 2-μm plasmid replication origin. These vectors are pDB248 derivatives (Beach *et al.*, 1982), pDB262 (Wright *et al.*, 1986b), and YEp13 (Broach *et al.*, 1979).

A second requirement for cross-species complementation is that gene expression mechanisms must be conserved enough so that a functional gene product is made. Although it has been shown that transcription of *S. pombe* genes in *Sacch. cerevisiae* is usually abnormal and inefficient (Russell, 1983, 1985), in practice poor transcription is unlikely to prevent complementation. This is because gene dosage can increase to 50 or more with genes cloned on multicopy plasmids in *Sacch. cerevisiae*. A more serious problem is intron splicing. *S. pombe* introns lack the intron consensus sequences that are essential for intron excision in *Sacch. cerevisiae* (see Section VI,A). Since about 25% of the *S. pombe* genes sequenced to date have introns, it is likely that about this proportion of attempts at cloning *S. pombe* genes by complementation in *Sacch. cerevisiae* will fail simply because the genes contain introns.

Two fission yeast genes which have been cloned by complementation in *Sacch. cerevisiae* are those encoding alcohol dehydrogenase (Russell and Hall, 1983) and triose-phosphate isomerase (Russell, 1985). In addition, the *S. pombe* cyc^+ and $top2^+$ genes complement corresponding *Sacch. cerevisiae* mutants (Russell and Hall, 1982; Yanagida and Wang, 1987), and *S. pombe* acid phosphatase is expressed in *Sacch. cerevisiae* (Elliott *et al.*, 1986).

C. Cloning by Complementation in *Schizosaccharomyces pombe*

Cloning by complementation of fission yeast mutations is the most common method of isolating *S. pombe* genes. The details of transformation, vectors, and gene manipulation are presented in later sections; here some of the principles of complementation cloning in *S. pombe* are considered.

Transformation in fission yeast is sufficiently efficient to allow routine screening of 25 or more *S. pombe* genome equivalents for complementing genes. Most *S. pombe* gene banks have an average insert size of 5–10 kb (kilobases); therefore, a single *S. pombe* genome of about 20,000 kb (Bostock, 1970) will be represented in 2000–4000 transformants. With gene banks, transformation frequencies are routinely better than 1×10^4 transformants/μg of plasmid/4×10^7 cells. Hence, it is common to screen 25–50 genome equivalents using 10 μg of plasmid gene bank DNA and 5×10^8 cells (~50 ml of mid-log phase culture).

There are several schemes for identifying complementing clones, but, when possible, selection should first be maintained for the vector marker, and then once colonies have formed they are screened for complementation by replica plating onto media in which selection for the vector marker is relaxed. However, if the mutation being screened has a significant reversion frequency it is best to initiate selection or screening sooner.

Having identified complementing clones it is necessary to show that complementation is due to a gene function present on a plasmid. Here advantage is taken of the fact that most *S. pombe* cloning vectors are rapidly lost during mitotic growth. After a few generations of growth under nonselective conditions, cells are plated for single colonies, grown without selection, and then replica-plated onto two plates. One replica plate screens for the vector marker gene, and the other screens for the complementation phenotype. All cells which have lost the vector marker should also have a complementation-negative phenotype. If some complementation-negative colonies still retain the vector marker this could simply indicate that the original transformed cell received more than one type of plasmid. This presents no difficulty other than making it necessary to recover more than one type of plasmid from these transformed cells.

Plasmids are recovered by transforming *E. coli* with DNA preparations made from the yeast transformants. The plasmids that are able to complement the mutation of interest when transformed back into *S. pombe* are retained. At this stage it is necessary to confirm that the cloned gene is in fact the wild-type version of the gene identified by the mutation. This is done by showing that the plasmid integrates at the chromosomal locus of the mutation via a homologous recombination event.

It is necessary to genetically map genes cloned by complementation because they often turn out to be extragenic suppressor genes. These genes are able to suppress when their level of expression is increased as a result of being present on a multicopy plasmid. Genes identified by mutation and their extragenic suppressors can be related in one of several interesting ways. The extragenic suppressors of *pho1*$^-$ (acid phosphatase) and *nda2*$^-$ (α-tubulin) mutations are close structural and functional homologs of the mutant genes they suppress (Maundrell *et al.*, 1985a; Adachi *et*

al., 1986), the gene product of the cell cycle control gene *cdc2*$^+$ appears to physically interact with the gene product of its extragenic suppressor *suc1*$^+$ (Draetta *et al.*, 1987), and loss of the mitotic inducer *cdc25*$^+$ can be suppressed by increased expression of *nim1*$^+$, which bypasses the *cdc25*$^+$ function and induces mitosis by a mechanism different from that of *cdc25*$^+$ (Russell and Nurse, 1987b).

III. *SCHIZOSACCHAROMYCES POMBE* CLONING VECTORS

A useful set of plasmids has been constructed for cloning in *S. pombe*. They include vectors for general purpose cloning, vectors designed for making gene banks, and expression vectors. The vectors are described in detail later in this section. First are considered the genes and mutations which form the plasmid selection systems in *S. pombe* and the autonomous replication sequences (*ars*) responsible for high transformation ability.

A. Selection Markers

Two plasmid selection systems have been extensively used in *S. pombe*. The first consists of the *Sacch. cerevisiae LEU2* gene complementing the *S. pombe leu1-32* mutation. *LEU2* encodes 3-isopropylmalate dehydrogenase. The degree of complementation is usually quite good, although *leu1-32* strains containing only a single integrated copy of *LEU2* often have reduced growth rates in selective media (Russell and Nurse, 1987a). This probably indicates that *LEU2* expression is rather weak in *S. pombe*, since it is known that plasmids which have expression-defective clones of *LEU2* fail to transform *S. pombe* at a high frequency (Beach and Nurse, 1981). The *leu1-32* mutation is quite stable, having a reversion frequency of less than 1 in 10^7 (Beach and Nurse, 1981).

The second popular *S. pombe* plasmid selection system consists of *ura4*$^-$ mutations which are complemented by *S. pombe ura4*$^+$ and *Sacch. cerevisiae URA3*. These genes encode orotidine-5′-phosphate decarboxylase. The *URA3* gene complements only when cloned on multicopy plasmids, again indicating poor expression of a *Sacch. cerevisiae* gene in *S. pombe*.

Other marker selection systems that have been employed in *S. pombe* include complementation of *ura1*$^-$ with the cloned *ura1*$^+$ gene (Sakaguchi and Yamamoto, 1982) and resistance to the antibiotic G418 provided by the *kan*^{r} gene of transposon Tn*903* (Sakai *et al.*, 1984). A selection system

based on complementation of the *ade6-704* mutation by the cloned suppressor tRNA gene *sup3-5* has also been described (Carr, 1987). The *ade6-704* mutants produce red pigment when grown in limiting concentrations of adenine. The *ade6-704* cells containing a chromosomal copy of *sup3-5* are white, while cells containing a plasmid-borne copy of *sup3-5* are pink owing to the subpopulation of cells that have lost plasmid and accumulate red pigment.

B. Autonomous Replication Sequences

In *S. pombe*, autonomous replication sequence (*ars*) elements are defined as sequences which promote high-frequency transformation and yield mitotically unstable transformants containing unrearranged plasmid (Maundrell *et al.*, 1985b). There is no evidence regarding whether *ars* elements in *S. pombe* actually function as replication origins. It appears that *ars* function is only partly conserved between *S. pombe* and *Sacch. cerevisiae*, since only a subset of sequences identified as *ars* elements in one yeast will function in the other.

The *ars* most commonly used in *S. pombe* cloning vectors is derived from the 2-μm circle plasmid of *Sacch. cerevisiae*. The *ars* activity of 2-μm plasmids that function in *S. pombe* is not dependent on any 2-μm-encoded gene function (Gaillardin *et al.*, 1983). These plasmids behave in *S. pombe* in a fashion similar to the way they do in *Sacch. cerevisiae* cells that lack endogenous 2-μm plasmid; they have a relatively low copy number, and transmission through mitosis and meiosis is poor. Surprisingly, the region of 2-μm plasmid that functions as an *ars* in *S. pombe* does not include the complete replication origin that functions in *Sacch. cerevisiae* (Gaillardin *et al.*, 1983). It is unclear if the two yeasts recognize the same *ars* in 2-μm circle, and the issue is further complicated by the observation that at least some plasmids which contain the complete 2-μm circle fail to transform *S. pombe* at a high frequency. Nevertheless, in some plasmids 2-μm sequences function quite adequately as *ars* elements in *S. pombe*.

Screening of *S. pombe* genomic DNA for sequences that promote high-frequency plasmid transformation in *S. pombe* has identified sequences analogous to *Sacch. cerevisiae ars* (Losson and Lacroute, 1983; Maundrell *et al.*, 1985b). The prototype *S. pombe ars* is called *ars1*; it is present on a 1.1-kb *Eco*RI fragment. *ars1* plasmids behave much like 2-μm *ars* plasmids in *S. pombe* except that they tend to have a higher copy number, around 30 versus 2–10 (Heyer *et al.*, 1986). The fidelity of mitotic and meiotic transmission of *ars1* plasmids can be greatly improved by an *S.*

pombe 1.3-kb *Eco*RI fragment called *stb*. The *stb* sequence is not derived from a centromere, and although it increases *ars1* plasmid copy number to about 100/cell, *stb* itself is not an *ars*. The copy number increase caused by *stb* was not sufficient to account for the increased plasmid stability; thus, it seems that *stb* must have a partitioning function distinct from that of centromeres (Heyer *et al.*, 1986).

In *S. pombe* it is possible to achieve high-frequency transformation with non-*ars* plasmids if they are cotransformed with *ars* plasmids. This has been investigated by Sakai *et al.* (1984) who showed that the transformation efficiency of the non-*ars* plasmid YIP5 could be increased from 100 transformants/μg to 28,000 transformants/μg by cotransforming with an *ars* plasmid. Analysis of plasmid structure by Southern hybridization indicated that the establishment of cotransformants occurred by copolymerization of the *ars* and non-*ars* plasmids; the copolymeric plasmids were presumably formed by homologous recombination between the plasmids. This property can be exploited to transform *S. pombe* at high efficiency with gene banks constructed in non-*ars* plasmids. This important method has been demonstrated by Lee and Nurse (1987) who cloned a complementing human homolog of $cdc2^{+}$ by cotransformation of a $cdc2^{ts}$ strain with a human cDNA library made in a non-*ars* plasmid together with an *ars* plasmid.

C. Cloning Vectors

1. General Purpose Cloning Vectors

a. pDB248X. Plasmid pDB248X, a *LEU2*/2 μm/pBR322 vector containing no *S. pombe* DNA, has been widely used for cloning in *S. pombe* (Beach and Nurse, 1981; Beach *et al.*, 1982). It has unique restriction sites for *Eco*RI, *Hin*dIII, *Bam*HI, and *Pst*I in the pBR322 sequences (Fig. 1). Plasmid pDB248X transforms efficiently (2×10^4 transformants/μg) and has a copy number of 5–10 plasmids/cell (Heyer *et al.*, 1986). Like most *S. pombe* cloning vectors it is frequently lost during mitotic growth; generally less than 10% of the cells retain the plasmid after 20 generations in nonselective medium. Transmission through meiosis is also quite poor; in a cross between a transformed and untransformed strain 6% of the progeny had the plasmid (Heyer *et al.*, 1986). Several *S. pombe* gene banks have been constructed with pDB248X (Beach *et al.*, 1982; Toda *et al.*, 1984).

b. YEp13. Another *LEU2*/2 μm/pBR322 vector, YEp13 (Broach *et al.*, 1979), has frequently been used for cloning in *S. pombe*. It has unique

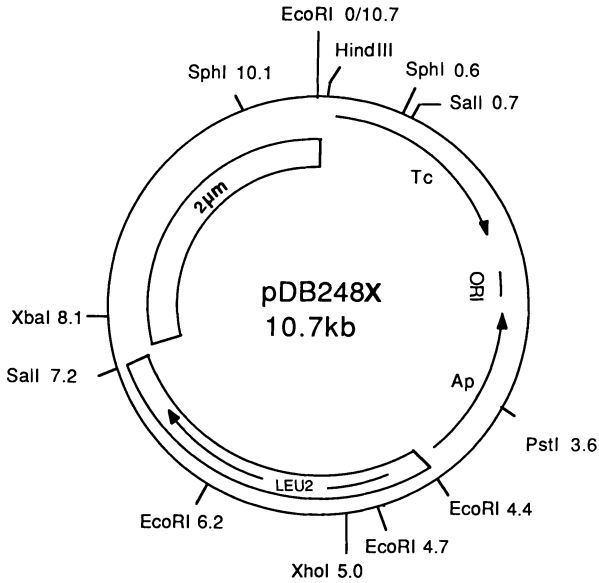


Fig. 1. pDB248X. Several derivatives of pDB248 (Beach and Nurse, 1981) have been described. Plasmids pDB248X and pDB248′ (Beach *et al.*, 1982) contain an approximately 1-kb duplication near the 5′ end of the *LEU2* region. In pDB248X this duplication has been removed (Durkacz *et al.*, 1985). pSAB1 is a derivative of pDB248X in which the *Sal*I site at map position 7.2 has been destroyed. Kilobase map positions of important restriction enzyme sites are shown.

restriction enzyme sites for *Xba*I, *Hin*dIII, *Bam*HI, *Sph*I, and *Sal*I. YEp13 appears to be a less efficient *ars* plasmid than pDB248X: it transforms at a lower frequency (3×10^3 transformants/μg), is more unstable through mitotic growth and meiosis, and is often more difficult to recover into *E. coli* (Heyer *et al.*, 1986). An *S. pombe* gene bank constructed in YEp13 (Russell and Hall, 1982) has been used to clone several *S. pombe* genes. In addition, a *Sacch. cerevisiae* gene bank constructed in YEp13 (Nasmyth and Reed, 1980) has been used to clone *Sacch. cerevisiae* genes by complementation in *S. pombe* (P. Russell, unpublished data).

c. pFL20. Plasmid pFL20 consists of pBR322, *Sacch. cerevisiae URA3*, and *S. pombe* sequences *ars1* and *stb* (Losson and Lacroute, 1983). This plasmid has a few useful cloning sites (Fig. 2), but its primary attributes are its high copy number (~80/cell) and relatively high mitotic and meiotic stability (Heyer *et al.*, 1986). In cells transformed with *ars* plasmids pDB248X or YEp13 approximately 30–45% of mitotic divisions produce a cell lacking plasmid, whereas in pFL20-transformed cells only

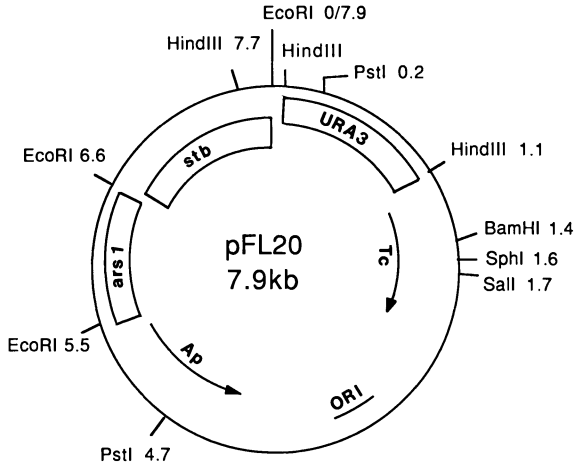


Fig. 2. pFL20.

13% of mitotic divisions produce a cell lacking plasmid. Likewise, in meiosis, pFL20 was found to be retained in around 16% of spores in crosses between transformed and untransformed cells, while other *ars* plasmids were present in 6% or less of the spores. An *S. pombe* gene bank has been constructed in pFL20 (Clarke *et al.*, 1986).

Because pFL20 has a high copy number and mitotic stability it is a potentially useful vector for expression of foreign genes in *S. pombe*. Broker *et al.* (1987) have used pFL20 successfully to express active human antithrombin III in *S. pombe*.

d. pIRT2. Plasmid pIRT2 consists of *LEU2* and *ars1* cloned into pUC118 (Fig. 3) (Hindley *et al.*, 1987). It contains the intergenic region of bacteriophage M13, enabling it to replicate to produce single-strand DNA template suitable for DNA sequence analysis by the dideoxynucleotide method. It contains unique restriction enzyme sites for *Sph*I, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, and *Sst*I in a polylinker.

2. *Vectors for Constructing Gene Banks*

pDB262 and pWH5. Plasmids pDB262 and pWH5 were developed for the purpose of constructing *S. pombe* libraries (Wright *et al.*, 1986b). These *LEU2*/2 μm vectors contain the λcI repressor gene and the tetracycline resistance gene fused to the λPr promoter (Fig. 4). Insertion of DNA fragments into a restriction enzyme site located in the λcI gene relieves repression of the tetracycline resistance gene, thereby providing positive

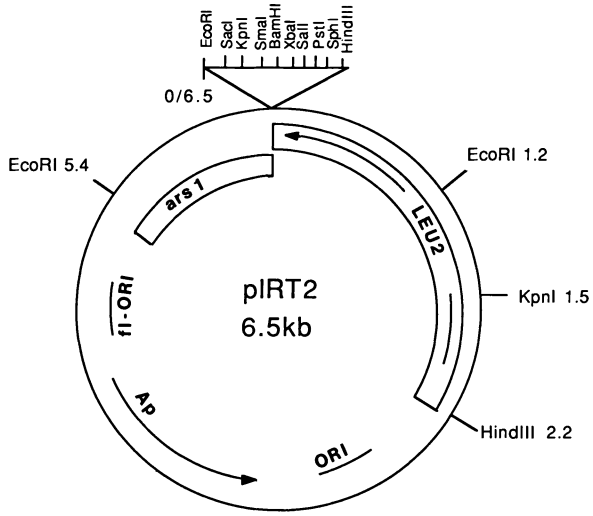


Fig. 3. pIRT2.

selection for recombinant plasmids. In plasmid pDB262 the λ*cI* gene contains unique cloning sites for *Hin*dIII and *Bcl*I, the latter of which accepts *Bam*HI, *Bgl*II, *Sau*3A, *Mbo*I, and *Xho*II cohesive ends. Intact pDB262 does not express antibiotic resistance, therefore it is easiest to select for it in a *leuB*$^-$ strain of *E. coli*. Plasmid pWH5 is an improved version of pDB262 which contains a unique *Sma*I site in the λ*cI* gene in addition to

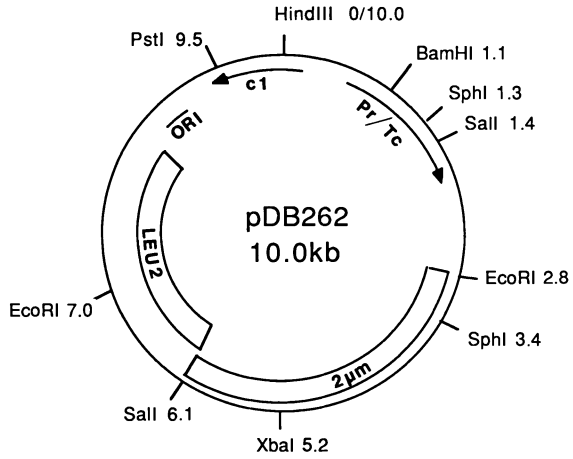


Fig. 4. pDB262.

the *Hin*dIII and *Bcl*I sites and also contains the ampicillin resistance gene. The transformation frequency and mitotic stability properties of these plasmids are similar to those of pDB248X. Gene libraries constructed using pDB262 have been described (Beach *et al.*, 1982; Russell and Nurse, 1986).

3. Expression Vectors

a. pEVP11. Plasmid pEVP11 contains the *S. pombe adh* promoter cloned into the *LEU2*/2 μm/pBR322 vector YEp13 (Fig. 5). Starting about 10 nucleotides downstream of the transcription initiation site is a polylinker containing unique restriction enzyme sites for *Sac*I, *Bam*HI, and *Hin*dIII (Russell and Nurse, 1986). The *adh* gene is highly expressed in *S. pombe*; it is estimated that 0.5–2% of the total soluble protein is alcohol dehydrogenase in glucose-grown cells (Russell and Hall, 1983). The *adh* gene is expressed constitutively in glucose- and glycerol/ethanol-grown cells (Russell, 1983). The 700-nucleotide *Eco*RI/*Sph*I fragment containing the *adh* 5′ flanking region in pEVP11 promotes strong transcription. The $cdc2^+$ encoded protein kinase was overproduced greater than 50-fold when the $cdc2^+$ gene was placed behind the *adh* promoter in pEVP11 (Simanis and Nurse, 1986).

b. pART1. Plasmid pART1 is another plasmid containing the *adh* promoter (McLeod *et al.*, 1987). In this case it is cloned into pIRT2 (described above), a *LEU2*/*ars1*/pUC118 plasmid. Unique cloning sites

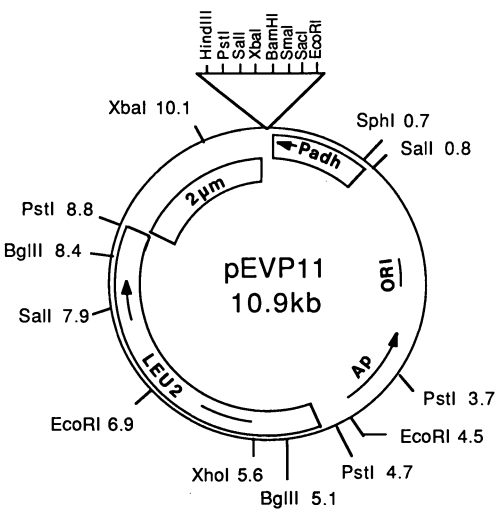


Fig. 5. pEVP11.

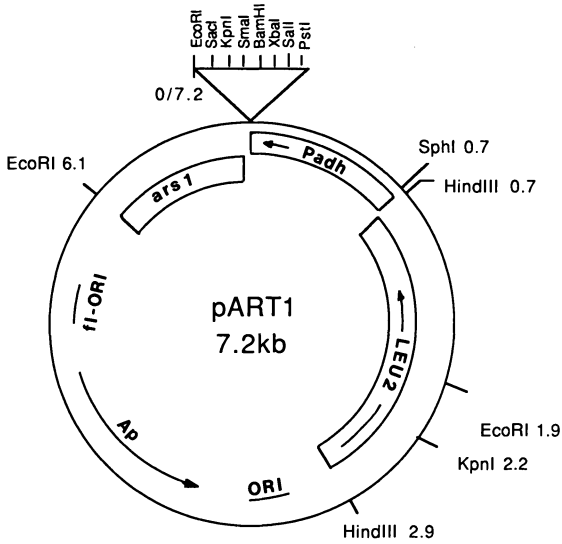


Fig. 6. pART1.

downstream of the *adh*-promoted transcription initiation site are *Pst*I, *Sal*I, *Bam*HI, *Sma*I, and *Sac*I (Fig. 6).

c. pSM-1. A 0.34-kb fragment containing the simian virus 40 (SV40) early promoter has been cloned upstream of a poly-restriction enzyme

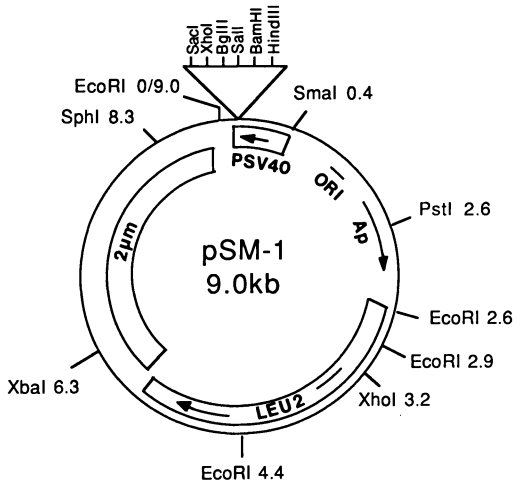


Fig. 7. pSM-1.

site sequence into pDB248X to form expression vector pSM-1 (Fig. 7). The SV40 early promoter is functional in *S. pombe* (Kaufer *et al.*, 1985), and the transcripts initiate at the same position in *S. pombe* as they do in mammalian cells (Jones *et al.*, 1988). The SV40 early promoter is weaker than the *adh* promoter, therefore pSM-1 is useful when it is not important to achieve maximum levels of expression or when strong overexpression of a gene is toxic to the cell.

IV. TRANSFORMATION, PLASMID RECOVERY, AND PLASMID INTEGRATION

A. Transformation Procedures

Two methods of transformation have been applied to *S. pombe*. A protoplast method was first described by Beach and Nurse (1981), and improved methods have been reported (Beach *et al.*, 1982; Sipiczki *et al.*, 1985). These methods are similar to the *Sacch. cerevisiae* protoplast transformation procedures but differ in the way the cell wall is degraded and later regenerated. The *S. pombe* cell wall contains α-1,3-glucans not found in *Sacch. cerevisiae*; as a consequence, best protoplasting is achieved with Novozym SP234 (Novo Industri, Bagsvaerd, Denmark), a *Trichoderma* extract rich in α-1,3-glucanase activity (Kopecka, 1975). Regeneration of *Sacch. cerevisiae* protoplasts is usually done in a soft agar overlay because the protoplasts require a matrix to support the newly made cell wall, but this was found to be unnecessary for *S. pombe* protoplasts (Necas *et al.*, 1968).

The primary attribute of the protoplasting method is that it gives the highest transformation frequency. With plasmids containing a 2-μm autonomous replication sequence (*ars*) transformation frequencies of 1–4 $\times$ 10^4 transformants/μg of plasmid are typically achieved (Beach and Nurse, 1981; Russell, 1983; Sakai *et al.*, 1984; Maundrell *et al.*, 1985b; Heyer *et al.*, 1986). In these experiments only about 0.01–0.1% of the cells were transformed, but in a typical transformation about 5×10^8 cells are transformed with 10 μg of plasmid DNA, yielding about 200,000 transformants.

It has been reported that transformation procedures using the cell wall-degrading extracts Glusulase (Dupont, Boston, Massachusetts) or Zymolyase (ICN ImmunoBiologicals, Lisle, Illinois), are also effective (Sakaguchi and Yamamoto, 1982; Chevallier and Lacroute, 1982; Russell, 1983). However, it is not known if these methods are as efficient as the Novozym SP234 method.

The alternative method of *S. pombe* transformation utilizes lithium salts to make whole cells competent to take up DNA. This method is identical to that described for *Sacch. cerevisiae* (Ito *et al.*, 1983). Its advantage is that it is much easier and faster than the protoplast method. In most cases, however, it is only 5–10% as efficient as the protoplast method, and therefore it is used when it is not important to obtain the maximum number of transformants (Heyer *et al.*, 1986). The whole cell method is also very useful for transforming certain mutant cells which tend to lyse by the protoplast method, such as is the case with some *cdc* mutants.

B. DNA Preparation and Plasmid Recovery

High molecular weight DNA from *S. pombe* can be easily made by protoplast methods similar to those used in *Sacch. cerevisiae* (Beach *et al.*, 1982; Russell and Hall, 1982; Maundrell *et al.*, 1985a; Clarke *et al.*, 1986). Zymolyase is a suitable glucanase extract for this procedure; Novozym SP234 can also be used, but it appears to have abundant nuclease activity as well as components which inhibit DNA restriction enzymes (Heyer *et al.*, 1986).

Plasmids can be recovered from high molecular weight DNA by transformation of *E. coli*. For the purpose of plasmid recovery DNA may also be made by a method in which cells are broken with glass beads (Beach *et al.*, 1982). In some cases it has been difficult or impossible to recover plasmid into *E. coli* (Sakaguchi and Yamamoto, 1982). This is a plasmid-specific phenomenon which may be due either to loss of vector DNA essential for replication or selection in *E. coli* or, more commonly, to the plasmid assuming a polymeric form in *S. pombe*. In such cases it is worthwhile to examine the state of the plasmid DNA by Southern hybridization. Recovery of plasmids which tend to be polymeric in *S. pombe* can be greatly enhanced by first cutting the *S. pombe* DNA preparation with a restriction enzyme that has one site in the plasmid and then ligating at low DNA concentration.

Certain *E. coli* strains appear to be better for recovering plasmid DNA from *S. pombe*. In particular, the *recBC* strains BJ5183 (Losson and Lacroute, 1983) and JA226 (Aves *et al.*, 1985) consistently yield more transformants than *recA* strains (Beach *et al.*, 1982).

C. Plasmid Integration

Having obtained a cloned gene from a plasmid library by functional complementation of a mutation, the first priority is to determine if the

genes identified by mutation and complementation of the mutation are the same. The genetic mapping of a cloned gene is done by standard genetic methods (Gutz *et al.*, 1974), using a strain in which the plasmid has integrated into the genome via a homologous recombination event between the gene of interest on the plasmid and its homolog on the chromosome. A fortunate characteristic of *S. pombe* that it shares with *Sacch. cerevisiae* is that homologous recombination events are at least a thousandfold more common than homology-independent events. Potential complications such as recombination between dispersed repeat sequences (i.e., 5 S RNA or tRNA genes), or between the *Sacch. cerevisiae* marker genes and their *S. pombe* homologs, have not been observed at an appreciable frequency. On average about 0.1% of transformant clones transformed with *ars* plasmids will have an integrated copy of the plasmid. Integration events occuring via homologous recombination at specific regions in the plasmid can be greatly enhanced by transforming plasmid DNA that has been linearized by a single cut in the region of interest, as was first described in *Sacch. cerevisiae* (Orr-Weaver *et al.*, 1983).

There are two potential problems concerning selection markers that can complicate any experiment involving DNA integration in *S. pombe*. The first involves using the fission yeast *ura4*$^+$ gene as a marker. Surprisingly, a high proportion of stable *ura4*$^+$ clones obtained by transforming *ura4-294* (point mutation) or *ura4-D6* (600-nucleotide deletion) mutants with *ura4*$^+$ plasmids are the result of gene conversion events (Grimm and Kohli, 1988). Therefore, it is best to use the *ura4-D18* mutation in which all sequences contained in the cloned 1.8-kb *Hin*dIII *ura4*$^+$ fragment are deleted from the chromosome.

The second problem sometimes encountered when integrating DNA in *S. pombe* occurs when the marker gene is poorly expressed. This is a problem mostly specific to the *Sacch. cerevisiae* genes *LEU2* and *URA3* which complement the *S. pombe* mutations *leu1*$^-$ and *ura4*$^-$, respectively. In both genes the *Sacch. cerevisiae* promoters function poorly in *S. pombe*, and so expression is greatly affected by adjacent sequences. Most integrated versions of *URA3* fail to complement *ura4*$^-$ mutations. This is less a problem with *LEU2*, but in experiments in which *LEU2* vectors are integrated in *leu1-32* strains very frequently the fastest growing clones contain two or three tandem integrated copies of the plasmid (Russell and Nurse, 1986, 1987a). This type of problem can also occur with the *S. pombe ura4*$^+$ gene (Hayles *et al.*, 1986). A convenient system for detecting integrants based on suppression of the *ade6-704* mutation by the nonsense suppressor tRNA gene *sup3-5* has been described (Carr, 1987).

V. MANIPULATION OF CLONED GENES

A. Cloning by Repair of Gapped Plasmids

In *Sacch. cerevisiae* a rapid method of cloning specific DNA regions from the genome is the gapped-plasmid repair technique (Orr-Weaver *et al.*, 1983). Double-stranded gaps on plasmids are repaired efficiently with homologous chromosomal sequences as a template. This method works well in *S. pombe* (Russell and Nurse, 1987a; Szankasi *et al.*, 1988). The poor ability of certain marker genes to complement when integrated, like *Sacch. cerevisiae URA3*, can be utilized to screen out clones which have integrated the plasmid after gap repair.

B. Gene Disruption and Replacement

Gene disruption and gene replacement are particularly important techniques because they provide a method to construct strains with unambiguous null mutations as well as other specific mutations made *in vitro*. Most useful is the one-step gene disruption method (Rothstein, 1983) in which linear DNA fragments containing a marker gene or other alteration constructed *in vitro* are transformed into a strain and then replace the chromosomal locus by a gene conversion event. This method works well in *S. pombe* although it is subject to the same possible complications involving marker genes as is plasmid integration (Section IV,C).

Gene disruptions and replacements are usually done in diploids which are then sporulated to produce haploids. A minor problem with *S. pombe* is that h^+/h^- diploids sporulate spontaneously after a few days growth on minimal medium. One way to overcome this problem is to conduct the disruptions in *ade6-210/ade6-216* diploids (Beach *et al.*, 1985; Fukui *et al.*, 1986). These two *ade6* mutations complement in a diploid and recombine very rarely; therefore, selection for adenine prototrophy excludes haploid spores from growing. A second solution is to do the disruption in nonsporulating h^+/h^+ or h^-/h^- diploids and then to mate them to a diploid homozygous for the opposite mating type (Russell and Nurse, 1986). Most of the diploid progeny will be h^+/h^- sporulators, about half of which will contain the disrupted allele on one chromosome. This is somewhat more cumbersome than the first method but is useful when it is desired to examine the effect of the gene disruption in a variety of genetic backgrounds.

The same recombination events used to make one-step gene disruptions can also be employed to introduce precise genetic alterations into the

genome. To facilitate this technique it is useful to be able to select for gene replacement. This can be done using a diploid strain in which one copy of the gene is disrupted with *ura4*$^+$, because *ura4*$^-$ cells are resistant to the drug 5-fluoroorotic acid (5-FOA) (Boeke *et al.*, 1984; Grimm and Kohli, 1988; Grimm *et al.*, 1988). Replacement of the *ura4*$^+$ disrupted gene with a linear DNA fragment containing the *in vitro* altered gene will convert the cells to a 5-FOA-resistant phenotype.

VI. PROPERTIES OF GENE EXPRESSION AND GENE STRUCTURE

More than 30 *S. pombe* protein-encoding genes have been cloned and sequenced, and in many cases mRNA start sites have been mapped. From these data some of the basic properties of intron splicing, codon usage, and promoter function in *S. pombe* can be ascertained. This information is useful both to those examining the structure and expression of *S. pombe* genes and to those concerned with expression of heterologous genes.

A. Introns

Genes with introns are relatively common in *S. pombe*: 8 of the first 30 *S. pombe* protein-encoding genes that have been sequenced have intervening sequences. There are five introns in the *nda3*$^+$ gene, four in *cdc2*$^+$, three in *ypt2*$^+$, two in *suc1*$^+$ and *cdc17*$^+$, and there is one intron each in *ras1*$^+$, *nda2*$^+$, and *cam1*$^+$. One remarkable feature of the known fission yeast introns is that they are all quite short, ranging from 36 to 129 nucleotides in total length. They contain short consensus sequences at the 5′ and 3′ splice junctions that are similar to those found in metazoans and most other fungi (Table II) (Mount, 1982; Guthrie, 1986). In addition, all but one of the *S. pombe* introns contain a short internal consensus sequence CTPuAPy starting 11 to 23 nucleotides upstream of the 3′ end of the intron. (The exception is the *cam1*$^+$ intron which contains the similar sequence CTGAA starting 34 nucleotides upstream of 3′ end of the intron.) The fission yeast internal consensus sequence is very similar to those identified in a variety of other eukaryotes (Keller and Noon, 1984; Guthrie, 1986). It has been shown that in *S. pombe*, like other eukaryotes, the internal consensus sequence is essential for splicing and that the A at position 4 is used for branch formation (Mertins and Gallwitz, 1987).

The available evidence indicates that fission yeast and budding yeast

TABLE II

Intron Consensus Sequences[a]

	5′ Splice site						Branch site								3′ Splice site		
A	—	—	95	37	5		32	56	—	—	74	100	5		21	100	—
G	100	—	—	16	95		6	32	—	—	26	—	—		—	—	100
C	—	—	—	5	—		6	—	100	—	—	—	79		5	—	—
T	—	100	5	42	—		56	12	—	100	—	—	16		74	—	—
	G	T	A	N	G	...19–99...	N	N	C	T	Pu	A	N	...3–16...	N	A	G

Consensus sequence

[a] Consensus sequences are compiled from 19 *S. pombe* introns in the following genes: *nda3*$^+$ (Hiraoka *et al.*, 1984), *cdc2*$^+$ (Hindley and Phear, 1984), *ypt2*$^+$ (Mertins and Gallwitz, 1987), *suc1*$^+$ (Hindley *et al.*, 1987), *cdc17*$^+$ (Barker *et al.*, 1987), *ras1*$^+$ (Fukui and Kaziro, 1985), *nda2*$^+$ (Toda *et al.*, 1984), and *cam1*$^+$ (Takeda and Yamamoto, 1987). Nucleotide-usage frequencies are expressed as a percentage value.

intron splicing systems are sufficiently different to block effective splicing of *S. pombe* pre-mRNA transcripts in *Sacch. cerevisiae*. It has been shown that the failure of the genomic copy of the *S. pombe cdc2*$^+$ gene to complement *Sacch. cerevisiae cdc28-ts* mutants can be corrected by expressing an intronless cDNA copy of the *cdc2*$^+$ gene (Booher and Beach, 1986). Likewise, the *S. pombe* DNA ligase gene *cdc17*$^+$, which contains two introns, fails to complement *Sacch. cerevisiae* mutants defective in the *CDC9*-encoded DNA ligase (polydioxyribonucleotide synthase), while the natural intronless *CDC9* gene complements *S. pombe cdc17*$^-$ mutations (Barker *et al.*, 1987). The failure of *Sacch. cerevisiae* to splice out *S. pombe* introns is almost certainly related to the fact that all known *Sacch. cerevisiae* introns contain the strictly conserved consensus sequences TACTAAC shortly upstream of the 3′ end of the intron and GTAPyGT at the 5′ end of the intron (Guthrie, 1986). None of the known *S. pombe* introns contain both of these consensus sequences. On the other hand, it would be expected that fission yeast should splice *Sacch. cerevisiae* introns since the budding yeast consensus sequences match the less stringent consensus sequences of *S. pombe* introns. Indeed, it has been shown that the *Sacch. cerevisiae* actin gene intron is spliced in *S. pombe*, albeit inefficiently (Mertins and Gallwitz, 1987). The inefficiency of splicing might be due to the fact that the budding yeast actin intron is larger (309 nucleotides) than *S. pombe* introns and its TACTAAC box is located further upstream (42 nucleotides) of the 3′ splice site.

Schizosaccharomyces pombe is quite similar to most other fungi and metazoans in terms of the frequency and distribution of introns within

genes and the consensus sequences of introns. The only major difference would appear to be intron size which is generally much bigger in metazoans. *Schizosaccharomyces pombe* can in fact properly excise the SV40 small T-antigen 67-nucleotide intron and produce the 20-kDa small T-antigen (Kaufer *et al.*, 1985). It remains to be seen if the *S. pombe* pre-mRNA splicing system is sufficiently similar to other fungi and higher eukaryotes to be of practical use in cloning heterologous genes by complementation from genomic DNA libraries. However, the remarkable similarity of the pattern and structure of the snRNA components of the splicing mechanism between *S. pombe* and metazoans (Ares, 1986; Tollervey and Mattaj, 1987) indicates that *S. pombe* should be an excellent model system with which to study intron splicing.

B. Codon Usage

Degenerate codons are not used at an equal frequency in *S. pombe* genes (Table III). Codon-usage bias is most extreme in genes which are highly expressed such as *adh*, encoding alcohol dehydrogenase, and *tpi*, encoding the glycolytic enzyme triose-phosphate isomerase. In these two genes only 43 of the potential 61 codons are used. A useful metric of the degree of codon usage bias in a gene is the codon bias index (CBI) (Bennetzen and Hall, 1982; Russell and Hall, 1983). The *adh* and *tpi* genes have CBI values above 0.8, the cytochrome *c* gene *cyc* has an intermediate CBI value of 0.51, and the cell cycle control genes *cdc2*, *cdc25*, and *wee1* have values below 0.2 (Table IV). Northern analysis of mRNA levels of transcripts from these genes has shown that *adh* and *tpi* mRNA levels are high, *cyc* levels are intermediate, and the *cdc2*, *cdc25*, and *wee1* mRNA levels are low (P. Russell, unpublished data). This indicates that in *S. pombe* there is a strong correlation between high mRNA levels and high CBI values. Therefore, the CBI value of a gene is probably a useful parameter to use in estimating the likely expression level of that gene.

One striking feature of the pattern of codon-usage bias in *S. pombe* is that twofold degenerate codon sets in genes with low CBI values do not simply exhibit random codon usage but instead actually prefer the codon which is not preferred in genes with high CBI values (Table III). For example, genes with high CBI values used the TTC Phe codon in 76% of the cases while genes with low CBI values used the alternate Phe codon TTT 72% of the time. The only exception is Gln codons. This difference could indicate that there is selective advantage for having genes which are highly expressed use different isoaccepting tRNA pools than those which are expressed at a lower level. Another pattern in all the twofold degener-

TABLE III

Codon Usage in *Schizosaccharomyces pombe*[a]

Lo	Hi	Codon	Amino acid	Lo	Hi	Codon	Amino acid	Lo	Hi	Codon	Amino acid	Lo	Hi	Codon	Amino acid
72	24	TTT	Phe	39	41	TCT	SER	67	13	TAT	Tyr	60	7	TGT	Cys
28	76	TTC	PHE	19	52	TCC	SER	33	88	TAC	TYR	40	93	TGC	CYS
26	5	TTA	Leu	18	0	TCA	Ser								
23	34	TTG	LEU	9	0	TCG	Ser							TGG	Trp
32	39	CTT	LEU	56	50	CCT	PRO	74	19	CAT	His	32	88	CGT	ARG
8	22	CTC	LEU	18	50	CCC	PRO	26	81	CAC	HIS	12	12	CGC	Arg
8	0	CTA	Leu	19	0	CCA	Pro	76	88	CAA	GLN	21	0	CGA	Arg
8	0	CTG	Leu	7	0	CCG	Pro	24	12	CAG	Gln	11	0	CGG	Arg
66	51	ATT	ILE	47	41	ACT	THR	63	13	AAT	Asn	10	4	AGT	Ser
18	49	ATC	ILE	30	59	ACC	THR	37	87	AAC	ASN	9	4	AGC	Ser
16	0	ATA	Ile	19	0	ACA	Thr	66	2	AAA	Lys	17	0	AGA	Arg
		ATG	Met	8	0	ACG	Thr	34	98	AAG	LYS	7	0	AGG	Arg
53	42	GTT	VAL	48	50	GCT	ALA	76	32	GAT	Asp	40	87	GGT	GLY
12	58	GTC	VAL	22	47	GCC	ALA	24	68	GAC	Asp	16	13	GGC	Gly
21	0	GTA	Val	22	4	GCA	Ala	67	23	GAA	Glu	27	0	GGA	Gly
14	0	GTG	Val	7	0	GCG	Ala	33	78	GAG	GLU	16	0	GGG	Gly

[a] Codon-usage frequencies are expressed as a percentage value among each degenerate codon set. Hi is a compilation of genes *adh* and *tpi* exhibiting high codon-usage bias, and Lo is a compilation of genes *cdc2*, *wee1*, and *cdc25* exhibiting low codon-usage bias. Amino acids designated by all capital letters are for preferred codons (see Table IV).

TABLE IV

Codon Bias Index[a]

Gene	CBI value
adh	0.88
tpi	0.82
cyc	0.51
wee1	0.19
cdc25	0.16
cdc2	0.00

[a] The codon bias index (CBI) value is a measure of the degree of codon-usage bias in a gene; high values indicate strong codon-usage bias. The CBI is calculated by the equation CBI = $P - R/T - R$, where P is the number of times preferred codons are used, R the expected number of times the preferred codons would be used if codon usage was random, and T the total number of codons in the gene not including Met, Asp, and Trp codons. Preferred codons were chosen as those which were used over 75% of the time in the *adh* and *tpi* genes; in most 3-, 4-, and 6-fold degenerate sets pairs of codons met this criterion. The 24 preferred codons in *S. pombe* are those in Table III for which the corresponding amino acid is in capital letters.

ate codon sets, again with the exception of Gln, is that the bias is in favor of G/C nucleotides at the third position in high-CBI genes and A/T nucleotides at the third position in low-CBI genes. This trend is also apparent in the overall frequency of third position nucleotides: high-CBI genes are 59% G/C while low-CBI genes are 34% G/C. Therefore, the differences between high- and low-CBI genes are twofold degenerate codon sets is likely to be due to selection acting in favor of G/C nucleotides at the third position in high-CBI genes or A/T nucleotides in low-CBI genes as opposed to selection acting in favor of high- and low-CBI genes using different isoaccepting tRNA pools. The basis of this presumed selective pressure is not known, but one possibility is that G/C nucleotides in the third position of codons in some way enhance gene expression.

C. Promoters

In *Sacch. cerevisiae* it is well established that promoters of protein-encoding genes, which are transcribed by RNA polymerase II, are com-

posite structures made up of at least three functional elements: (1) upstream activation sites (UAS) including enhancer-like elements and regulatory regions, (2) TATA boxes which specify the region of transcription initiation, and (3) specific transcription initiation sites (reviewed by Guarente, 1984). RNA polymerase II promoters have not been well investigated in *S. pombe*; however, the available evidence indicates that at least the first two promoter elements described in *Sacch. cerevisiae* are also present in *S. pombe*. Transcription generally starts within 200 nucleotides upstream of the open reading frame in *S. pombe*, and often there are two start sites located within a 10-nucleotide region. In genes which are transcribed at moderate or high levels there is a TATA sequence motif located about 35–45 nucleotides upstream of the start sites (Table V). TATA boxes are less obvious in genes which are transcribed at lower levels. Deletions located as far as 1 kb upstream of the start sites can impair promoter function (P. Russell, unpublished data), indicating that UAS sequences are also present in *S. pombe*. Specific UAS sequences have not been identified in *S. pombe*; however, the nine histone genes of *S. pombe* contain a 17-nucleotide consensus sequence located upstream of the TATA box. This sequence is a good candidate for the regulatory region involved in controlling the periodic transcription of these genes during the cell cycle (Matsumoto and Yanagida, 1985).

To evaluate the structure and function of promoters in *S. pombe* it has been informative to investigate the transcription of *Sacch. cerevisiae*

TABLE V

Putative TATA Boxes and Distance to mRNA Start Sites[a]

Gene	TATA sequence	Distance to 5′ end of mRNA
adh	TATAAATAG	45
tpi	TATATATAA	41
cyc	TAAATTTAA	42
ran1	TAGAAAATC	41
suc1	AAATTTAGC	46
mei3	TATAAGTAT	34
cdc17	TACAAATAT	36

[a] References for mRNA mapping are as follows: *adh* (Russell, 1983), *tpi* (Russell, 1985), *cyc* (Russell and Hall, 1982), *ran1* (McLeod and Beach, 1987), *suc1* (Hindley *et al.*, 1987), *mei3* (McLeod *et al.*, 1987), and *cdc17* (Barker *et al.*, 1987).

genes in *S. pombe* and vice versa. In no case has the heterologous transcription pattern of a gene been identical to the homologous transcription pattern (Russell, 1983, 1985; Losson *et al.*, 1985). The yeasts clearly differ in the mechanism which determines the 5′ end of the mRNA relative to the TATA box. In *Sacch. cerevisiae* the transcription of *S. pombe* genes generally initiates downstream of the *S. pombe* start sites. This is consistent with the observations that in *Sacch. cerevisiae* transcription can initiate in a wide region approximately 35 to 150 nucleotides downstream of the TATA box. In most cases heterologous transcription appears to be inefficient (Russell, 1985).

VII. CONCLUSIONS

Most of the important gene cloning methods available for studying *Sacch. cerevisiae* have now also been adapted to *S. pombe*. However, two major techniques are still lacking in *S. pombe*. One serious problem is the absence of functioning centromere plasmids. In *Sacch. cerevisiae*, *cen* DNA fragments cause circular *ars* plasmids to be inherited in a relatively stable fashion while maintaining a copy number of approximately one (Carbon, 1984). The stable low copy number property has been invaluable in making it possible to clone genes which are lethal at high or even moderate copy numbers (Rose and Fink, 1987). *Saccharomyces cerevisiae* centromeres fail to stabilize plasmids in *S. pombe*, a fact now readily understood by the finding that centromere regions are much larger in *S. pombe* (>50–100 kb) than in *Sacch. cerevisiae* (~1 kb) (Clarke *et al.*, 1986; Nakaseko *et al.*, 1986). Unfortunately, the *S. pombe cen* sequences do not stabilize circular plasmids in *S. pombe*. Currently then, there is no plasmid that can be straightforwardly used to clone *S. pombe* genes which are lethal at elevated copy number, as is the case with *nda2*$^+$ encoding β-tubulin (Toda *et al.*, 1984).

The second tool lacking in the repertoire of *S. pombe* cloning methods is an inducible promoter which could be used to regulate expression of cloned genes. The portable promoters that have been used so far are all constitutively transcribed. Transcription of *pho1*$^+$ encoding secreted acid phosphatase is induced in media containing low concentrations of inorganic phosphate; however, the significant level of uninduced transcription largely negates the potential use of this promoter (Maundrell *et al.*, 1985a). Recently two genes have been cloned which are strongly transcribed when switched from maltose to glucose medium (K. Maundrell, personal communication). It is hoped that the promoters of these genes will be useful for regulating expression of cloned genes.

VIII. ADDENDUM

Plasmid pDB248X (Fig. 1) contains a *Bam*HI site at position 0.4. Plasmid pFL20 (Fig. 2) contains an additional *Sph*I site at map position 6.9 and an additional *Sal*I site at map position 7.4.

ACKNOWLEDGMENTS

The author thanks Jackie Hayles, Jurg Kohli, Kinsey Maundrell, Paul Nurse, Viesturs Simanis, Aresa Toukdarian, and members of the Cell Cycle Control Laboratory at the Imperial Cancer Research Fund Laboratory for discussions and comments and Clare Middlemiss and Laurel Kelly for preparing the manuscript.

REFERENCES

Adachi, Y., Toda, T., Niwa, O., and Yanagida, M. (1986). Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **6,** 2168–2178.

Ares, M., Jr. (1986). U2 RNA from yeast is unexpectedly large and contains homology to vertebrate, U4, U5 and U6 small nuclear RNAs. *Cell* **47,** 49–59.

Aves, S. J., Durkacz, B. W., Carr, A., and Nurse, P. (1985). Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc10* "start" gene. *EMBO J.* **4,** 457–463.

Barker, D. G., and Johnston, L. H. (1983). *Saccharomyces cerevisiae cdc9,* a structural gene for yeast DNA ligase which complements *Schizosaccharomyces pombe cdc17*. *Eur. J. Biochem.* **134,** 315–319.

Barker, D. G., White, J. H. M., and Johnston, L. H. (1987). Molecular characterization of the DNA ligase gene, *CDC17,* from the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **162,** 659–667.

Beach, D., and Nurse, P. (1981). High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature* (*London*) **290,** 140–142.

Beach, D., Piper, M., and Nurse, P. (1982). Construction of a *Schizosaccharomyces pombe* gene bank in a yeast bacterial shuttle vector and its use to isolate genes by complementation. *Mol. Gen. Genet.* **187,** 326–329.

Beach, D., Rodgers, L., and Gould, J. (1985). RAN1$^+$ controls the transition from mitotic division to meiosis in fission yeast. *Curr. Genet.* **10,** 297–311.

Bennetzen, J. L., and Hall, B. D. (1982). Codon selection in yeast. *J. Biol. Chem.* **257,** 3026–3031.

Boeke, J. D., Lacroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-Fluoroorotic acid resistance. *Mol. Gen. Genet.* **197,** 345–346.

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of *cdc2*$^+$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **6,** 3523–3530.

Bostock, C. J. (1970). DNA synthesis in the fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **60,** 16–26.

Broach, J. R., Strathern, J. N., and Hicks, J. B. (1979). Transformation in yeast: Development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8,** 121–131.

Broker, M., Ragg, H., and Karges, H. E. (1987). Expression of human antithrombin III in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **908,** 203–213.

Carbon, J. (1984). Yeast centromeres: Structure and function. *Cell* **37,** 351–353.

Carr, A. M. (1987). Analysis and characterisation of the *cdc2* gene region of fission yeast. Doctoral thesis, University of Sussex.

Chevallier, M. R., and Lacroute, F. (1982). Expression of the cloned uracil permease gene of *Saccharomyces cerevisiae* in a heterologous membrane. *EMBO J.* **1,** 375–377.

Choe, J., Shuster, T., and Grunstein, M. (1985). Organization, primary structure, and evolution of histone H2A and H2B genes of the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **5,** 3261–3269.

Clarke, L., Amstutz, H., Fischel, B., and Carbon, J. (1986). Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8253–8257.

Draetta, G., Brizuela, L., Potashkin, J., and Beach, D. (1987). Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by $cdc2^+$ and $suc1^+$. *Cell* **50,** 319–325.

Durkacz, B., Beach, D., Hayles, J., and Nurse, P. (1985). The fission yeast cell cycle control gene *cdc2:* Structure of the *cdc2* region. *Mol. Gen. Genet.* **201,** 543–545.

Elliott, S., Chang, C. W., Schwingruber, M. E., Schaller, J., Rickli, E. E., and Carbon, J. (1986). Isolation and characterization of the structural gene for secreted acid phosphatase from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **261,** 2936–2941.

Fantes, P. A. (1981). Isolation of cell size mutants of a fission yeast by a new selective method. *J. Bacteriol.* **146,** 746–754.

Fukui, Y., and Kaziro, Y. (1985). Molecular cloning and sequence analysis of a *ras* gene from *Schizosaccharomyces pombe*. *EMBO J.* **4,** 687–691.

Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986). Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. *Cell* **44,** 329–336.

Gaillardin, C., Fournier, P., Budar, F., Kudla, B., Gerbaud, C., and Heslot, H. (1983). Replication and recombination of 2-μm DNA in *Schizosaccharomyces pombe*. *Curr. Genet.* **7,** 245–253.

Grimm, C., and Kohli, J. (1988). Observations on integrative transformation in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **215,** 87–93.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe:* A system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. Gen. Genet.* **215,** 81–96.

Guarente, L. (1984). Yeast promoters: Positive and negative elements. *Cell* **36,** 799–800.

Guthrie, C. (1986). Finding functions for small nuclear RNAs in yeast. *Trends Biol. Sci.* **11,** 430–434.

Gutz, H., Heslot, H., Leupold, U., and Lopreno, N. (1974). *Schizosaccharomyces pombe*. *Handb. Genet.* **1,** 395–446.

Hayles, J., Aves, S., and Nurse, P. (1986). *suc1* is an essential gene involved in both the cell cycle and growth in fission yeast. *EMBO J.* **5,** 3373–3379.

Heyer, W.-D., Sipiczki, M., and Kohli, J. (1986). Replicating plasmids in *Schizosaccharomyces pombe:* Improvement of symmetric segregation by a new genetic element. *Mol. Cell. Biol.* **6,** 80–89.

Hindley, J., and Phear, G. A. (1984). Sequence of the cell division gene *CDC2* from *Schizosaccharomyces pombe;* patterns of splicing and homology to protein kinases. *Gene* **31,** 129–134.

Hindley, J., Phear, G., Stein, M., and Beach, D. (1987). *suc1*$^+$ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **7,** 504–511.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes β-tubulin: A cold-sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39,** 349–358.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163–168.

Jones, R., Moreno, S., Nurse, P., and Jones, N. (1988). Expression of the SV40 promoter in fission yeast: Identification and characterization of an AP1-like factor. *Cell* **53,** 659–667.

Kaufer, N. F., Simanis, V., and Nurse, P. (1985). Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. *Nature (London)* **318,** 78–80.

Keller, E. B., and Noon, W. A. (1984). Intron splicing: A conserved internal signal in introns of animal pre-mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7417–7420.

Kopecka, M. (1975). The isolation of protoplasts of the fission yeast *Schizosaccharomyces* by *Trichoderma viride* and snail enzymes. *Folia Microbiol.* **20,** 273–276.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature (London)* **327,** 31–35.

Losson, R., and Lacroute, F. (1983). Plasmids carrying the yeast OMP decarboxylase structural and regulatory genes: Transcription regulation in a foreign environment. *Cell* **32,** 371–377.

Losson, R., Fuchs, R. P. P., and Lacroute, F. (1985). Yeast promoters *URA1* and *URA3*. Examples of positive control. *J. Mol. Biol.* **185,** 65–81.

McLeod, M., and Beach, D. H. (1987). Homology between the *ran1*$^+$ gene of fission yeast and protein kinases. *EMBO J.* **5,** 3665–3671.

McLeod, M., Stein, M., and Beach, D. (1987). The product of the *mei3*$^+$ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J.* **6,** 729–736.

Mao, J., Schmidt, O., and Soll, D. (1980). Dimeric transfer RNA precursors in *S. pombe*. *Cell* **21,** 509–516.

Matsumoto, S., and Yanagida, M. (1985). Histone gene organization of fission yeast: A common upstream sequence. *EMBO J.* **4,** 3531–3538.

Maundrell, K., Nurse, P., Schonholzer, F., and Schweingruber, M. E. (1985a). Cloning and characterisation of two genes restoring acid phosphatase activity in *pho1*$^-$ mutants of *Schizosaccharomyces pombe*. *Gene* **39,** 223–230.

Maundrell, K., Wright, A. P. H., Piper, M., and Shall, S. (1985b). Evaluation of heterologous ARS activity in *S. cerevisiae* using cloned DNA from *S. pombe*. *Nucleic Acids Res.* **13,** 3711–3722.

Mertins, P., and Gallwitz, D. (1987). Nuclear pre-mRNA splicing in the fission yeast *Schizosaccharomyces pombe* strictly requires an intron-contained, conserved sequence element. *EMBO J.* **6,** 1757–1763.

Mount, S. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res.* **10,** 459–472.

Nadin-Davis, S. A., Yang, R. C., Narang, S. A., and Nasim, A. (1986). The cloning and characterisation of a *RAS* gene from *Schizosaccharomyces pombe*. *J. Mol. Evol.* **23,** 41–51.

Nakanishi, N., and Yamamoto, M. (1984). Analysis of the structure and transcription of the *aro3* cluster gene in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **195,** 164–169.

Nakaseko, Y., Adachi, Y., Funahashi, S., Niwa, O., and Yanagida, M. (1986). Chromo-

some walking shows a highly homologous repetitive sequence present in all the centromeric regions of fission yeast. *EMBO J.* **5,** 1011–1021.

Nasmyth, K. A., and Reed, S. I. (1980). Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2119–2123.

Necas, O., Svoboda, A., and Havelkova, M. (1968). Mechanism of regeneration of yeast protoplasts. V. Formation of the cell wall in *Schizosaccharomyces pombe*. *Folia Biol. (Praha)* **14,** 80–85.

Orr-Weaver, T. K., Szostak, J. W., and Rothstein, R. J. (1983). Genetic applications of yeast transformation with linear and gapped plasmids. *In* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, pp. 228–245. Academic Press, New York.

Rose, M. D., and Fink, G. R. (1987). *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell* **48,** 1047–1060.

Rothstein, R. J. (1983). One-step gene disruption in yeast. *In* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, pp. 202–211. Academic Press, New York.

Russell, P. R. (1983). Evolutionary divergence of the mRNA transcription initiation mechanism in yeast. *Nature (London)* **301,** 167–169.

Russell, P. R. (1985). Transcription of the triose-phosphate isomerase gene of *Schizosaccharomyces pombe* initiates from a start point different from that in *Saccharomyces cerevisiae*. *Gene* **40,** 125–130.

Russell, P. R., and Hall, B. D. (1982). Structure of the *Schizosaccharomyces pombe* cytochrome *c* gene. *Mol. Cell. Biol.* **2,** 106–116.

Russell, P. R., and Hall, B. D. (1983). The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **258,** 143–149.

Russell, P., and Nurse, P. (1986). *cdc25*$^+$ functions as an inducer in the mitotic control of fission yeast. *Cell* **45,** 145–153.

Russell, P., and Nurse, P. (1987a). Negative regulation of mitosis by *wee1*$^+$, a gene encoding a protein kinase homolog. *Cell* **49,** 559–567.

Russell, P., and Nurse, P. (1987b). The mitotic inducer *nim1*$^+$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49,** 569–576.

Sakaguchi, J., and Yamamoto, M. (1982). Cloned *ura1* locus of *Schizosaccharomyces pombe* propagates autonomously in this yeast assuming a polymeric form. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 7819–7823.

Sakai, K., Sakaguchi, J., and Yamamoto, M. (1984). High-frequency cotransformation by copolymerization of plasmids in the fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **4,** 651–656.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene *cdc2*$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45,** 261–268.

Sipiczki, M., Heyer, W.-D., and Kohli, J. (1985). Preparation and regeneration of protoplasts and spheroplasts for fusion and transformation of *Schizosaccharomyces pombe*. *Curr. Microbiol.* **12,** 169–174.

Szankasi, P., Heyer, W.-D., Schuchert, P., and Kohli, J. (1988). DNA sequence analysis of the *ade6* gene of *Schizosaccharomyces pombe:* Wild-type and mutant alleles including the recombination hot spot allele *ade6-M26*. *J. Mol. Biol.* **204,** 917–925.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **9,** 6429–6437.

Takeda, T., and Yamamoto, M. (1987). Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3580–3584.

Toda, T., Adachi, Y., Hiraoka, Y., and Yanagida, M. (1984). Identification of the pleiotropic cell division cycle gene *NDA2* as one of two different α-tubulin genes in *Schizosaccharomyces pombe*. *Cell* **37,** 233–242.

Tollervey, D., and Mattaj, I. W. (1987). Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNAs. *EMBO J.* **6,** 469–476.

Uemura, T., Morikawa, K., and Yanagida, M. (1986). The nucleotide sequence of the fission yeast DNA topoisomerase II structural gene: Structural and functional relationships to other DNA topoisomerases. *EMBO J.* **5,** 2355–2362.

Wright, A. P. H., Maundrell, K., and Shall, S. (1986a). Transformation of *Schizosaccharomyces pombe* by non-homologous unstable integration of plasmids in the genome. *Curr. Genet.* **10,** 503–508.

Wright, A., Maundrell, K., Heyer, W.-D., Beach, D., and Nurse, P. (1986b). Vectors for the construction of gene banks and the integration of cloned genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Plasmid* **15,** 156–158.

Yamamoto, M., Asakura, Y., and Yanagida, M. (1981). Cloning of a gene from the fission yeast *Schizosaccharomyces pombe* which complements *Escherichia coli PyrB* the gene for aspartate transcarbamylase EC 2.1.3.2. *Mol. Gen. Genet.* **182,** 426–429.

Yanagida, M. (1987). Yeast tubulin genes. *Microbiol. Sci.* **4,** 115–118.

Yanagida, M., and Wang, J. C. (1987). Yeast DNA topoisomerases and their structural genes. *Nucleic Acids Mol. Biol.* **1,** 196–209.

8

General Cytology of Fission Yeasts

C. F. ROBINOW* AND J. S. HYAMS†

*Department of Microbiology and Immunology
The University of Western Ontario
London, Ontario, Canada N6A 5C1

†Department of Biology
University College London
London WC1E 6BT, England

I. GENERAL CYTOLOGY OF FISSION YEASTS

Fission yeasts, growing and dividing under optimal conditions, have simple, regular shapes that invite quantitative studies of cell growth of the kind initiated by Mitchison (1957) and reviewed by him in Chapter 6 (this volume). A visible activity of cells (and we are here concerned with

The authors have performed their work independently. Section I, General Cytology of Fission Yeasts is provided by C. F. Robinow, and Section II, Fluorescence Microscopy, is provided by J. S. Hyams.

visible structures and events) is growth which in fission yeasts is accompanied by increase in length. When the cell has nearly doubled its length (and volume) division of the nucleus takes place, an event which is soon followed by the division of the cell itself, an activity that requires the addition of new sections to its cell wall. Having this natural order of things prescribed for us, we start our morphological overview with certain inclusions in the cytoplasm, describe normal mitosis and nuclear division, and conclude our brief account of the vegetative life of fission yeasts with a short sketch of cell division. There remains another course of life to be considered. Fission yeasts, in common with haploid lines of certain budding yeasts, are able to conjugate and generate diploid synkaryons that undergo meiosis followed by the generation of haploid offspring in the form of ascospores. These events are briefly described, and our account of them ends with the return to the vegetative life by the germination of the spores.

A. Cytoplasmic Features

Large-scale structures within the cytoplasm such as droplets of lipid, vacuoles filled with polyphosphate, and mitochondria are revealed by conventional light microscopy, but a more sophisticated tool, immunofluorescence microscopy, is required for the exploration of the more recently discovered elements of the cytoskeleton described in Section II of this chapter. Electron microscopy has been particularly useful in resolving the organization of the spindle of mitosis and fine-structure details of cell division, conjugation, and spore formation (described later) but has revealed few features not also encountered and already described in budding yeasts and mycelial fungi.

1. Lipid

Growing cells of *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* tend to contain clusters of small, shiny droplets largely consisting of lipid (see Figs. 1 and 2), to judge by the blue-green color imparted to them by Sudan black B. Most numerous near the ends of the cell, they are also found elsewhere in the cytoplasm, particularly near its surface where they are likely to superimpose their unwanted images on that of the nucleus. These are the small, dark or shiny, round droplets scattered about the living cells in phase-contrast micrographs of fission yeasts by McCully and Robinow (1971) and Tanaka and Kanbe (1986). During life the droplets oscillate in a sedate kind of Brownian movement, a sure sign that the cell harboring them is in good condition and may be expected to grow and in due course to divide. In electron micrographs of

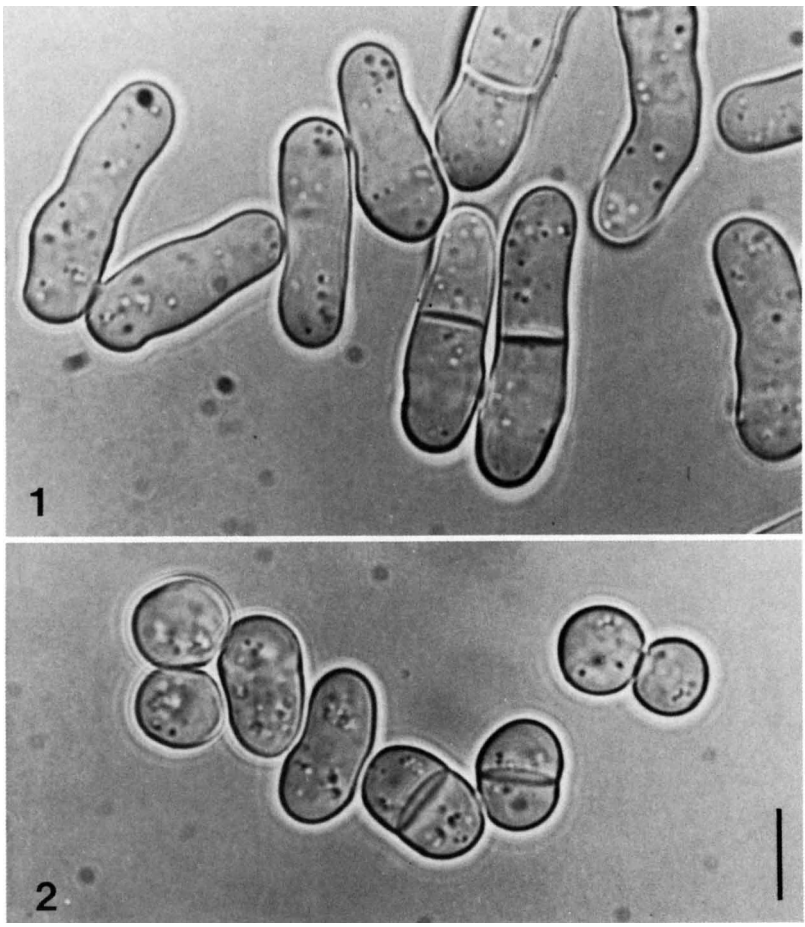


Fig. 1. *Schizosaccharomyces japonicus* after 4 hr on yeast extract/glucose agar at 30°C.
Fig. 2. Cells from the culture used for Fig. 1 after 3 days at room temperature. Bar, 5 μm.

sections the sites of lipid present as the sharply contoured round or ovoid blank spaces seen at the poles of the cell in Fig. 3b. See also equivalent "white" profiles in electron micrographs of sections of *S. pombe* in Walker *et al.* (1982) and Tanaka and Kanbe (1986).

2. *Polyphosphate Vacuoles*

The subject of the present section is conveniently introduced by quotations from the opening paragraph of a review paper by Guilliermond

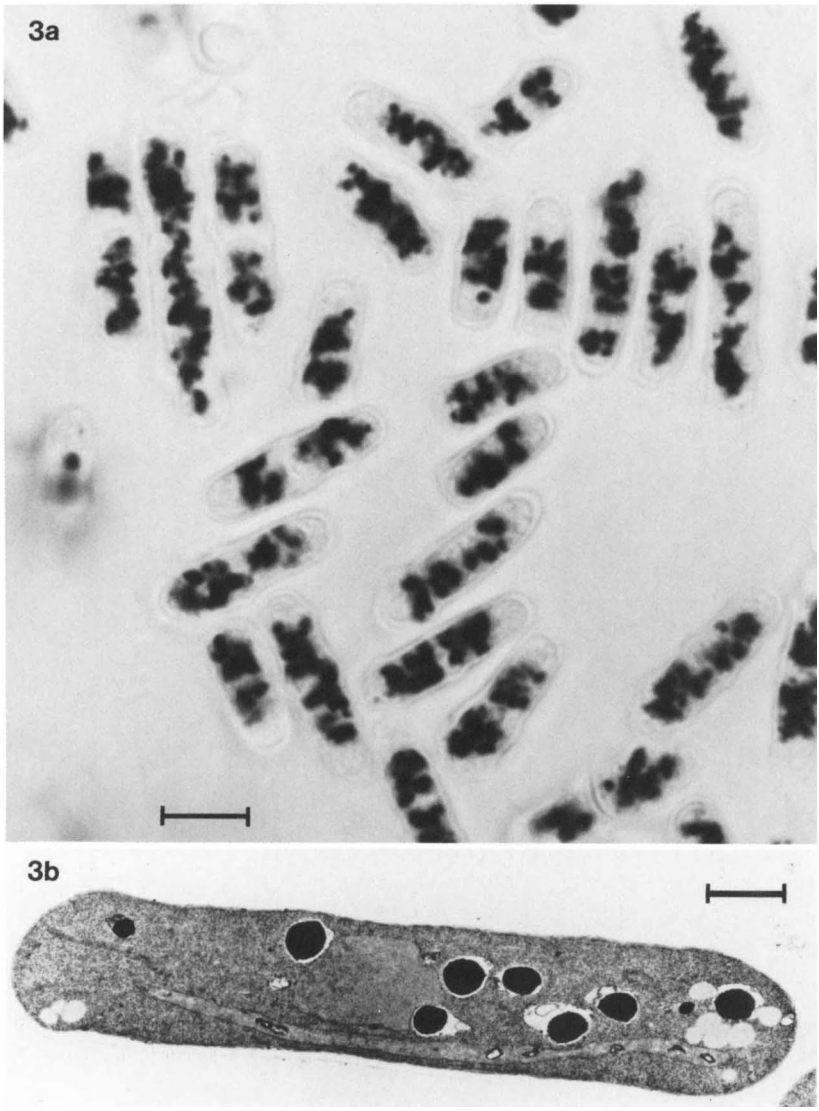


Fig. 3. (a) *Schizosaccharomyces pombe* after 4 hr on yeast extract/glucose agar at 30°C. Cells were fixed with ethanol–formalin–acetic acid, 40/5/5% (v/v), in water and stained for 2 min with 0.025% toluidine blue in 0.5% acetic acid. The cells are crammed with selectively stained polyphosphate (volutin) vacuoles. Bar, 5 μm. (b) Electron micrograph of a section of *S. pombe* that had been fixed with glutaraldehyde–formaldehyde. Dark inclusions represent polyphosphate. Clusters of small, empty vacuoles at the poles correspond to lipid droplets. Bar, 1 μm. [From McCully and Robinow (1971), with permission.] (c) Electron micrograph of a section of *S. pombe* that had been fixed with glutaraldehyde and postfixed with potassium permanganate as described in Miyata *et al.* (1985). Much of the polyphosphate has been preserved. Bar, 1 μm. (Courtesy of Dr. M. Miyata.)

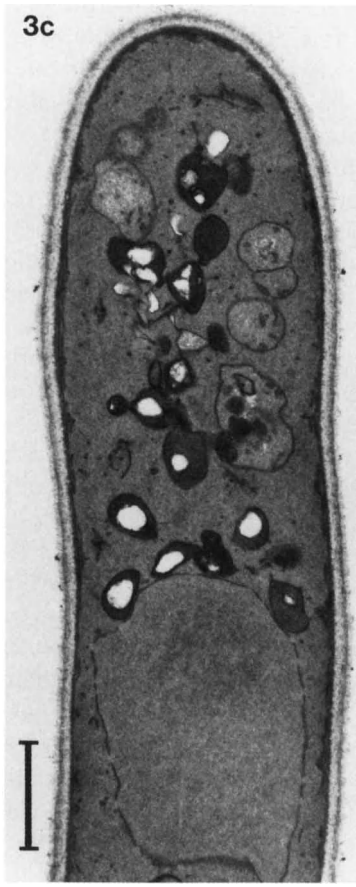


Fig. 3c.

(1910), who notes

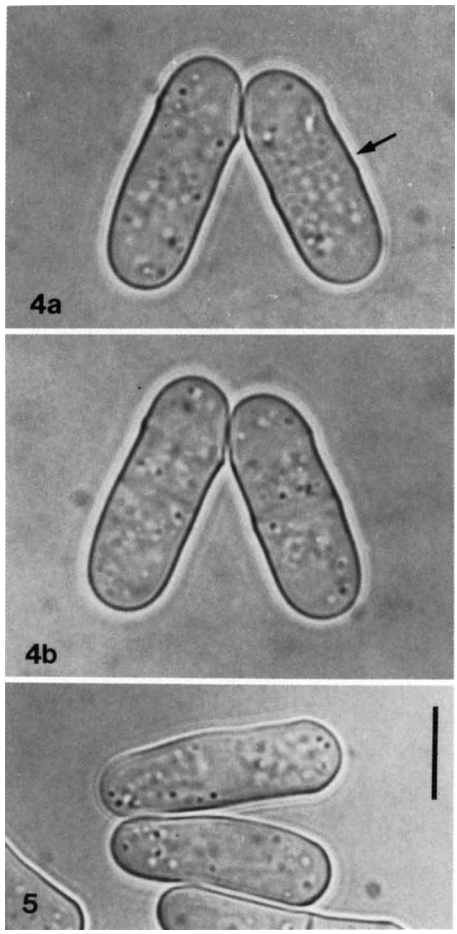
> Lorsqu'on colore une cellule de champignon ou d'algue, on est immédiatement frappé de rencontrer, en dehors du noyau, un nombre considérable de grains qui présentent pour les colorants une affinité beaucoup plus grande que le noyau. Ces grains ... ont réçu les désignations de corpuscules métachromatiques ou de grains de volutine. Ce sont des grains de sécretion situés dans le cytoplasme.... Leur forme est sphérique et leurs dimensions sont extrêmement variables.* (p. 289.)

*Having [suitably] stained fungal or algal cells, one is immediately struck to find, outside the nucleus, a considerable number of granules which display a much greater affinity for stains than the nucleus. These granules ... have been called metachromatic corpuscles or volutin granules. They are granules of secretion [stored] in the cytoplasm.... Their shape is spherical and their dimensions are extremely variable.

Prominent among the stains used by Guilliermond and contemporaries in later work was neutral red, which stains volutin (named after its occurrence in *Spirillum volutans*) in living cells. The metachromasia to which Guilliermond refers in the passage quoted is the ability of materials in the cytoplasm of chemically fixed cells to shift the color of dyes such as methylene blue and toluidine blue toward shades of purple and red.

Volutin has been seen in *S. pombe*. In a study of the visible effects on this yeast of deprivation of and inhibition by inositol, Schopfer *et al.* (1962) note in passing that normal, living cells of this yeast (from several days old cultures to judge by the photographs provided) contain groups of little vacuoles each containing a particle having affinity for neutral red. The authors took this observation to signify, indirectly, that these granules were of the metachromatic kind, that is, volutin. That *S. pombe* cells from cultures past the exponential phase of growth tend to be full of granules is the common experience. Less notice has been taken of the observation recorded by E. K. McCully in McCully and Robinow (1971) that growing and dividing cells from young cultures, notwithstanding their clear, translucent cytoplasm, are full of granules that are selectively and metachromatically stained by acidified toluidine blue (Fig. 3a). Granules with these properties, whose presence in fission yeasts we have, belatedly, confirmed, are still often referred to as volutin but are increasingly also called by the name of one of their constituents, namely, inorganic polyphosphate. Much analytical chemical work has been done on volutin from yeasts and bacteria, and the connection between metachromasia and polyphosphate is well established (Harold, 1966; Miller, 1984). This is not saying that volutin is plain polyphosphate. According to Smith *et al.* (1954) the volutin granules in bacteria "probably consist of other substances such as RNA and protein and are merely coated or permeated with the metaphosphate which confers their peculiar staining character." Another partner for polyphosphate is proposed by Allan and Miller (1980) and Miller (1984) who argue persuasively that a major component of polyphosphate granules in *Saccharomyces cerevisiae* may be *S*-adenosylmethionine (S-AM).

The composition of the metachromatic granules in fission yeasts remains to be determined, as does their role in the economy of these organisms. The neglect of this item of the morphological inventory of fission yeasts is largely due to the low visibility of what we believe to be the equivalent of the volutin-bearing vacuoles in living, growing cells from young cultures. Their density, it seems, is too close to that of the cytoplasm (Figs. 4 and 5) to attract—to judge by our personal experience—the attention of observers unacquainted with the images produced by staining with toluidine blue. Volutin vacuoles are clearly visible in many



Figs. 4 and 5. Living cells of *S. japonicus* displaying lipid droplets, small, dark, or shiny, and larger translucent inclusions (arrow) corresponding in numbers and distribution to polyphosphate vacuoles. See also Fig. 15. Cell division has started in Fig. 4b. Bar, 5 μm.

phase-contrast photomicrographs (though not necessarily, before development, to the photographer); Fig. 15 is an example of this. Regrettably, the conditions favoring their predictable emergence into view have not yet been determined.

Various kinds of vacuoles in *S. pombe* have been recorded by electron microscopy. There are, first, the many empty vacuoles of irregular shape that are prominent features of sections of the permanganate-fixed cells

studied (for other reasons) by Osumi and Sando (1969), Oulevey *et al.* (1970), and Johnson *et al.* (1973). The second kind of inclusion, regularly seen after glutaraldehyde–formaldehyde fixation, takes the form of a round, ovoid, or angle-cornered slice of solidly dense matter set eccentrically in a clear, partly membrane-lined surround, presumably a vacuole. The larger ones of these enclose also granular matter and shreds and whorls of membranes, a sign, perhaps, of damage sustained during fixation and/or sectioning. A few profiles of this variety of inclusion appear, but are not commented on, in two modern studies of *S. pombe,* those by Heslot *et al.* (1970) and Walker *et al.* (1982), whose authors were concerned with mitochondria, not vacuoles, and whose micrographs may therefore be accepted as unselected regarding the latter. Numerous profiles of vacuoles with dark contents of the kind described above are seen scattered all over the cytoplasm in the low-power electron micrographs of sections of *S. pombe* obtained by E. K. McCully and described and interpreted in McCully and Robinow (1971). One of these sections, reduced in size, is reprinted as Fig. 3b in this chapter. McCully regarded these prominent vacuoles with their dense contents as representing the volutin granules of light microscopy (McCully and Robinow, 1971). We agree but find even more plausible likenesses of the latter in the profiles of perfectly round vacuoles, more or less evenly filled with spongy, dense matter in sections of cells of *S. pombe* preserved by freeze–substitution as described by Tanaka and Kanbe (1986).

When electron micrographs of *S. pombe* illustrating the three kinds of vacuoles described above are placed alongside light micrographs of fission yeasts stained with acidified toluidine blue, it becomes evident that the inclusions in the cytoplasm of all of them are but different images of the same thing, namely, vacuoles containing volutin granules differentially affected by different fixatives of which freeze–substitution must be adjudged good, permanganate unsuitable, and glutaraldehyde/formaldehyde partly successful. The contrasting effects of permanganate and glutaraldehyde on the polyphosphate vacuoles have, inadvertently, been well illustrated by differences in the appearance of the vacuoles in one and the same cell from a sample of *S. pombe* (Fig. 3c) that had been subjected to 2 hr of preliminary fixation with glutaraldehyde followed by 30–40 min of "extraction" with potassium permanganate. In the micrograph, kindly supplied by Dr. Machiko Miyata, it is seen that under these conditions the ability of permanganate to remove polyphosphate from the vacuoles in the cytoplasm has been much reduced.

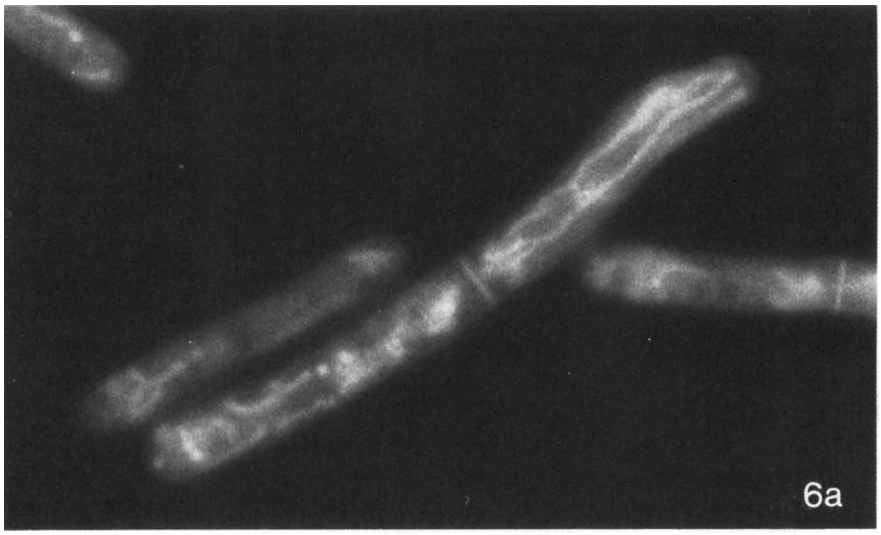
Light microscopy has a similar tale to tell. In that sphere, too, different fixatives affect volutin granules in different ways, thereby providing us with potential clues to their composition and, up to a point, justifying

Miller's skeptical surmise (1984), "The volutin granules demonstrated in cells by some common cytological procedures may be artifacts and not actually present in living cells." The photomicrographs published by Schopfer *et al.* (1962) and those comprising Figs. 4 and 5 show that vacuoles having the size and distribution of volutin granules do exist in living *S. pombe* cells. Even so, the occurrence of deceptive artifacts cannot be discounted. Certain fixatives reduce the size of volutin granules; at least one removes them altogether. We found that suspending growing *S. pombe* cells for 45 min in 0.02% potassium permanganate before fixation in the mixture used for the preparation of Fig. 3a leaves all but one or two in several thousands of cells totally bereft of granules stainable with acidified toluidine blue, thus accounting for the presence of numerous empty vacuoles in published electron micrographs of sections of *S. pombe* fixed with permanganate.

3. Mitochondria

It is now well known that mitochondria of budding yeasts undergo marked changes of shape during the cell cycle (Stevens, 1977, 1981; Miyakawa *et al.*, 1984). Currently these changes are demonstrated by computer reconstructions or by models built up from tracings of serial thin sections, see, for example, the work of Keddie and Barajas (1969), Davison and Garland (1977), Taylor and Wells (1979), and Tanaka *et al.* (1985). Use has also been made of the bright fluorescence that DAPI (diamidinophenylindole) imparts to the DNA of mitochondria (Williamson and Fennel, 1975; Williamson, 1976; Miyakawa *et al.*, 1984). Phase-contrast microscopy has, so far, failed to provide us with unambiguous images of the mitochondria in living cells of fission yeasts. Instead, relying on familiarity with the appearance of mitochondria in living cells of certain budding yeasts (Robinow, 1975, 1981a,b), we have studied fixed and stained preparations of fission yeasts to obtain information about the shape and distribution of their mitochondria at different stages of the cell cycle. Photomicrographs of mitochondria in living *S. pombe,* rendered fluorescent with DASPMI (dimethylaminostyrylmethylpyridinium iodine) according to Miyakawa *et al.* (1984), have recently been presented to us by Dr. Machiko Miyata (Fig. 6a). There is good agreement between these results and those of our independently conducted studies.

Our work has mainly been conducted on *S. japonicus* but we have satisfied ourselves that the mitochondria of *S. japonicus* var. *versatilis* and of *S. pombe* behave in the same way. Each of the configurations in Figs. 6b–10 has been seen by us many times in preparations from young cultures growing on agar media, but we readily admit that for a more



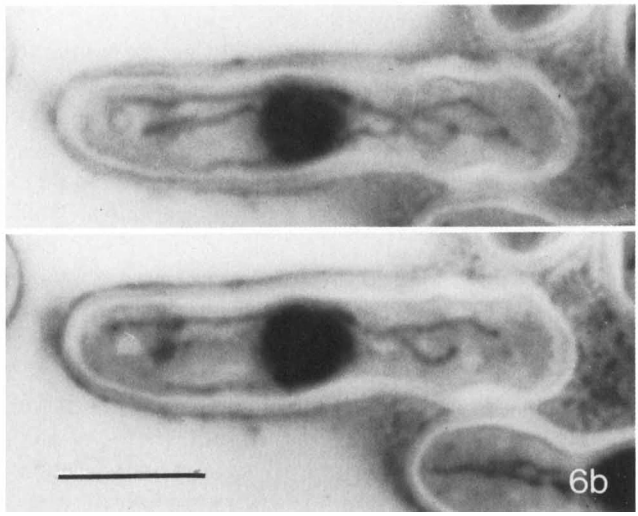
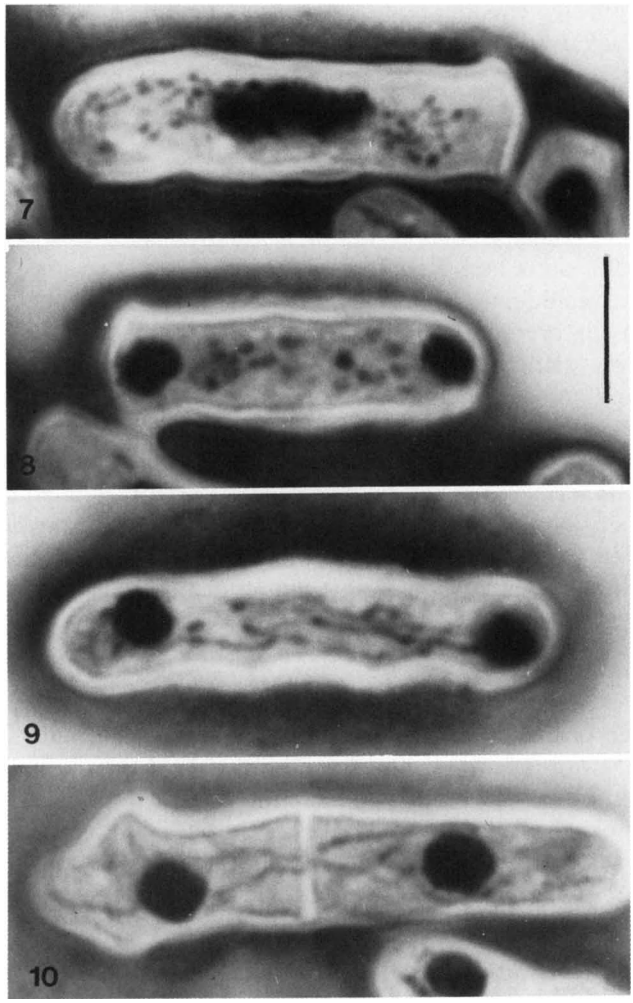


Fig. 6. (a) Mitochondria in long, living cells of *S. pombe* rendered fluorescent with DASPMI. (Courtesy of Dr. M. Miyata.) (b) Mitochondria in *S. japonicus* var. *versatilis*, shown at two levels of focus. Note also long mitochondrion below the nucleus in Fig. 3b. Cells were obtained from single-layer culture on yeast extract/glucose agar flooded with one-half strength Schaudinn's fixative and stained with Giemsa after acid hydrolysis. Bar, 5 μm.

compelling demonstration of the course of events samples would have to be taken from synchronized cultures.

The mitochondria of growing cells are few in number. Some are short but others are stretched over the full length of the cell. The mitochondria are slightly bent here and there but are aligned more or less parallel to each other in a pattern suggestive of the existence of an underlying ordering system in the cytoplasm. The start of nuclear division (Fig. 7) is



Figs. 7–10. Mitochondria in *S. japonicus* from anaphase of mitosis to an advanced state of cell division; treatment as for Fig. 6b. Bar, 5 μm.

accompanied by disarray and fragmentation which is complete by the time sibling nuclei reach the ends of the cell. Fusion of fragments soon follows, and the restoration of the normal pattern of long, snaky mitochondria is complete even before the septum between the new sister cells has formed (Fig. 10). Our findings are compatible with the three-dimensional reconstruction of a solitary cell of *S. pombe* by Davison and Garland (1977), who found "two large [long] reticular structures and two small rounded mitochondria" and with the increased number of mitochondrial profiles encountered by Osumi and Sando (1969) in cells with a dividing nucleus. The long mitochondrion extending over almost the entire length of a longitudinal section of *S. pombe* shown by McCully and Robinow (1971), considered a curiosity at the time, is exactly what one would now expect to find. That section is reprinted here as Fig. 3b. The disposition of the mitochondria in sections of normal *S. pombe* (see Figures in Walker *et al.*, 1982) has its precise counterparts in our stained preparations. Finally, McCully and Robinow (1971) noted the regular presence of a mitochondrion in a tangential position relative to the long axis of the spindle pole body. One may speculate that both partners have been passively brought into this close association by the local orientation of the fibrous skeleton of the cell.

The many studies of the behavior of mitochondria in budding yeasts that have been published in the 1980s have but confirmed the soundness of the views of Williamson (1976) that "it is not unreasonable to suppose that mitochondrial reproduction occurs by some sort of unequal fragmentation rather than by binary fission, and also that large mitochondria may be generated by coalescence and fusion of small ones." Our limited observations would seem to support just that conclusion.

B. Nucleus

Under an ordinary microscope the nucleus in living, growing cells of the larger fission yeasts is more readily identified than is the nucleus of most budding yeasts. This is evident in the first description of "*S. versatilis*" by Wickerham and Duprat† (1945) and is mentioned as an unusual feature by Sloof (1970). More remarkable than the ready visibility of the nuclei of *S. japonicus* and *S. japonicus* var. *versatilis* during life is the presence in them of chromosomes which become well separated from each other at mitosis and are of a size that permits rewarding light microscopy. One of the chromosomes is visible even in resting nuclei and can be

†C. F. Robinow owes his introduction to fission yeasts to E. Duprat.

followed through mitosis during life. The full complement is displayed in the constellations of anaphase in fixed and suitably stained preparations (Robinow, 1981a).

We now describe the resting nucleus with its two compartments, nucleolus and nucleoplasm, proceed to aspects of mitosis visible during life, and, last, deal with selected features of mitosis seen in stained preparations and in electron micrographs. Although following, on the whole, this breakdown of the subject, the discussion is not strictly limited to information obtained by one particular form of microscopy.

1. Resting Nucleus

The resting nucleus, to use the language of morphology rather than physiology, is normally at the center of the cell. The nucleus has smooth contours that are not always those of a regular geometrical shape such as a sphere or an ellipsoid. Electron microscopy of sections of *S. pombe* fixed with glutaraldehyde–formalin (McCully and Robinow, 1971) reveals that this fixative preserves the overall geometry of the nuclei fairly well but causes wrinkles to appear in the nuclear envelope. The natural smoothness of the envelope is preserved in freeze-etched specimens (Kopp, 1975) and by the freeze–substitution technique of fixation adopted by Tanaka and Kanbe (1986). The featureless, translucent contents of the nucleus of living cells surround a large, dense, eccentrically placed nucleolus deserving of special attention.

2. Chromosome in Nucleolus

Phase-contrast microscopy reveals that the nucleolus of living cells of *S. japonicus* and *S. japonicus* var. *versatilis* encloses an arc or "worm" or more complex arabesque of relatively low density (Fig. 11). The material at this site has no noticeable affinity for common nuclear stains such as hematoxylin or acetocarmine, but the Feulgen and HCl–Giemsa procedures identify the worm as a chromosome (Fig. 13) embedded over most of its length in the nucleolus in the manner of some of the chromosomes of the ciliate protozoan *Opalina,* so well described by Chen (1948).

There is nothing unusual about finding a nucleolus penetrated by a chromosome. It is the rule rather than an exception. What is remarkable about this instance is that the nucleolus-associated chromosome (NAC) of the larger fission yeasts displays (after Feulgen hydrolysis) a strong affinity for the basic components of the Giemsa stain at all stages of the nuclear cycle whereas the other chromosomes of the fission yeast nucleus

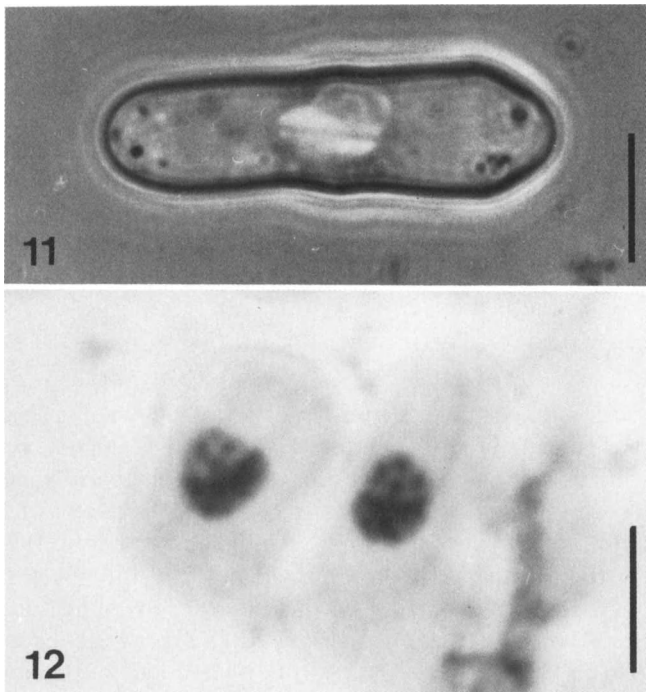


Fig. 11. Anaphase of mitosis in nucleus of a living cell of *S. japonicus*. Spindle and nucleolus-associated chromosome (translucent arc) are clearly visible. Bar, 5 μm. (Reprinted with permission from *Proc. Int. Yeast Symp., 5th, 1980*, C. F. Robinow, The view through the microscope, Copyright 1981, Pergamon Journals Ltd.)

Fig. 12. Vegetative cells of *Schizosaccharomyces octosporus*, Helly, HCl–Giemsa. In both nuclei, the nucleoplasm occupies the lower half of the image and is heavily stained. Above it is the region occupied by the nucleolus, which is seen to contain beads of chromatin, too small for reliable optical resolution, but possibly representing turns of a nucleolus-associated chromosome. Bar, 5 μm.

acquire this degree of affinity (or basophilia) only in the course of mitosis. Chromosomes "dipping" into the nucleolus have also been found in *S. pombe* (McCully and Robinow, 1971; Toda *et al.*, 1981), but in *S. pombe* they have far less affinity for the Giemsa stain (after Feulgen hydrolysis) than has the chromosome in the nucleolus of the larger species of fission yeasts.

In electron micrographs of sections (Fig. 14) of *S. japonicus* var. *versatilis*, profiles of the NAC appear as tortuous translucent channels within the dense matter of the nucleolus. They recall, albeit in simplified form, the tortuous DNA-filled channels that harbor the "nucleolonema" of

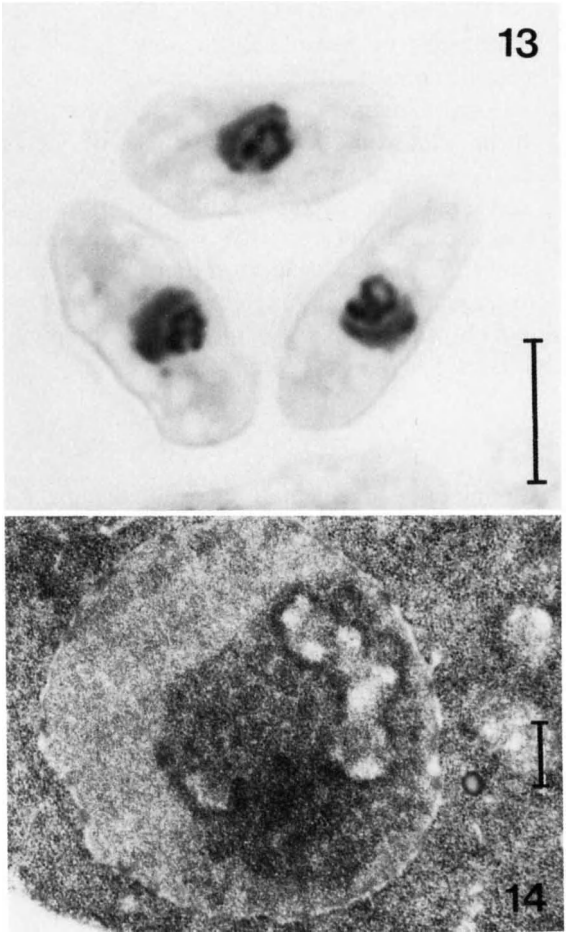


Fig. 13. Vegetative cells of *S. japonicus*, Helly, HCl–Giemsa. The chromatin in the nucleoplasm is softly stained in contrast to the heavily stained horseshoe-shaped nucleolus-associated chromosomes. Bar, 5 μm.

Fig. 14. Electron micrograph of a section of a nucleus of *S. japonicus* var. *versatilis*. The patches of low density in the upper right and the lower left quadrants of the nucleolus are profiles of the site of the chromosome that is regularly found embedded in the nucleolus of nuclei of *S. japonicus* and *S. japonicus* var. *versatilis*. Bar, 1 μm. (Courtesy of Dr. H. Bauer.)

plant nuclei discussed by, among others, Lafontaine and Lord (1973) and Chouinard (1974). Despite their obvious connection with a chromosome the meandering channels in the nucleoli of *S. japonicus* and *S. japonicus* var. *versatilis* have, so far, proved devoid of intelligible fine structure.

The "electron-lucid" zones which Ashton and Moens (1982) encountered so frequently in nucleoli of *Schizosaccharomyces octosporus* are undoubtedly of the same nature, that is, they are profiles of NACs whose presence in *S. octosporus* can, in fact, be demonstrated with the Giemsa stain (Fig. 12). In the electron microscope, tracks of NACs in *S. pombe* are less conspicuous than they are in *S. octosporus, S. japonicus,* and *S. japonicus* var. *versatilis*. The texture of the nucleolus of *S. pombe* appears to be that of a coarse sponge work of dense granular matter with seemingly randomly distributed "electron-lucid" interstices which suitable methods of image integration may yet reveal to represent twists and turns of nucleolar chromosomes (McCully and Robinow, 1971; Tanaka and Kanbe, 1986). (See also Figs. 15 and 16.)

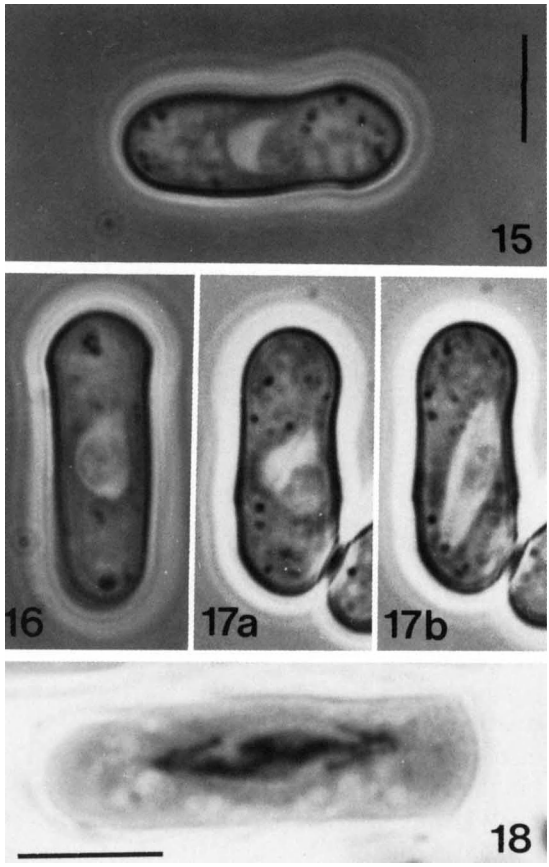
3. Nucleoplasm

During life the nucleoplasm, the material filling the space in the nucleus not occupied by the nucleolus, retains an even, low density throughout the nuclear cycle. This condition is reflected in the featureless or, at most, finely granular appearance of the nucleoplasm of resting nuclei in well-fixed, HCl–Giemsa stained preparations (Figs. 13 and 19). An exception of this is worth brief mention: At least one reagent mixture, formalin–acetic acid–alcohol (5/5/40%, v/v in water), induces the chromatin of the nucleoplasm to condense in the form of a few delicate, tangled filaments (Fig. 25). Exploration with the electron microscope of the fine structure of these artificially condensed elements, which presumably are chromosomes, might be interesting.

4. Features of Mitosis and Nuclear Division Visible during Life

In fission yeasts, budding yeasts, and many kinds of mycelial fungi, mitosis takes place within the crowded tent of the intact nuclear envelope and involves not only the segregation of chromosomes to opposite poles but also the more or less equitable partition of the substance of the nucleolus. In *S. japonicus* and *S. japonicus* var. *versatilis,* which we have studied most often, a nucleus approaching division becomes larger, and the shape of the NAC may change from a worm to that of a more complicated cipher; however, no chromosomes become visible in the nucleoplasm at this time, nor do they do so at later stages of mitosis.

Mobilization of the nucleolus for division frequently lags behind enlargement and elongation of the nucleus as a whole. When this happens the nucleolus briefly stays at one side of the rapidly elongating spindle



Figs. 15 and 16. Living vegetative cells of *S. japonicus*. The angular contours of the nuclei in both cells signal impending division. In Fig. 15, the cytoplasm contains both lipid droplets (dark) and polyphosphate vacuoles (light gray). The nucleolus-associated chromosome is distinct in Fig. 16. Bar, 5 μm.

Fig. 17. Two successive stages (a and b) of intranuclear mitosis in *S. japonicus*. Spindle and nucleolar chromosome(s) are clearly visible. (Reprinted with permission from *Proc. Int. Yeast Symp., 5th, 1980*, C. F. Robinow, The view through the microscope, Copyright 1981, Pergamon Journals Ltd.)

Fig. 18. Anaphase of mitosis in *S. japonicus*, Helly, HCl–Giemsa. The proximal ends of the two long, thick nucleolus-associated chromosomes have not yet disjoined. They are (and will remain) surrounded by nucleolar material. Two short, thin chromosomes are attached to the right nuclear pole. Bar, 5 μm.

that traverses the expanding nucleoplasm at this time (Figs. 11 and 17a). Somewhat later, presumably aided by a lowering of the viscosity of its substance, the nucleolus begins to flow along the spindle and to envelop it. The persisting high density of the nucleolus enhances the visibility of the chromosome embedded in it, which now divides into two chromatids. Their anaphase progress toward opposite poles may be readily followed during life (Fig. 17b). Evidence of continuity between the substance of the nucleolus of the mother cell and that fraction of it that is retained by the daughter nuclei is provided by the transient persistence in the nucleoli of the latter of a bundle of anaphase microtubules (McCully and Robinow, 1971; Tanaka and Kanbe, 1986). Such bundles of microtubules are not found in "adult" nucleoli. At telophase the newly formed sibling nuclei move rapidly apart to opposite ends of the cell, then gradually drift back to positions equidistant from the poles of the cell and its midline (Fig. 27b).

5. Mitosis as Seen in Fixed Preparations

It has to be borne in mind that the fixed preparations about to be discussed were subjected to Feulgen-style hydrolysis before the chromosomes were stained. This treatment destroys the spindle and removes most of the stainable material of the nucleolus. However, as we shall see, much can be learned from what is left behind.

The behavior of the chromosomes in dividing nuclei of vegetative cells is best studied in the larger species of fission yeasts, namely, *S. japonicus* and *S. japonicus* var. *versatilis*. There are three chromosomes of which one, that associated with the nucleolus (the NAC), is both longer and thicker than the other two. Early phases of mitosis are not easily resolved into their component chromatids (Figs. 20 and 21), but all becomes clear with the start of anaphase (Figs. 18 and 22–24). Frequently, as illustrated by Fig. 18, the two NACs, surrounded as they are by nucleolar matter and with their distal ends looped around each other, take longer than the other two chromosomes to let go of each other, to disjoin. At telophase the chromosomes are found attached to the poles of the nucleus at a site close to or identical with the poles of the spindle (Figs. 23 and 24). What may be unresolved images of spindle pole bodies (SPBs) are sometimes seen at this very location (e.g., Fig. 24, lower nucleus) but not sufficiently clearly, in our work, to warrant detailed discussion.

Judging from the work of Peterson and Ris (1976) on mitosis in *Saccharomyces*, it is highly probable that the attachment of chromosomes to the SPB region involves the aid of short microtubules, and the findings of Tanaka and Kanbe (1986) in *S. pombe* point to the same conclusion. We

believe it to be likely that the chromosomes of fission yeasts are permanently attached to, and that they and their attachments undergo replication, in the region of the SPBs, as seems to be the case in *Saccharomyces*. However that may be, there does not seem to exist, in fission yeast nuclei, a prophase/metaphase stage at which condensed chromosomes are floating about in the nucleoplasm waiting to be marshalled on a metaphase plate.

At this point in our account of the morphology of mitosis in the larger species of fission yeasts a note of warning needs to be sounded: The deeply stained chromosomes in Figs. 19–26 look not unlike chromosomes of mitotically dividing nuclei of tissue cells of plants and animals. So to regard them would be a grave error, however, for the chromosomes in our photomicrographs had not undergone the condensation that is a regular feature of mitosis in higher eukaryotes. The condensed mitotic chromosomes of *Tradescantia* are visible during life, unstained, and because of this began to attract the attention of microscopists as early as 1849 (see Hughes, 1959), but not so in yeasts. The slender chromosomes that are seen at either side of the NAC at anaphase, distinct as they are in HCl–Giemsa preparations, are not visible at anaphase in the nucleoplasm of the nuclei of living cells. This point is well illustrated by Fig. 11 where, to judge by the length of the spindle, anaphase may be assumed to have advanced to a stage comparable to that of Fig. 21. The chromosomes of fission yeasts undoubtedly undergo chemical and physical changes during mitosis, but, whatever the nature of these changes, they are not accompanied by a degree of condensation which raises the optical density of the chromosomes above that of the nucleoplasm in which they are immersed. It should be remembered that the NAC owes its exceptional visibility during life (at all stages of the nuclear cycle) to the contrast between its own unvarying low density and the relatively high density of the substance of the nucleolus.

The state of affairs described in the preceding paragraph is paralleled by the frustrating absence from electron micrographs of any images identifiable as parts of the chromosomes of normal nuclei of fission yeasts. It is electron micrographs, however, that have to be studied for detailed information about the spindle pole bodies and the microtubules of the spindle itself. For this the reader is referred, once again, to McCully and Robinow (1971) and Tanaka and Kanabe (1986).

Genetics apart, the clearest evidence that haploid *S. pombe* has three chromosomes has been provided by Umesono *et al.* (1983) in the form of fluorescence photomicrographs of DAPI-stained chromosomes arrested at mitosis under the influence of thiabendazole. Electron microscopy of sections reveals that thiabendazole induces a state of extreme condensa-

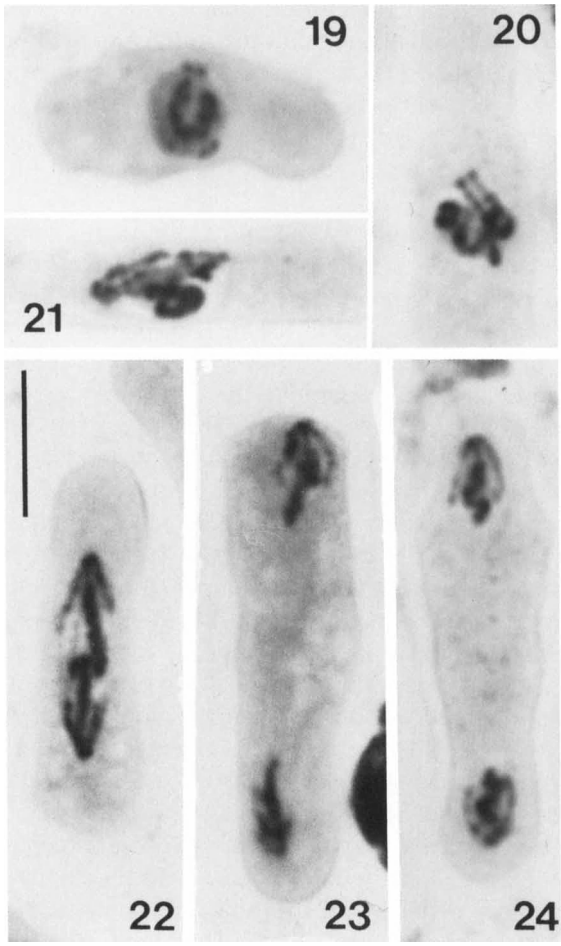


Fig. 19. The nucleus of this *S. japonicus* cell displays a crescent of weakly and diffusely stained nucleoplasm, a heavily stained chromosome within the nucleolus, and what appears to be spindle pole bodies or nucleus-associated organelles at opposite sides of the nuclear contours.

Figs. 20–24. The three chromosomes of *S. japonicus* at various phases of mitosis. Note in Figs. 22, 23, and 24 that the long chromosome is tied to the upper pole of the nucleus by a short, straight cord. Bar, 5 μm. [(Figures 20–24) Reprinted with permission from *Proc. Int. Yeast Symp., 5th, 1980*, C. F. Robinow, The view through the microscope, Copyright 1981, Pergamon Journals Ltd.]

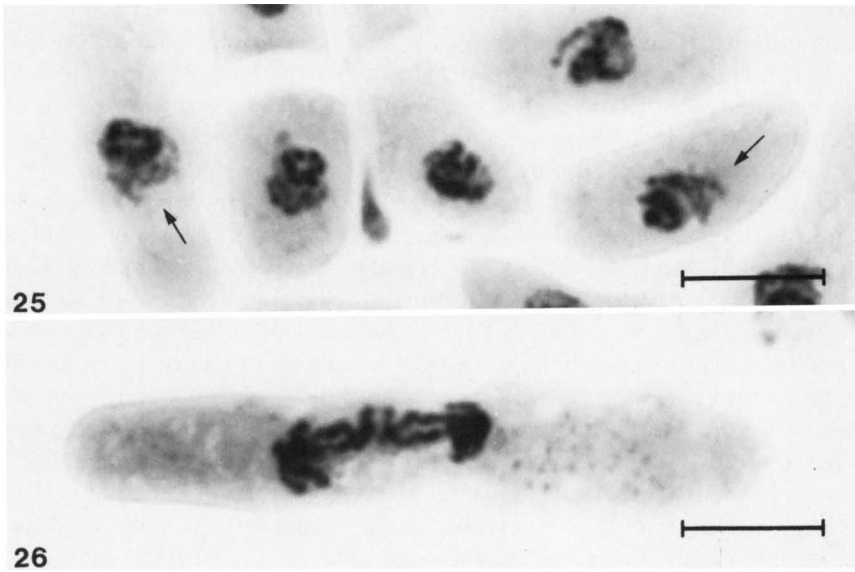


Fig. 25. In the "resting" nuclei of this sample of *S. japonicus* cells, fixation with alcohol–formalin–acetic acid (see legend to Fig. 3a) has led to condensation of the chromosomes in the nucleoplasm (arrows). The big nucleolus-associated chromosome has retained its usual appearance. Bar, 5 μm.

Fig. 26. Anaphase of mitosis in the nucleus of a cell from an apparently diploid (unstable) strain of *S. japonicus*. Bar, 5 μm.

tion of the chromosomes unlike anything ever encountered in the course of the normal nuclear cycle. The fine structure of these weird productions has been meticulously analyzed by Erard and Barker (1985).

6. *Dynamics of Mitosis and Nuclear Division in Schizosaccharomyces pombe Inferred from Conventional Light Microscopy and Electron Microscopy*

The two modern papers concerned with the dynamics of mitosis and nuclear division are those of McCully and Robinow (1971) and Tanaka and Kanbe (1986), the first of which was written before its authors had become aware of the presence of a cytoskeleton in fission yeasts. McCully and Robinow (1971) regarded active expansion of the nuclear envelope as the principal motor of nuclear division, while Tanaka and Kanbe (1986) assigned this role to the intranuclear spindle. It would not be profit-

able to discuss the relative merits of these proposals without consideration of the part so obviously played by the cytoskeleton, but it may still be worthwhile to remind ourselves that it would have been feasible to accommodate both contending views in a single consistent program of nuclear division by allowing that nuclear division may be achieved by two different mechanisms coming into play one after the other.

To begin with, it is well known that in *Sacch. cerevisiae* the spindle inside a stretched nucleus that is in the process of entering a newly formed bud is usually short and tilted at a steep angle away from the main axis of the nucleus, thus suggesting that at this time the spindle does not act as a motor of nuclear form change. Then again, marked elongation of a dividing nucleus "at a time when the spindle is quite short and tucked away in one corner of it" is the rule in *Mucor* according to McCully and Robinow (1973). Before the end of karyokinesis, however, the spindle there has increased more than 10-fold in length, has become the main axis of the now dumbbell-shaped nucleus, and by its combination of rigidity with steady extension could, conceivably, have become the principal agent in the pushing apart of the prospective daughter nuclei to opposite poles. Thus, in *Mucor* a process of nuclear division that began with the expansion of the envelope may in the end be achieved by the work of the intranuclear spindle.

Similarly, in fission yeasts, the asymmetrically bulging contours of nuclei entering division (e.g., that of the nucleus in Fig. 11) could be interpreted as indicating active, autonomous expansion of the envelope, while pointed, diamond shaped nuclei, with conspicuous wirelike spindles only seconds from final division, present an equally plausible case for division as accomplished by pressure exerted on the nuclear poles by the central spindle. And so the arguments might be advanced and turned around and around; however, all have become (more or less) idle speculations since the publication, duly noted by Tanabe and Kanbe (1986), of the important, elegant work of Aist and Berns (1981) who used laser microbeam surgery to investigate the role of the spindle in the segregation of mitotic chromosomes and arrived at the hitherto unsuspected conclusion that (in *Fusarium*) "extranuclear forces, presumably involving astral microtubules pull on the incipient daughter nuclei and that *the central spindle limits the rate of separation*" (our italics).‡ Work on the behavior of the cytoskeleton in *Saccharomyces* and *S. pombe* by Kilmartin and Adams (1984) and Marks

‡Astral microtubules are outside the nucleus, originating or inserted in the region of the SPB. The cytoplasmic tubules described by Hereward (1974) and Streiblová and Girbardt (1980) are of this kind. For fine examples of astral tubules in a mycelial fungus other than *Fusarium*, see Olson (1974).

et al. (1986) has strengthened the possibility that in yeasts, too, nuclear division is achieved with the aid of Aist–Berns mechanics.

It is to be hoped that the large size of *S. japonicus* and *S. japonicus* var. *versatilis* will permit experiments to be undertaken in which laser or ultraviolet microbeams are used as tools of surgery as they were in the work of Aist and Berns (1981) and McKerracher and Heath (1986). Experiments of this kind might eventually provide us with a full and satisfying account of mitosis in fission yeasts.

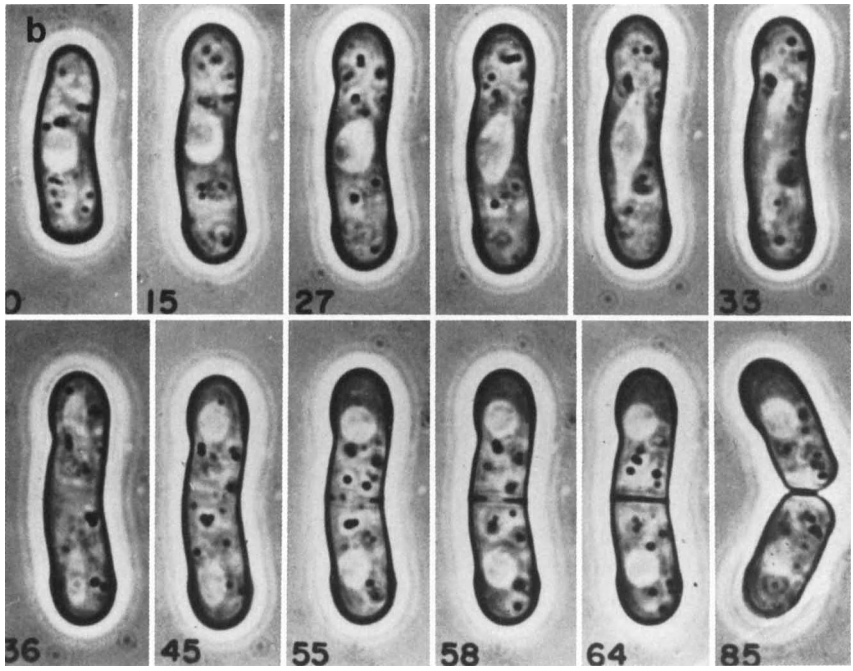
C. Cell Division

1. Overview

Observed in the phase-contrast microscope, the division of fission yeasts appears to be accomplished by a thin, sharply defined diaphragm which, arising from the cylindrical cell wall and at right angles to it, steadily closes in on the central region of the cell. Soon after, cells are seen breaking away from each other, the products of a process of fission that has given the genus its name.

Before the two cells were able to part contact a crack must have arisen in the old wall; if so, why was cytoplasm not extruded there? The wall did indeed crack all around, but the cytoplasm was held in by a patch of new wall material pressed up against it. To illustrate this point, in contrast to the expensive achievements of "invisible mending," a patch put upon leather or woven fabric always shows because its edge presents a sharp discontinuity. If the patch is put on from underneath then it will be the original edge of the hole or tear that provides the visible discontinuity. That is what happens in dividing fission yeasts, where the task is not only to repair a crack that runs around the cell wall tube but also to seal the newly formed open ends. After division the break in the old wall persists as the plainly visible rim of a shallow crater the floor of which is aptly named a scar plug (Streiblová *et al.*, 1966).

Let us now trace the process of division in greater detail. As reported by Marks and Hyams (1985) and Marks *et al.* (1986), a premonitory event happens during nuclear division when a broad ring of actin, rendered visible by fluorescence microscopy, is formed, temporarily, at the site where the growth of a transverse septum will soon be initiated. According to K. Tanaka (personal communication) the actin is associated with a population of vesicles, the "dots" of Marks *et al.* (1986), whose progress from the poles to the future site of cell division Tanaka has been able to chart in computer-drawn reconstructions of serial sections of *S. pombe*


b
0
15
27
33
36
45
55
58
64
85

from different stages of the cell cycle. An alignment of elements of the endoplasmic reticulum in the future plane of division, noted in several sections by Deshusses *et al.* (1970), may reflect activities connected with the accumulation of actin in this region at that time. Another hitherto unsuspected structure, a mat of microfilaments involved in cell division in *S. pombe*, has been found by K. Tanaka (personal communication). It is laid down in the cytoplasm ahead of and in the plane of the rim of the gradually closing transverse septum. This "red carpet" device calls to mind the "microfilamentous septal belt," described by Girbardt (1979), that makes its appearance just before cell division in the basidiomycete *Trametes versicolor*. While newly discovered structures and events are still *sub judice*, we do not attempt to illustrate them here. What follows is based on the morphological analysis of normal cell division in *S. pombe* by Johnson *et al.* (1973) and others to be mentioned later, whose accounts we briefly paraphrase.

In electron micrographs of permanganate-fixed *S. pombe* the event signalling the start of cell division is the emergence of an annular ridge, the primary septum. Arising in the periplasmatic space just outside the plasma membrane, the primary septum is from the beginning sheathed in a thick layer of periplasmatic "dark material" further discussed below. The septum itself is less electron-dense than the old cell wall but far exceeds the latter in the brilliance of fluorescence in the presence of Calcofluor (Johnson *et al.*, 1974). Sheets of electron-dense proper wall material next arise within the dark matter on both sides of the primary septum [Fig. 27a (A)] to form the beginning of two secondary septa. In due course, expanding centrifugally, the secondary septa reach the cell's periphery and become bonded there to the inner face of the sound edge of the cell wall close but distal to the site, all around the equator of the cell, where the old wall is now beginning to crumble and be eroded. That state of affairs (the "patch" in place) has just been reached in the cell of Fig. 27a (B). At the same time what has remained of the primary septum in the narrow space between the two secondary septa also suffers erosion and dissolution mediated, presumably, by enzymes akin to the "zipperase" allegedly at work in dividing bacteria in the laboratory of Murray (1988).

Fig. 27. (a) Diagrams of some phases of the process of cell division in fission yeasts. All structures, except dense round bodies, are outside the plasma membrane. PS, Primary septum; SS, secondary septa; MDT, "materiél dense triangulaire"; F, fuscannel. Consult the text. (b) Time-lapse photomicrographs of nuclear and cell division in *S. japonicus* var. *versatilis*. Numbers indicate minutes passed since the first photograph was taken. Slide culture in 21% gelatin containing yeast extract and glucose. Consult Section I,B. [From Robinow and Bakerspigel (1965).]

As pointed out by Johnson *et al.* (1974), it is now "after lytic removal of the primary septum, that turgor pressure can cause the new ends of the cell to round up [Fig. 27a (C)]. The scars left by these events persist as chronological markers through subsequent growth cycles. Good examples of smoothed division scars, to mention only two sets of them, are to be found among most of the profiles of *S. pombe* in electron micrographs presented (in a different context) by Osumi and Sando (1969) and along the walls of many of the conjugating cells in Calleja *et al.* (1977a).

2. *Other Players*

Much of the electron microscopy of dividing *S. pombe* has been done on samples fixed with potassium permanganate. This reagent, as is well known, extracts much from the cytoplasm but for that very reason gives us a good view of membranous organelles which are not always equally clearly visible after glutaraldehyde fixation. The electron micrographs in four contemporary studies of *S. pombe* in which permanganate had been used, those of Osumi and Sando (1969), Deshusses *et al.* (1970), Oulevey *et al.* (1970), and Johnson *et al.* (1973) as well as others since, show mitochondria, Golgi elements (dictyosomes), and, invariably close to the cell surface and arranged there in single file, long lines of flat cisternae of the endoplasmic reticulum (ER). There is nothing unusual in this, but there is one piece of inventory, conspicuous only when permanganate had been used, that calls for comment. This is the periplasmic dense matter at the base of the cell wall, just outside the plasma membrane. In many places, especially along the sides of the primary and, later, the secondary septa, thick accumulations of dense matter indent the plasma membrane to the extent of giving it a wavy, scalloped profile. Small round particles of similar density, fairly uniform in size, are scattered about the cytoplasm and are often seen in close contact with periplasmic dense matter, which may mean that they are either budding off it or fusing with it (Johnson *et al.*, 1973), and on reexamination the possibility has to be conceded that some of them may be identical with members of the crowd of (actin?) particles which, as Marks *et al.* (1985) and K. Tanaka (personal communication) have shown, migrate to these sites prior to cell division.

A consistent feature of the sections illustrated, in various degrees of clarity, in the four papers quoted above, are particularly voluminous accumulations of dense matter that anchor and buttress the primary septum when it first emerges as an annular ridge. These masses are themselves cast in the form of shallow ridges, one on each side of the base of the primary septum. They are triangular in cross section, hence the name of "materiél dense triangulaire" given to their profiles. These supporting

ridges are made of remarkably durable material that seems affected neither by the erosion and dissolution of the old cell wall (at whose base they are located) nor by the breakdown of the primary septum for which they had originally provided lateral support. The diagram in Fig. 27a (B) shows they are shouldered aside by the secondary septa (about to become the new end walls) on their way to making contact with the inner face of the sound edge of the old wall. The rounding up of the cell ends, following the dissolution of the primary septum, carries remnants of the matériel triangulaire, the "fuscannel" of Johnson *et al.* (1973), some slight distance away from the broken edge of the old cell wall. However, several growth cycles later remnants of "dense triangular matter" may still be recognized as a distinctive part of old bud scars. Readers trying to sort out ridges, septa, plugs, and scars are advised to take a look at the instructive scanning electron micrograph image of a recently divided *S. pombe* which B. F. Johnson and colleagues supplied to Prescott (1976). Periplasmic dense matter needs to be illuminated in terms of cell wall chemistry.

3. Equality among Sibs

Precision in the placing of the transverse septum is the impression conveyed by life cycle diagrams of fission yeasts, one that would be reinforced by a cursory glance at a sample of cells from the most recent in a series of daily subcultures. This would be especially true of *S. pombe*, the neatest, perhaps, and most orderly of fission yeasts. This impression would be a wrong one, however. Impeccable quality control, exercised by Johnson *et al.* at the Ottawa laboratories (1979), has established that "the new septum is sited asymmetrically at division by length parameters.... The volumes of the resultant sibs, however, are equal." Thus, fission is binary, after all.

4. Anomalies

It is to be expected that a delicately coordinated sequence such as the maneuvers of cell division will occasionally experience miscarriage. Changes in the parameters of cultivation, as well as spontaneous and chemically induced mutations, may indeed lead to departures from the normal behavior of fission yeasts. Cells that elaborate several closely spaced transverse septa in succession or none at all and conditions that encourage the formation of multiseptate, branching hyphae have all been reported and are dealt with in an appropriate chapter in this volume (Chapter 5). One common variant form is the frequent occurrence of what Johnson *et al.* (1982) refer to as "the stable hypha, a branching anomaly."

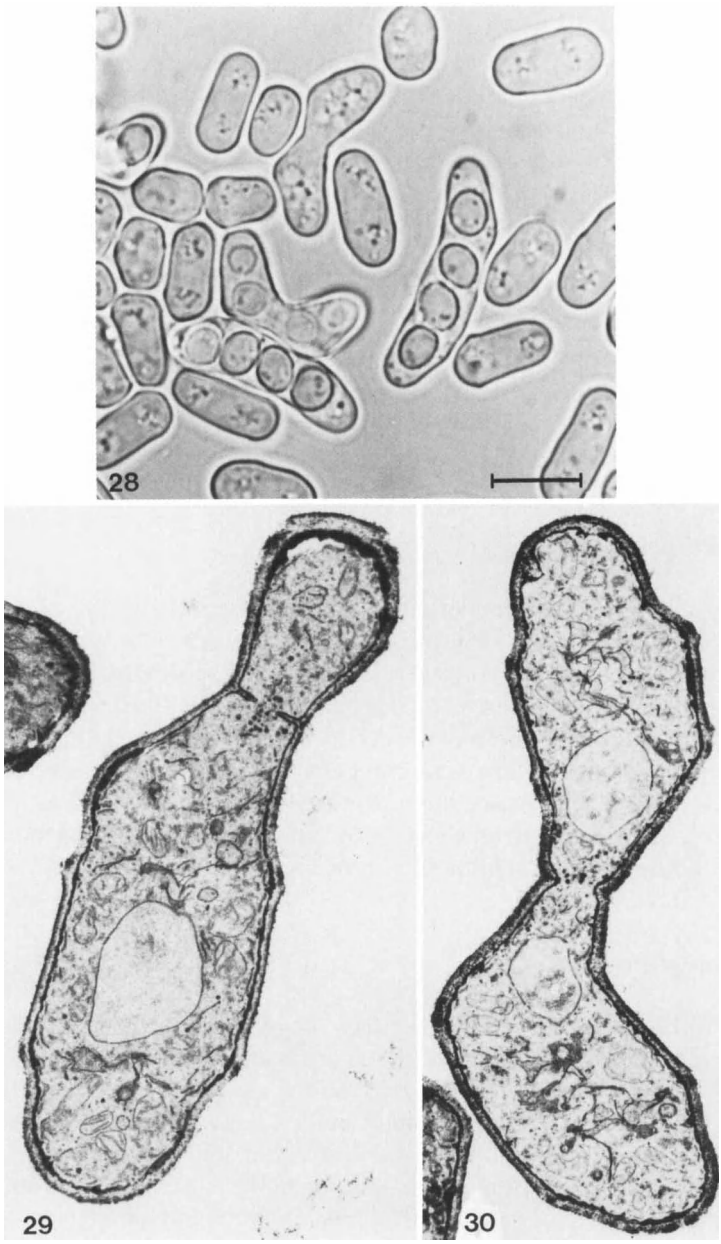


Fig. 28. Conjugation/sporulation in *S. pombe* from a culture on malt extract/agar. Bar, 5 μm.

Students of fission yeasts are familiar with the sight of a number of short, separate hyphae emerging from underneath colonies on agar. Occasionally, however, one or two among hundreds of ordinary colonies will be seen surrounded by a phalanx of hyphae so densely spaced as to simulate the growth of a mold. On agar this anomaly remains stable through frequent transfers, but, stable or not, the sight of such colonies [first encountered in "*S. versatilis*" by the discoverers of the yeast, Wickerham and Duprat (1945)] raises the question of what difference there really is between life at the surface of agar and 10–20 μm below it in the same medium. The commonly given answer, that it is a matter of the reduced availability of oxygen "down there," does not seem entirely credible. For a broad discussion of cell division, readers are referred to Johnson *et al.* (1982).

D. Conjugation

1. Building Up and Tearing Down

Yeasts do not move on their own accord, and to achieve conjugation those growing in a liquid medium have to be allowed to flocculate while those expected to conjugate on agar have to be plated out at a density which ensures that multiplication will provide adequate numbers of chance encounters of cells of suitable mating type. In *S. pombe* cell ends about to make contact are tapering and slightly bent and are referred to as conjugation tubes (see Figs. 28–30). More pronounced "beaks," as Ashton and Moens (1982) have shown, are sprouted by cells of *S. octosporus* about to fuse. Conjugants may be closely related. In their original account of "*S. versatilis*" Wickerham and Duprat (1945) mention that "the hinge which held sister cells together was the point at which fusion often began." And according to Ashton and Moens (1982) conjugation in *S. octosporus* "frequently occurs among cells of common ancestry." The physiological parameters of conjugation have been treated extensively by Calleja *et al.* (1977b, 1981) and McDonald *et al.* (1982). Here we restrict ourselves to matters of anatomy.

Fig. 29. The cross wall separating conjugants in process of demolition. Note accumulation of dark bodies (vesicles). Note also that no scars mark the site where the outer walls of the conjugants have been seamlessly welded together. [From Calleja *et al.* (1977a), with permission.]

Fig. 30. Migrating nuclei are approaching the site of the former cross wall. The crimp in the outer wall marks the site of fusion of the walls of the conjugants. [From Calleja *et al.* (1977a), with permission.]

Accounts of the details of conjugation and of the events following it must draw on the results of electron microscopy. For this method of investigation two commonly used fixatives, permanganate and glutaraldehyde, complement each other in usefulness. The former, as we have already seen, serves well in the study of cell walls and of membranous organelles of the cytoplasm; glutaraldehyde achieves, among other things, the preservation of intra- and extranuclear microtubules and of SPBs, all with parts to play from conjugation to spore formation. The chromosomes of the synkaryon and their behavior during meiosis have, so far, proved inaccessible to electron microscopy but something can be learned about them from light microscopy.

Readers of the well-illustrated study of conjugation in *S. pombe* by Calleja *et al.* (1977a) must wonder at the apparent skill with which conjugating cells achieve the fusion, well nigh seamless on these occasions, of the cylindrical side walls of the two partners only micrometers distant of activities of the very opposite kind. The latter are carried out at sites where conspicuous accumulations of "dense particles" (once again) seem involved in the demolition of the contiguous end walls of conjugants which stand in the way of the fusion of the protoplasts of the two partners that will lead to the formation of a synkaryon and, in due course, a zygote. Here glutaraldehyde scores a point. When this fixative is used the particles at "the conjugation isthmus connecting a pair of cells" become "abundant vesicles" with clear centers as shown for *Sacch. cerevisiae* by Byers and Goetsch (1975), by Hirata and Tanaka (1982) for *S. pombe*, and by Ashton and Moens (1982) for *S. octosporus*. Seeing these, microscopists cannot but be reminded of the clouds of vesicles of similar dimensions found at the tips of growing hyphae of several kinds of fungi by Grove and Bracker (1970), in growing bud cells of *Leucosporidium scottii* and similar yeasts by McCully and Bracker (1972), and in the emergent bud of zygotes of *Sacch. cerevisiae* by Byers and Goetsch (1974). Believed to be carrying enzymes and precursors of materials required in the construction of cell walls, vesicles of this kind may equally well be imagined to be charged with enzymes capable of breaking walls down where this is required. We encounter them again in the cytoplasm of asci where their kind is found close to developing forespore walls.

Success in carrying out construction and demolition at the same time and at close quarters depends on perfect coordination. In *S. pombe* that is not always achieved; demolition then overwhelms construction, gaps in the cell wall are not properly sealed, and conjugants (or half-conjugants) succumb to lysis. According to Calleja *et al.* (1977b) up to 15% of cells attempting conjugation may be so affected. The safety record of the break-and-patch method of vegetative cell division is a far better one but is not impeccable. At a certain population density many cells of *S. pombe*

lyse in an otherwise satisfactory medium containing slightly less than adequate amounts of asparagine or phosphate (Johnson, 1967). The breakdown appears to be due to faulty connections between old cylindrical wall and secondary septa.

2. *Karyogamy, Meiosis, and After*

Nuclear behavior in *S. octosporus* up to the moment of karyogamy has been the subject of a thorough study by Ashton and Moens (1982). A shorter work by Hirata and Tanaka (1982) deals with karyogamy and meiosis in *S. pombe*. Electron microscopy has not yet provided views of any of the intricate maneuvers chromosomes engage in at meiosis, but it has added new players to the performance in the shape of thin "linear elements" whose function is at present unknown. They have been described by Olson *et al.* (1978) and Hirata and Tanaka (1982). Electron microscopy becomes more rewarding when used to study the formation of the envelopes of the ascospores of fission yeasts and the events of their germination, subjects that have attracted the attention of Yoo *et al.* (1973), Tanaka and Hirata (1982), and Johnson *et al.* (1982). We briefly comment on what has been learned.

Ashton and Moens (1982) show that the nuclei in conjugants of *S. octosporus*, their chromatin rendered fluorescent by mithramycin, have teardrop shapes with a small bright spot at the tip of the narrow leading portion (the trailing edge of real tears). The authors regard these shapes as strongly suggestive of migration as do many others whose works are quoted. They illustrate this notion with photomicrographs of nuclei confronting each other in the conjugation isthmus and of synkaryons moving toward the end of the zygote. Comparison of the fluorescent images with their own electron micrographs of the same material encourages the authors to suggest that the bright bead at the tip of migrating nuclei represents the SPB. There is much evidence in favor of this interpretation in the literature on ascomycetes. Girbardt (1960) has charted the courses of many fungal nuclei during life and has shown those of *Polystictus*, a basidiomycete, to have indeed an elongated teardrop shape with an SPB of a particular kind, called nucleus-associated organelle (NAO) by Girbardt and Haedrich (1975), at the tip.

The bright fluorescence of mithramycin-stained SPBs indicates that they are the site of DNA. Ashton and Moens accept this interpretation and cite in its support the finding of Zickler (1973) that the SPB of *Ascobolus* is Feulgen positive and that its density in electron micrographs is much decreased after digestion with DNase. We have sometimes been able to stain the SPBs of fission yeast nuclei by the HCl–Giemsa method (see Figs. 19 and 24) which would be compatible with the presence there

of DNA. On the other hand, Robinow and Marak (1966) found that the SPB of *Sacch. cerevisiae* stands out as a colorless, sharply defined "bite" or dimple against the distinctly Feulgen-positive nucleoplasm. We have not seen published DAPI fluorescent images indicating otherwise. More work on this matter appears to be needed.

Ashton and Moens (1982) did not find a nucleolar chromosome in fluorescent mithramycin-stained nuclei of *S. octosporus*. This is both disappointing and puzzling because their own excellent electron micrographs strongly suggest the presence of just such a chromosome. Actually, an extra bright speck of light is detectable within the fluorescent glow of the left-hand nucleus of Fig. 10 and in both the nuclei of Fig. 12.

3. Cytoplasmic Microtubules at Meiosis

For reasons not yet understood meiosis is the time when bundles of microtubules associated with nuclei are found in the cytoplasm of fission yeasts after the same processing for electron microscopy which generally fails to reveal all but a very few tubules in the cytoplasm of vegetatively growing and dividing cells (where immunofluorescence microscopy now reveals large tracts of them). Records of isolated microtubules in the cytoplasm of fission yeasts appeared in the literature in the mid-1970s. No doubt correctly observed, the earliest of them still were of the character of "rare bird" notices in the local paper. Things changed when Byers and Goetsch (1975), working with *Sacch. cerevisiae*, proposed that cytoplasmic microtubules might have a part to play in the interactions between conjugating nuclei "because stages are found in which they clearly interconnect the plaques of the nuclei." Their surmise was to be amply confirmed by Ashton and Moens (1982). Even earlier, bundles of microtubules had been found by Olson *et al.* (1978) to be running along one side of *S. pombe* nuclei at meiosis I supporting, perhaps, the elongated shape of the nuclei at this stage. In cross sections many tubules of this bundle were seen to be in close contact with the outer face of the SPB.

These findings, too, are supported by Ashton and Moen's (1982) topographical survey of the disposition of cytoplasmic tubules during conjugation and syngamy in *S. octosporus*. The authors found bundles of microtubules flanking migrating prefusion nuclei as well as synkaryons, making contact here and there with the nuclear envelope and often and distinctly with the SPB. The authors do not doubt that the microtubules they detected were instrumental in nuclear migration and aptly quote the work of Pickett-Heaps and Fowke (1970) who showed that postmitotic nuclei in the unicellular alga *Closterium littorale* migrate to their appointed distant stations along narrow grooves lined with microtubules. In

fungi, too, there is strong evidence implicating microtubules in nuclear migration, but unsolved problems are also to be found there.

Among the conditional, temperature-sensitive *cdc* mutants of *Aspergillus nidulans* studied by Morris (1980) were several in which nuclear division, a process requiring spindle tubules, is performed in normal fashion but postmitotic migration of nuclei is inhibited. These observations established, though they did not define its nature, that there is a special mechanism for nuclear migration and indirectly implied that there might be two kinds of microtubules answering to two different administrations, something we now know to be true. Next, Oakley and Morris (1980) showed by means of an elegant combination of genetics and drug action that mutation in a β-tubulin gene, in another of their conditional mutants, left nuclear migration immune to the strongly inhibitory action of benomyl and thus that β-tubulin, most likely as a constituent of microtubules, must be involved in nuclear movement.

The arrangements of Girbardt (1968) allowed him to say with certainty that a certain nucleus in an electron micrograph had been moving this way or that at the moment of fixation and, "Observation on the living material suggests that the KCE [a now obsolete acronym for SPB or NAO] is pulled." With this anyone who has read the account of Aist and Berns (1981) of the results of their laser microbeam surgery on dividing *Fusarium* nuclei can but agree. But, writing now of oscillating mitotically dividing nuclei, closely observed and recorded during life and of the same nuclei later studied in the immobility of an electron micrograph, Girbardt (1968) says, "Microtubules seem not to act directly.... This is indicated by their behavior during oscillatory movements when microtubules are bent against the direction of movement, indicating that they are passively dislocated as soon as movement is induced. The force therefore seems to originate in the cytoplasm." Obviously much remains to be learned, and it is to be expected that the contribution of immunofluorescence microscopy will be great. Meanwhile we conclude this excursion into nuclear dynamics with the thought from Oakley and Morris (1980), "Microtubules might function solely as a framework which serves to anchor the active elements of the organellar transport system, or they might form an active part of the system, perhaps as part of a dynein-like microtubule arrangement."

4. Meiosis

Electron microscopy shows little about the behavior of the chromosomes at meiosis in fission yeasts. The nuclei preserve a ground glass blandness and indifference which even Peter Moens, an expert with syn-

aptonemal complexes, has not been able to shatter. Actually, something is to be seen at meiosis. At the time when the nuclei attain an elongated shape, they "become populated by numerous thin electron-dense structures, which tend to be oriented parallel to the nuclear axis, though some may run across it" (Olson *et al.*, 1978). Reconstruction from serial sections revealed the presence of 25–30 of these elements, most of them without connection with the nuclear envelope. Their apparent thickness varied from 10 to 70 nm which could be due to their having the shape of a twisted ribbon. The authors discuss the possibility that the linear elements might be comparable to lateral elements of normal synaptonemal complexes but did not arrive at definite conclusions. One wonders whether the large number of short separate linear elements might perhaps be accounted for by "sea serpent geometry," with one and the same element dipping in and out of a given level of sectioning. We have mentioned before the discovery by Olson *et al.* (1978) of a bundle of microtubules, attached to the SPB, and running outside of and along one side of the nucleus. Hirata and Tanaka (1982) who have confirmed the observations of the Danish workers point out that this bundle of tubules may account for the smoothness and straightness of the contours of the nucleus on its side, compared with the irregular profile of the nuclear envelope on the opposing side.

5. Formation and Germination of Ascospores

In *S. pombe* the spores are set free by the gradual breakdown of the ascus, which is none other than the cell wall of the zygote under another name. In other words, the envelope of the spores resists lysis by enzymes which degrade the cell wall. This, in turn, suggests that the wall of ascospores differs in its chemistry from the wall of vegetative cells. If so, it is probably not produced via the usual route across the plasma membrane but by a different organ of the cell and at a different site. There is general agreement that this is indeed what happens.

Much work had already been done on the development of ascospores in higher ascomycetes and in *Sacch. cerevisiae* before attention turned to fission yeasts. You *et al.* (1973), relying, as did most of the early workers in this field, on permanganate as fixative, clearly established that in *S. pombe* the spore wall primordium can be traced back to a membranous element more densely stained than other cytoplasmic membranes, one which is first seen in the vicinity of the nuclei after meiosis I and proceeds to encircle the nuclei while they are undergoing meiosis II. At telophase of that division each haploid nucleus finds itself more or less completely

enclosed by the spore wall primordium or "forespore membrane" together with a portion of cytoplasm and a few mitochondria. The spore membrane can now be resolved into two closely applied unit membranes. The space between the latter gradually widens and begins to be filled by visibly unstructured, increasingly dense material that will become the spore wall. Much the same situation of future wall material being deposited in the space between the two leaves of the forespore membrane was described for *Sacch. cerevisiae* at just the same time by Beckett *et al.* (1973).

As Yoo *et al.* (1973) rightly pointed out: "Although the forespore membrane can be easily identified ... it is difficult to differentiate it from other cytoplasmic membranes at its incipient stages of development. For this reason the origin of this membrane is yet to be discovered and, to this end, both the conventional chemical fixation and freeze-etching [electron] microscopy seems to be inadequate." Fixation with glutaraldehyde was soon to provide the missing information and had in fact already done so for *Sacch. cerevisiae* in work by Moens (1971) which was elegantly confirmed by Zickler and Olson (1975). These workers established that in *Sacch. cerevisiae* the spore wall primordium is generated from clusters of vesicles of endoplasmic reticulum that arise in the vicinity of enlarged and modified pole bodies of the spindles of meiosis II. The vesicles coalesce to form a narrow bell-shaped cisterna (i.e., the forespore membrane), and, *mutatis mutandis*, what follows takes the course outlined for *S. pombe* by Yoo *et al.* (1973). The complete story from endoplasmic reticulum to ascospore wall of *S. pombe* and *S. japonicus* was told for the first time by Tanaka and Hirata (1982) (see Fig. 31).

The NAO or spindle pole body, familiar to us as a generator of intranuclear spindle tubules (and short, stubby cytoplasmic ones), surprises us with a versatility that permits it at the same time to play an active part in the assembly of the spore primordium. The account we have just presented implies that the inner leaf of the spore primordium of endoplasmic reticulum origin becomes the plasma membrane of the dormant spore. In this character it is later able to function as a mediator, a channel, in the construction of standard *S. pombe* cell wall, a task it is called on to perform when the ascospore germinates. Contrary to what is still to be found in the literature, awakening ascospores do not simply swell, elongate, and thus transform themselves into active vegetative cells. As Johnson *et al.* (1982) have shown, they push off the spore envelope by secreting a proper cell wall beneath it and emerge from the spore at the rate at which the synthesis of this wall proceeds (see Fig. 32).

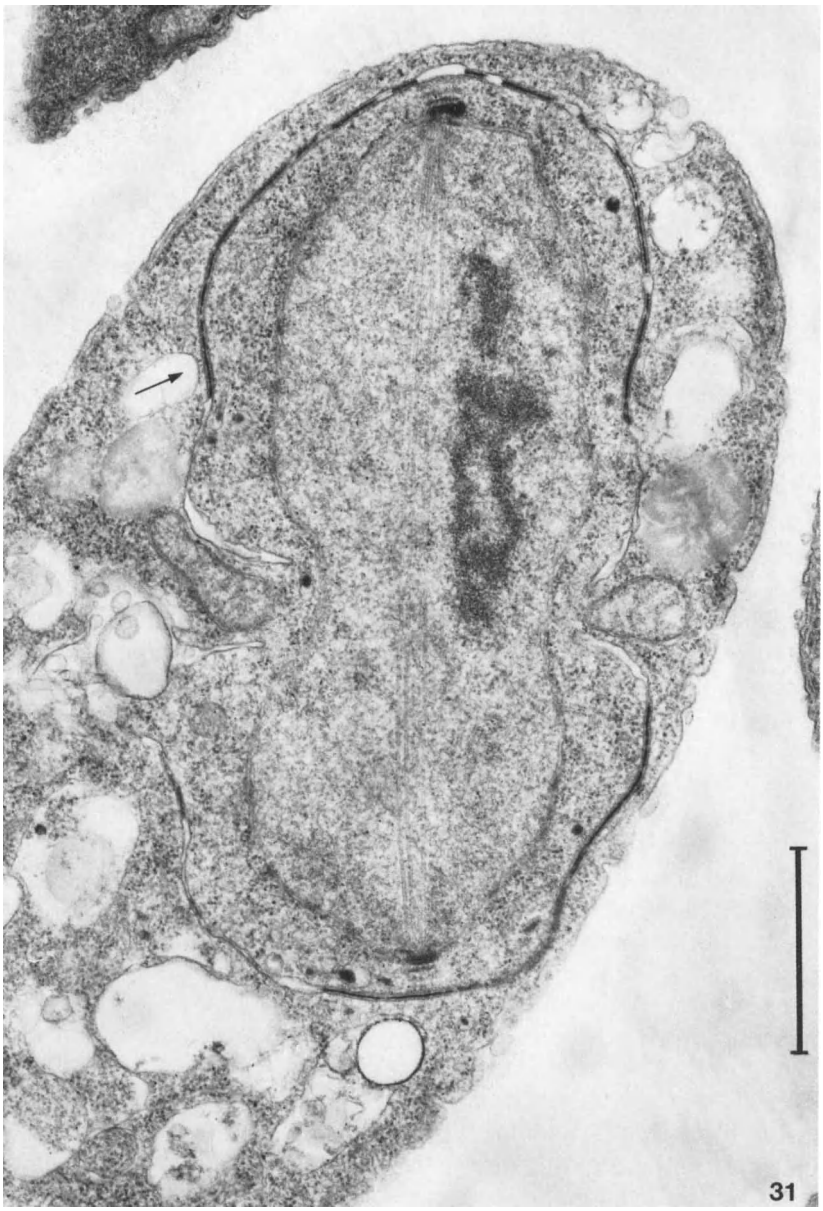


Fig. 31. An early stage of the development of the wall of ascospores of *S. japonicus*. Anaphase of a postmeiotic mitosis is in progress. Bell-shaped two-ply membranes, the spore wall primordia, are preparing to envelop sister spore cells. The arrow points to dense material being deposited in the space between the two leaves of the wall primordium. Bar, 1 μm. [From Tanaka and Hirata (1982), with permission.]

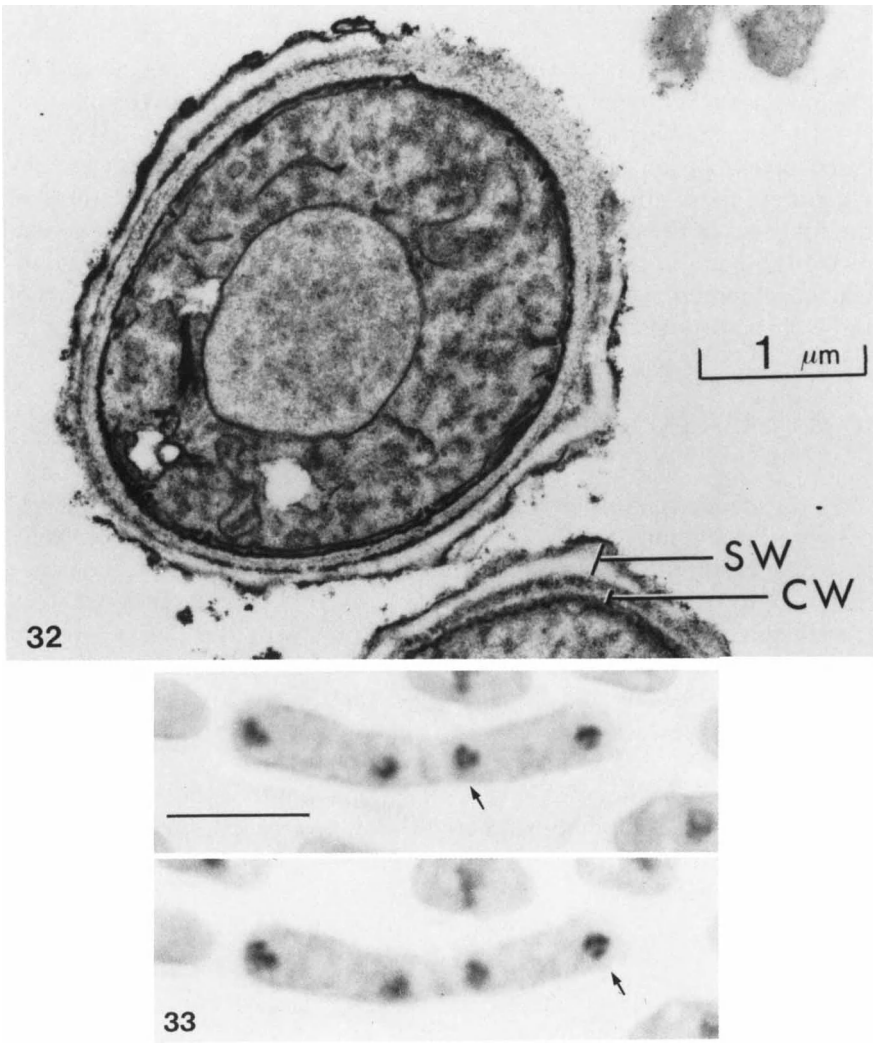


Fig. 32. Electron micrograph of a section of a germinating ascospore of *S. pombe*. New cell wall (CW) has developed underneath the spore wall (SW). This is clearly seen to the left of the bar. [From Johnson *et al.* (1982), with permission.]

Fig. 33. Telophase II of meiosis in an ascus of *S. pombe* h^{90}. The same ascus is shown at two levels of focus. Arrows point to sets of three chromosomes. Bar, 5 μm. [From Robinow (1977), with permission.]

6. *Meiosis in Light Microscopy*

Schizosaccharomyces pombe undergoing meiosis has been stained for chromatin by Schopfer *et al.* (1963), Robinow (1977), and Olson *et al.* (1978). The smallness and compactness of the constellations to be analyzed have imposed caution on the interpretation of the photomicrographs illustrating these efforts, but $n = 3$ has been suggested by the authors of the first two of them. The least ambiguous images were obtained at anaphase II where, according to Robinow, four compact, evenly spaced nuclei in a common cytoplasm could sometimes be resolved into neat sets of three chromosomes. Examples are provided in Fig. 33.

II. FLUORESCENCE MICROSCOPY

Fluorescence microscopy combines the best features of light microscopy with the ability to resolve structures of dimensions more typically associated with the electron microscope. The application of fluorescence methods to yeast cytology dates back over a quarter of a century (Streiblová and Beran, 1963), although it is only with the relatively recent development of indirect immunofluorescence techniques (Kilmartin and Adams, 1984; Adams and Pringle, 1984) that profound insights into the cytological organization of yeast cells have emerged. What follows does not pretend to be an exhaustive review of what has been achieved in *S. pombe* thus far. Rather, we have focused on a limited number of fluorescent probes, and combinations thereof, that have been used to characterize changes in the nucleus, cell wall, and cytoskeleton through the fission yeast cell division cycle.

A. Nucleus

The highly sensitive fluorescent DNA stain 4′,6-diamidino-2-phenylindole (DAPI) was originally introduced to yeast cell biology as a method for resolving mitochondrial DNA (Williamson and Fennell, 1975), although it is also a convenient nuclear stain. The most extensive use of DAPI staining in *S. pombe* has been the work of Yanagida and colleagues (Toda *et al*, 1981; Umesono *et al.*, 1983). Interphase haploid nuclei (~2 μm in diameter) consist essentially of staining and nonstaining hemispheres. Two short protrusions extend from the former into the latter which, based on its intense fluorescence with acridine orange and sensitivity to RNase, is the nucleolus.

Fission yeast cells will grow in the presence of DAPI with little or no loss of viability. This property enabled Toda *et al.* (1981) to follow nuclei through the cell division cycle by means of video fluorescence microscopy. Throughout interphase, the hemispherical appearance was maintained, albeit increasing in size during S phase. At mitosis the DAPI-staining area transformed, first into a condensed ellipsoid and then a U-shaped intermediate. This separated into two smaller hemispheres which moved to the ends of the cell (Toda *et al.*, 1981; see also Yanagida *et al.*, 1986).

The above study did not resolve the three chromosomes of *S. pombe* (Robinow, 1977). However, in thiabendazole-treated wild-type cells or in the β-tubulin mutant *nda3*, three DAPI-staining bodies were clearly seen (Umesono *et al.*, 1983; Yanagida *et al.*, 1986). Emerging from the smallest of these was a fine fibrous loop associated with the nucleolus and hence, presumably nucleolar organizer DNA. The loop appeared to be derived from the two protrusions which are a feature of interphase chromatin. In marked contrast to *S. pombe*, mitotic chromosomes are readily resolved by DAPI staining in *S. japonicus* var. *versatilis*, owing to their larger size (Fig. 34), the images being strikingly similar to those observed in Giemsa-stained preparations (Robinow, 1981a).

Perhaps the most important long-term consequence of studies using DAPI in fission yeasts is the characterization of mutants of *S. pombe* defective in either chromosome structure and/or segregation (Hiraoka *et al.*, 1984; Uemura and Yanagida, 1984, 1986; Uemura *et al.*, 1987; Hirano *et al.*, 1986, 1988; Okhura *et al.*, 1988; for a review, see Hirano and Yanagida, 1988). Nowhere is the power of DAPI shown better than in the study of Niwa *et al.* (1986), who were able to visualize a 500-kb (kilobase)

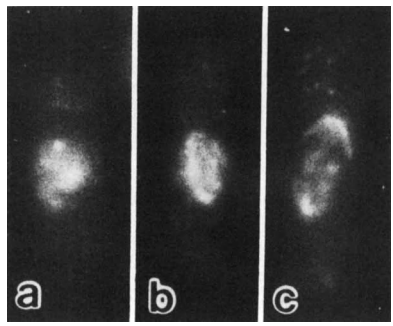


Fig. 34. DAPI staining of *S. japonicus* var. *versatilis*. (a) Interphase, (b) early mitosis, and (c) late mitosis. The speckled background in c is due to mitochondrial DNA. ×1875.

minichromosome of *S. pombe* consisting essentially of just the the centromere of chromosome III.

B. Cell Wall

Fission yeast cells grow by elongation in an asymmetric manner. This results in regions of the cell surface having architectural and compositional differences. These show different affinities for so-called brighteners such as Calcofluor white, which thus become useful probes for changes in cell surface organization through the cell division cycle (Streiblová, 1984). The precise nature of growth of *S. pombe* cells varies with growth conditions (growth medium, temperature, oxygen tension, etc.) (Johnson and McDonald, 1983) and even among wild-type strains (Mitchison and Nurse, 1985). These subtle differences have not always been universally appreciated as witnessed by the arguments that have occurred regularly in the literature since the first analysis of *S. pombe* growth by Knaysi (1940). It should be stressed that the observations presented in this and the subsequent section were obtained with strain 972 h^- grown at 25°C in liquid Edinburgh minimal medium plus phthalate (Nurse, 1975). Other strains grown in other ways will almost certainly behave differently.

Schizosaccharomyces pombe cells double stained with Calcofluor and DAPI are shown in Fig. 35. Following cytokinesis, each daughter cell has two ends which differ in age, namely, an old end which it inherited from its parent and a new end which is created by the cleaving action of the intensely fluorescent septum (Mitchison and Nurse, 1985). Although the old end stains strongly with Calcofluor the new end does not and appears as a dark hemisphere (Fig. 35).

In newly divided cells there is a lag, corresponding to about one-tenth of the cell cycle, before growth begins. Initially, growth is an almost exclusive prerogative of the old end. At a point in G_2 almost exactly one-third of the way through the cell division cycle, new end growth, which hitherto contributed insignificantly to cell elongation, is accelerated. This event is referred to by Mitchison and Nurse (1985) as NETO (for new end take off). The newly synthesized wall material is Calcofluor positive. This results in the non-Calcofluor-staining region being displaced from the pole to form the birth scar, a dark band which thus marks cells which have passed this point in the cell cycle (Fig. 35). Passage through NETO requires not only that cells have spent some obligatory period of time in G_2 but also that they have achieved a critical cell length; in the case of 972 h^-, this is 9.5 μm.

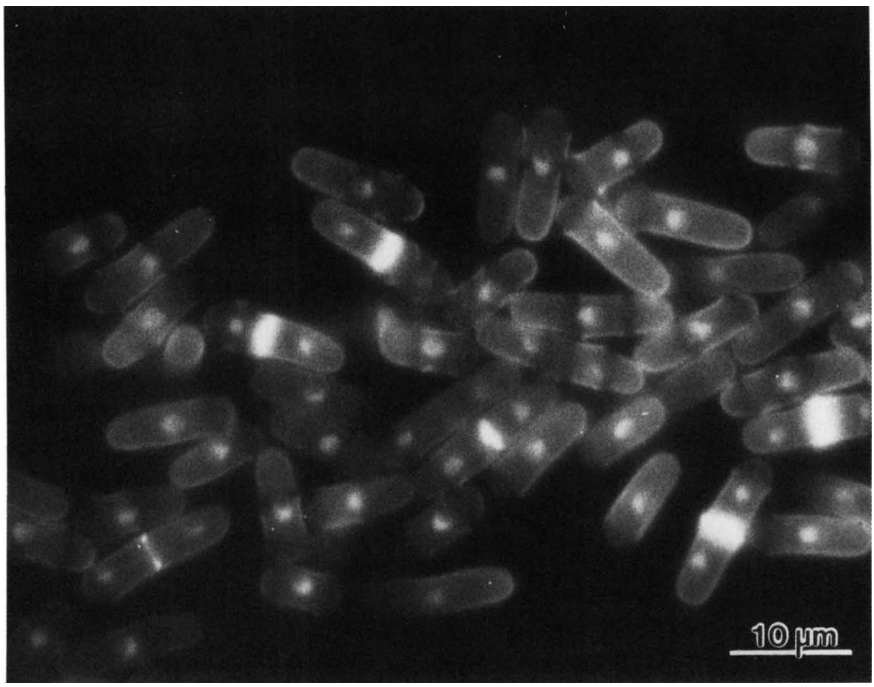


Fig. 35. Calcofluor–DAPI staining of *S. pombe*.

Acceleration of new end growth following NETO is accompanied by a slight reduction in the rate of elongation at the opposite end although this still remains faster than that at the new end (Mitchison and Nurse, 1985). Bipolar, but unequal, growth is maintained until three-quarters of the way through the cell division cycle, whereupon cells achieve the constant volume state which preceeds cell division.

Since new end growth begins later than that at the old end and proceeds at a slower rate, it accounts for a relatively small proportion of total cell elongation. This is evident from the Calcofluor-stained images which show that, in cells which have reached maximum length (most clearly, those having a septum), the cap of fluorescent staining distal to the birth scar is only of the order of 1–2 μm in length (Fig. 35). Because this contribution to total cell growth is relatively small it is easy to see how it could be overlooked and consequently how some of the controversies concerning the mechanism of growth of *S. pombe* have arisen.

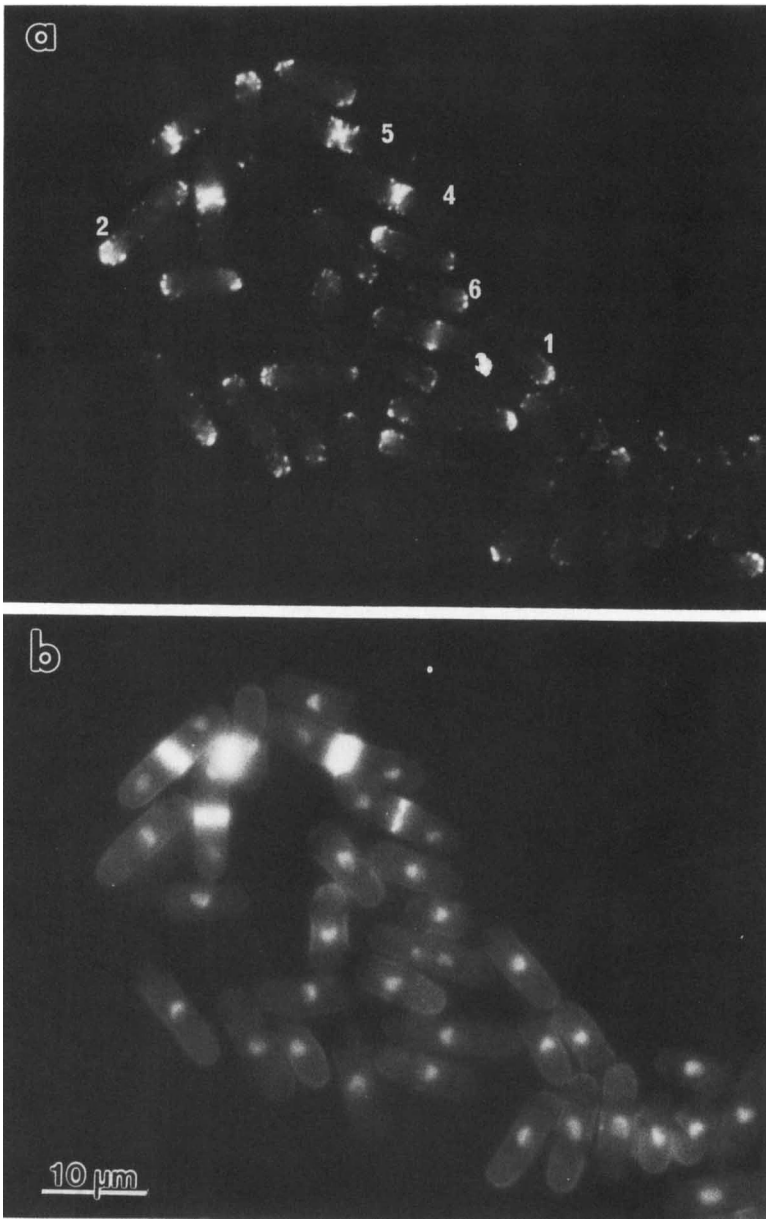


Fig. 36. (a) Cells from an asynchronous culture of *S. pombe* stained with rhodamine-conjugated phalloidin. (b) The same field stained with Calcofluor and DAPI. By comparing the two images, the colocalization of F-actin and cell wall deposition is evident. For details, see the text.

C. Cytoskeleton

1. Actin

Fluorescence microscopy using rhodamine-conjugated phalloidin as a specific probe provided the first unambiguous demonstration of actin in *S. pombe* (Marks and Hyams, 1985). Phalloidin staining of fission yeast typically reveals accumulations of F-actin, either at one or both ends of the cell or at the equator (Fig. 36a). Localization of actin in *S. pombe* by means of indirect immunofluorescence microscopy, using an anti-actin antibody instead of phalloidin, yields identical results (Marks *et al.*, 1986, 1987), as does phalloidin staining following freeze–substitution, a technique which avoids the use of chemical fixatives (Fig. 37; J. Marks and J. S. Hyams, unpublished observations). However, phalloidin staining following aldehyde fixation is by far the most convenient since it involves

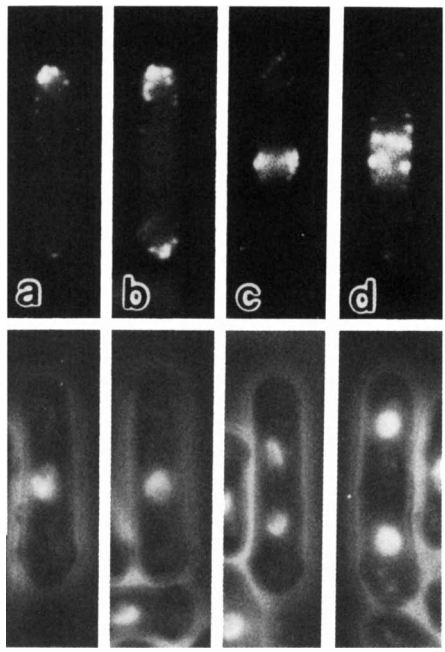


Fig. 37. Localization of F-actin in *S. pombe* following freeze–substitution. Top row, Phalloidin staining; bottom row, phase contrast–DAPI. (a) Single (old) end staining (pre-NETO), (b) double end staining (post-NETO), (c) tight equatorial actin ring typical of early anaphase cells, and (d) strong spotty equatorial staining marking the end of mitosis/start of cytokinesis. ×2400.

the fewest preparative steps. Indeed, from living cell to observation through the microscope need take no more than 1.5 hr (Marks and Hyams, 1985).

By comparing the phalloidin image with that obtained with Calcofluor and DAPI, it is possible to reconstruct the changes in actin distribution which occurs through the cell division cycle, even in an asychronous culture (Fig. 36a and b). In newly divided cells, actin is localized at the single growing (old) end, in the form of clusters of patches or dots (cell 1). Coincident with the switch to two-end growth, the actin dots also show a bipolar distribution, although biased toward the more rapidly growing old end and often associated with fine filaments extending along the long axis of the cell (cell 2). As cells reach the constant volume stage and cease elongation, actin rapidly disappears from the ends but reappears to form an equatorial ring overlying the nucleus and anticipating the subsequent site of septation (cell 3). The ring is a useful marker for the early stages of mitosis since its formation precedes any detectable distortion of the nucleus (i.e., the anaphase separation of the chromosomes). Formation of the ring also clearly precedes that of the septum (note the absence of equatorial Calcofluor fluorescence in cell 3). As the septum forms centripetally, the actin ring diminishes but fluorescence increases owing to the accumulation of dots reminiscent of those seen at the cell ends (cell 4). This becomes even more evident as the septum consolidates (cell 5). In very small cells immediately following cytokinesis, residual equatorial actin remains at the nongrowing (new) end (cell 6) and must relocate to the opposite pole for the elongation phase to resume. This redistribution may correspond to the observed lag in growth following cytokinesis. The changes in actin distribution through the cell division cycle of *S. pombe* are summarized in Fig. 38.

That a relationship between actin distribution and cell wall deposition in fission yeast exists now seems unarguable. It has been demonstrated not only in asynchronous cultures of wild-type cells as illustrated above but also in synchronous cultures (Marks and Hyams, 1985) as well as a variety of cell division cycle (*cdc*) mutants (Marks and Hyams, 1985; Marks *et al.*, 1986, 1987). These data are also consistent with findings from other yeasts (Kilmartin and Adams, 1984; Adams and Pringle, 1984; Anderson and Soll, 1986) and, indeed, other tip growing fungi (Hoch and Staples, 1983; Runeberg *et al.*, 1986; Heath, 1987).

In *Sacch. cerevisiae*, molecular genetics has allowed the link between actin and cell wall organization to be made even more forcibly. *Saccharomyces cerevisae* contains a single actin gene (Gallwitz and Seidel, 1980; Gallwitz and Sures, 1981; Ng and Abelson, 1980) containing a single, disposable intron (Ng *et al.*, 1985). Disruption of this gene is lethal to the

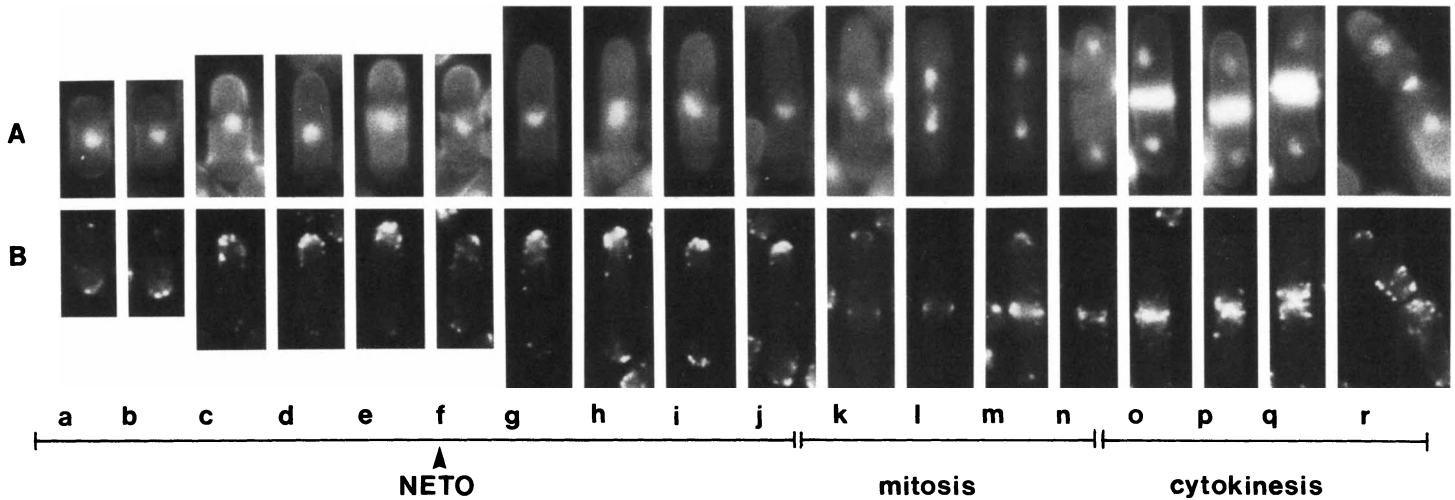


Fig. 38. Structural rearrangements of F-actin through the cell division cycle of *S. pombe*. (A) Calcofluor–DAPI and (B) rhodamine-conjugated phalloidin. The new end of the cell is toward the bottom in each case. ×1600. [From Marks and Hyams (1985), with permission.]

cell (Shortle *et al.*, 1982), but conditional lethal mutations (Shortle *et al.*, 1984) have shown that disorganization of the normal cellular disposition of actin results in a corresponding structural and functional disorganization of the cell surface (Novick and Botstein, 1985).

Schizosaccharomyces pombe has a single actin gene with no introns (Mertins and Gallwitz, 1987). Although less well studied than its budding yeast counterpart, strains of *S. pombe* carrying an extra copy of this gene have been created by integrative transformation. Asynchronous cultures of these strains contain a higher proportion of cells undergoing septation than do equivalent wild-type cultures, and the incidence of aberrent septation is higher. Nevertheless, and in contrast to the situation in *Sacch. cerevisae* (Shortle *et al.*, 1982), these strains are clearly viable (A. Schroeder-Lorenz, J. Marks, J. S. Hyams, and P. Fantes, unpublished observations).

Although the spatial and temporal coincidence between actin and cell wall deposition is clear, the nature of their relationship is not. We have speculated about this at length elsewhere (Marks *et al.*, 1987) and do not restate these arguments in detail here. Suffice it to say that the major obstacle to deciphering the cellular role of actin in *S. pombe* has been the lack of corroborative ultrastructural evidence for the observations described above. That is not to say that there have not been numerous electron microscope studies of *S. pombe*, rather that the cytoplasmic filament systems in fungi are poorly preserved for electron microscopy by chemical fixatives (Howard and Aist, 1979) and may only be adequately resolved by freeze–substitution (Howard, 1981), a technique which has not, as yet, been extensively applied to fission yeast (Tanaka and Kanbe, 1986). However, the omens are encouraging. A new freeze–substitution study of cytoplasmic organization in *S. pombe* has identified a system of Golgi-derived vesicles and filasomes (Hoch and Howard, 1980) associated with the sites of wall deposition (Fig. 39; K. Tanaka, personal communication). The distribution of this vesicle system through the cell division cycle thus parallels that of the phalloidin-stained dots, although whether these are different images of the same structures awaits information from immunoelectron microscopy. A clearer understanding of the role of actin in growth may well also emerge from a more extensive analysis of the cloned actin gene and the identification of proteins associated with actin *in vivo*.

2. *Microtubules*

Microtubules were first visualized by indirect immunofluorescence microscopy in *S. pombe* by Hiraoka *et al.* (1984). Although their study resolved mitotic spindles, no cytoplasmic microtubules were observed

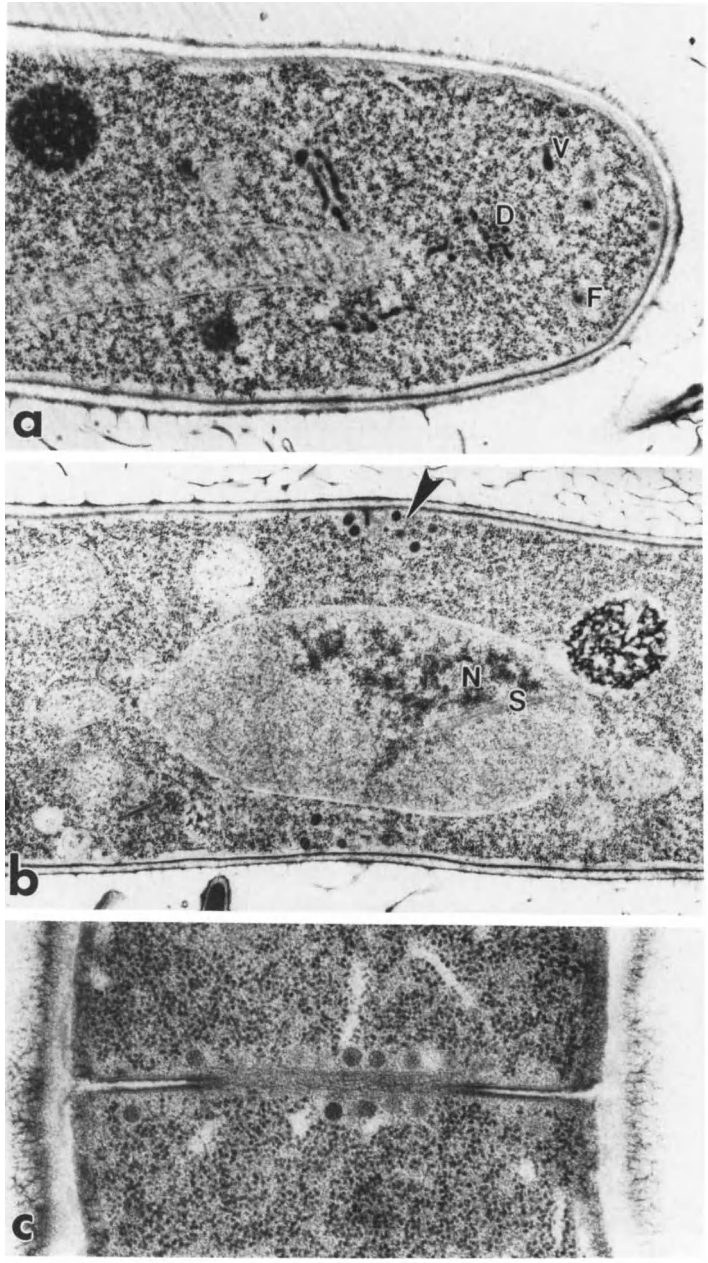


Fig. 39. *Schizosaccharomyces pombe* cells prepared for electron microscopy by freeze–substitution. (a) Cell pole showing clusters of dictyosome cisternae (D), associated vesicles (V), and filasomes (F). ×19,950. (b) Mitotic cell showing clusters of vesicles associated with the incipient septum (arrowhead). S, Mitotic spindle; N, nucleolus. ×16,150. (c) Cell in cytokinesis. Vesicles are arranged on either side of the microfilament bundle associated with the ingrowing septum. ×32,300. (Courtesy Dr. K. Tanaka.)

(see also Hirano *et al.*, 1986; Uemura and Yanagida, 1986). A more extensive analysis of microtubule staining patterns in *S. pombe* was recently reported by Hagan and Hyams (1988), and a field of cells prepared by their protocol is shown in Fig. 40. In interphase cells, between four and eight linear tubulin-staining elements, each probably a bundle of two or three microtubules, extend between the end of the cells (cell 1 in Fig. 40). At the G_2/M boundary this cytoplasmic array is replaced by an intranuclear spindle (cell 2). Initially, this is dumbell shaped due to the presence of two families of microtubules, those extending between the two spindle pole bodies (SPBs) and those extending between the poles and the chromosomal kinetochores (Hiraoka *et al.*, 1984; Tanaka and Kanbe, 1986; Ohkura *et al.*, 1988). The latter shorten (anaphase A) prior to the elongation of the pole-to-pole microtubules (anaphase B), during which astral microtubules oriented obliquely to the cytoplasmic face of the SPBs appear (cell 3). Spindle elongation proceeds at a rate of about 1.6 μm/min

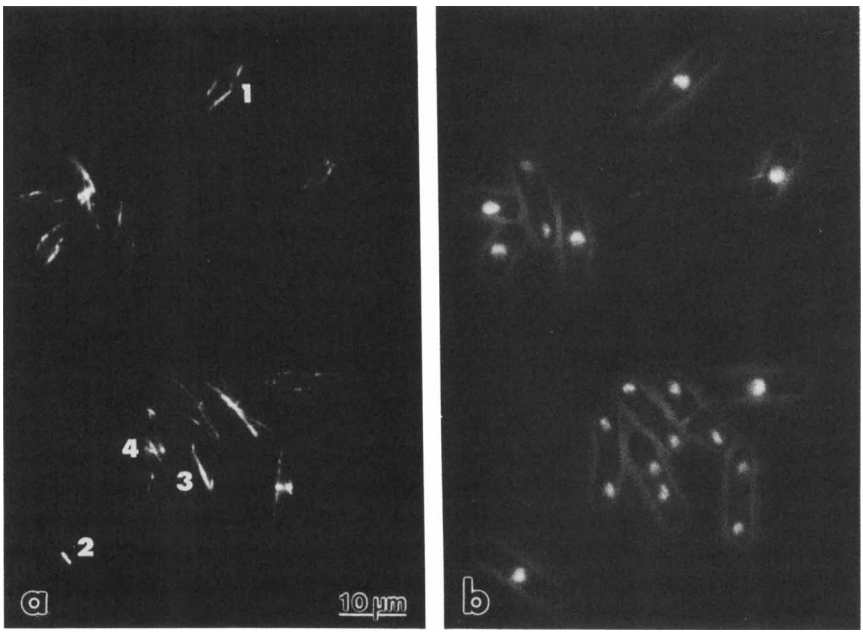


Fig. 40. (a) Indirect immunofluorescence microscopy of *S. pombe* using an antitubulin antibody. (b) The same field of cells viewed with phase contrast–DAPI. Note the following microtubule patterns: interphase (cell 1), early mitosis (cell 2), mid-mitosis (cell 3), late mitosis (cell 4). In cell 4 the fluorescent staining at the cell equator is due to the initiation of the microtubules that will reestablish the interphase array from two microtubule organizing centers. [From Marks *et al.* (1986), with permission.]

until the nuclei reach the ends of the cell (I. M. Hagan, P. Riddle, and J. S. Hyams, unpublished results). At this point the microtubules that will form the interphase array of the two daughter cells are initiated from two foci (microtubule organizing centers) at the cell equator (cell 4). These are distinct from the SPBs which appear to be associated with microtubules only during mitosis.

What is the function of cytoplasmic microtubules in *S. pombe*? One fairly clear role is in the positioning of the nucleus. In cells of the cold-sensitive β-tubulin mutant *nda3* grown at the restrictive temperature, the nucleus is displaced from the center (Toda *et al.*, 1983; Umesono *et al.*, 1983). This is also seen in thiabendazole-treated wild-type cells (Erard and Barker, 1985) and, even more clearly, in experiments in which *cdc25* cells underwent mitosis at exaggerated cell length. On depolymerization of the cytoplasmic microtubules at the G_2/M boundary, the mitotic nuclei were free to wander a considerable distance from their normal equatorial position (Hagan and Hyams, 1988).

Although the evidence for it is less convincing, cytoplasmic microtubules probably also play some role in establishing/maintaining cell polarity in *S. pombe*. Cold-grown *nda3* cells and wild-type cells treated with microtubule inhibitors exhibit various abnormal shapes including the formation of branches (Walker, 1982; Toda *et al.*, 1983; Umesono *et al.*, 1983). These observations imply that, in the absence of microtubules, the normal controls determining the cellular distribution of F-actin break down, and it is interesting that the time of disappearance of cytoplasmic microtubules in the normal *S. pombe* cell division cycle coincides with the redistribution of F-actin from the ends of the cell to the equator (see above). The inferred relationship between microtubules and polarity/shape gains further credence from indirect immunofluorescence microscopy of *S. japonicus*. These cells have a less defined shape than *S. pombe*, and their microtubule cytoskeleton is correspondingly less well organized (Fig. 41; Alfa and Hyams, 1989).

D. Summary and Conclusions

Although still in its infancy, fluorescence microscopy has contributed significantly to our understanding of cellular organization in *S. pombe*. Perhaps surprisingly, it has revealed features that were not suspected from previous investigations using electron microscopy. This is particularly true in the case of the cytoskeleton, where reports of both cytoplasmic microtubules and presumtive F-actin filaments have been sparse and confined to cells in fairly atypical circumstances (Hereward, 1974; Streiblová and Girbardt, 1980; Streiblová *et al.*, 1984; King and Hyams,

Fig. 41. Indirect immunofluorescence microscopy of *S. japonicus* var. *versatilis*. Cytoplasmic microtubules in interphase cells in particular show a greater number and complexity than those of *S. pombe*, perhaps consistent with the larger, more irregular cell shape of this yeast.

1982). The lability of cytoplasmic microtubules in *S. pombe* is shown by their variable preservation in different fixation protocols for immunofluorescence microscopy (Hagan and Hyams, 1988), and actin filaments are known to be destroyed by osmium tetroxide (Maupin-Szamier and Pollard, 1978). Clearly, there is a need to review the ultrastructure of fission yeast in the light of modern preparative techniques (Hoch, 1986), and the studies of K. Tanaka and colleagues (personal communication) promise to be particularly informative.

Remodeling of the cytoskeleton, in particular at the G_2/M boundary, provides new "landmarks" for the *S. pombe* cell division cycle. These may prove useful in further establishing the relationships between the many genes which function at this major cell cycle control point (Hayles and Nurse, 1986) and indeed in the identification of other genes acting at or close to it. A number of *cdc* genes encode protein kinases (Simanis and Nurse, 1986), and a number of cytoskeletal structures (mitotic spindle poles, nuclear lamina) contain phosphoproteins (Vandre *et al.*, 1986; Gerace, 1986). This suggests an obvious way in which rearrangements of the cytoskeleton may be integrated with other cellular events, although the reality may prove to be far more subtle and complex. Establishing the subcellular distribution of *cdc* gene products using antibody techniques may prove particularly informative. Studies of *ras* function may also hold important clues since cells carrying a null mutation of the *ras1* gene have distorted shapes (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986; see also Nadin-Davis *et al.*, Chapter 4, this volume).

Actin plays a major but as yet incompletely understood role in *S. pombe* growth and cytokinesis. A cytokinetic ring of actin is probably a ubiquitous feature of the division of cells having cell walls (Girbardt, 1979; Clayton and Lloyd, 1985), although whether this is contractile like the analogous structure in animal cells (Rappaport, 1986) awaits the definitive identification of myosin in *S. pombe*, as was recently achieved in *Sacch. cerevisiae* (Watts *et al.*, 1985, 1987). Extending our understanding of the secretory pathway in *S. pombe* may well contribute significantly to our understanding of the role of actin in growth. Localization of Golgi markers using antibody techniques may help in this regard. Calmodulin plays an essential role in the growth process (Takeda and Yamamoto, 1987) and determining its cellular distribution through the cell division cycle will also be informative.

More information is required regarding the physiology of growth in *S. pombe*. In filamentous fungi exogenous electrical currents induce hyphal branching (McGillivray and Gow, 1986) as do both calcium and proton ionophores (Harold and Harold, 1986). Interestingly, cytochalasins, which disrupt cellular processes based on actin, have a similar effect (Harold and Harold, 1986). Perhaps by following all these different lines

of investigation we may one day understand the growth of fission yeast cells and complete the study initiated by Knaysi and reported in 1940.

ACKNOWLEDGMENTS

J. S. Hyams is extremely grateful to John Marks, Iain Hagan, and Caroline Alfa for their contributions, both practical and cerebral, to this article. We also wish to thank Kenji Tanaka for generously providing electron micrographs and for sharing unpublished results. C. F. Robinow warmly thanks Professor R. G. E. Murray for defraying the expenses of most of the photographic work for Section I from his own research funds.

REFERENCES

Adams, A. E. M., and Pringle, J. R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* **98,** 934–945.

Aist, J. R., and Berns, M. W. (1981). Mechanics of chromosome separation during mitosis in *Fusarium* (fungi imperfecti): New evidence from ultrastructural and laser microbeam experiments. *J. Cell Biol.* **91,** 446–458.

Alfa, C. E., and Hyams, J. S. (1989). Submitted.

Allan, R. A., and Miller, J. J. (1980). Influence of *S*-adenosylmethionine on DAPI-induced fluorescence of polyphosphate in the yeast vacuole. *Can. J. Microbiol.* **26,** 912–920.

Anderson, J. M., and Soll, D. R. (1986). Differences in actin localization during bud and hypha formation in the yeast *Candida albicans*. *J. Gen. Microbiol.* **132,** 2035–2047.

Ashton, M.-L., and Moens, P. B. (1982). Light and electron microscopy of conjugation in the yeast *S. octosporus*. *Can. J. Microbiol.* **28,** 1059–1077.

Beckett, A., Illingworth, R. F., and Rose, A. H. (1973). Ascospore wall development in *Sacch. cerevisiae*. *J. Bacteriol.* **113,** 1054–1057.

Byers, B., and Goetsch, L. (1974). Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* **38,** 123–131.

Byers, B., and Goetsch, L. (1975). Behaviour of spindles and spindle plaques in the cell cycle and conjugation of *Sacch. cerevisiae*. *J. Bacteriol.* **124,** 511–523.

Calleja, G. B., Yoo, B. Y., and Johnson, B. F. (1977a). Fusion and erosion of cell walls during conjugation in the fission yeast (*S. pombe*). *J. Cell Sci.* **25,** 139–155.

Calleja, G. B., Yoo, B. Y., and Johnson, B. F. (1977b). Conjugation-induced lysis of *S. pombe*. *J. Bacteriol.* **130,** 512–515.

Calleja, G. B., Johnson, B. F., and Yoo, B. Y. (1981). The cell wall as sex organelle in fission yeast. *In* "Sexual Interactions in Eucaryotic Microbes" (D. H. O'Day and P. Horgen, eds.), pp. 225–259. Academic Press, Orlando, Florida.

Chen, T.-T. (1948). Chromosomes in Opalinidae (Protozoa, Ciliata) with special reference to their behaviour, morphology, individuality, diploidy, haploidy and association with nucleoli. *J. Morphol.* **83,** 281–385.

Chouinard, L. A. (1974). An electron-microscope study of the intranucleolar chromatin in root meristematic cells of *Allium cepa*. *J. Cell Sci.* **15,** 645–657.

Clayton, L., and Lloyd, C. W. (1985). Actin reorganization during the cell cycle in meristematic plant cells. *Exp. Cell Res.* **156,** 231–238.

Davison, M. T., and Garland, P. B. (1977). Structure of mitochondria and vacuoles of *Candida utilis* and *S. pombe* studied by electron microscopy of serial thin sections and model building. *J. Gen. Microbiol.* **98,** 147–153.

Deshusses, J., Oulevey, N., and Turian, G. (1970). Etude ultrastructurale des anomalies cellulaires produits chez la levure *S. pombe* par divers anti-inositols. *Protoplasma* **70,** 119–130.

Erard, M., and Barker, D. G. (1985). Electron microscopic studies of condensed mitotic chromosomes in the fission yeast *S. pombe*. *Biol. Cell.* **55,** 27–34.

Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986). Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. *Cell* **44,** 329–336.

Gallwitz, D., and Seidel, R. (1980). Molecular cloning of the actin gene from the yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **8,** 1043–1059.

Gallwitz, D., and Sures, I. (1981). Structure of a split gene: Complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2546–2550.

Gerace, L. (1986). Nuclear lamina and organization of nuclear architecture. *Trends Biochem. Sci.* **11,** 443–446.

Girbardt, M. (1960). Licht- und elektronenoptische Untersuchungen an *Polystictus versicolor* (L.) VI. Der nucleolus-Formenwandel. Ein Beitrag zur Frage der Kernbewegung. *Planta* **55,** 365–380.

Girbardt, M. (1968). Ultrastructure and dynamics of the moving nucleus. *Symp. Soc. Exp. Biol.* **22,** 249–259.

Girbardt, M. (1979). A microfilamentous septal belt (FSB) during induction of cytokinesis in *Trametes versicolor* (L. ex Fr.) *Exp. Mycol.* **3,** 215–228.

Girbardt, M., and Hädrich. (1975). Ultrastruktur des Pilzkernes III. Genese des Kern-assoziierten Organells (NAO="KCE"). *Z. Allg. Mikrobiol.* **15,** 157–173.

Grove, S., and Bracker, C. E. (1970). Protoplasmic organization of hyphal tips among fungi: Vesicles and Spitzenkörper. *J. Bacteriol.* **104,** 989–1009.

Guilliermond, A. (1910). A propos des corpuscules métachromatiques ou grains de volutine. *Arch. Protistenkd.* **19,** 279–309.

Hagan, I. M., and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **89,** 343–357.

Harold, F. M. (1966). Inorganic phosphates in biology: Structure, metabolism and function. *Bacteriol. Rev.* **30,** 772–794.

Harold, R. L., and Harold, F. M. (1986). Ionophores and cytochalasins modulate branching in *Achlya bisexualis*. *J. Gen. Microbiol.* **132,** 213–219.

Hayles, J., and Nurse, P. (1986). Cell cycle regulation in yeast. *J. Cell Sci. Suppl.* **4,** 155–170.

Heath, I. B. (1987). Preservation of a labile cortical array of actin filaments in growing hyphal tips of the fungus *Saprolegnia ferax*. *Eur. J. Cell Biol.* **44,** 10–16.

Hereward, F. V. (1974). Cytoplasmic microtubules in a yeast. *Planta* **117,** 355–360.

Heslot, H., Louis, C., and Goffeau, A. (1970). Segregational respiratory-deficient mutants of a "Petite-negative" yeast, *S. pombe* 972 h^-. *J. Bacteriol.* **104,** 482–491.

Hirano, T., and Yanagida, M. (1988). Controlling elements in the cell division cycle of *Schizosaccharomyces pombe*. *In* "The Molecular Biology of Yeasts." Blackie, London, in press.

Hirano, T., Funahashi, S., Uemura, T., and Yanagida, M. (1986). Isolation and characterisation of *Schizosaccharomyces pombe cut* mutants that block nuclear division but not cytokinesis. *EMBO J.* **5,** 2973–2979.

Hirano, T., Hiraoka, Y., and Yanagida, M. (1988). A temperature-sensitive mutation of the

Schizosaccharomyces pombe gene *nuc2*$^+$ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. *J. Cell Biol.* **106,** 1171–1183.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes β-tubulin. A cold sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39,** 349–358.

Hirata, A., and Tanaka, K. (1982). Nuclear behaviour during conjugation and meiosis in the fission yeast *S. pombe*. *J. Gen. Appl. Microbiol.* **28,** 263–274.

Hoch, H. C. (1986). Freeze–substitution of fungi. *In* "Ultrastructure Techniques for Microorganisms" (H. C. Aldridge and W. J. Todd, eds.), pp. 183–212. Plenum, New York.

Hoch, H. C., and Howard, R. J. (1980). Ultrastructure of freeze substituted hyphae of the basidiomycete *Laetisaria arvalis*. *Protoplasma* **103,** 281–297.

Hoch, H. C., and Staples, R. C. (1983). Visualization of actin *in situ* by rhodamine-conjugated phalloidin in the fungus *Uromyces phaseoli*. *Eur. J. Cell Biol.* **32,** 52–58.

Howard, R. J. (1981). Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkorper, cytoskeleton and endomembranes after freeze–substitution. *J. Cell Sci.* **48,** 89–103.

Howard, R. J., and Aist, J. R. (1979). Hyphal tip ultrastructure of the fungus *Fusarium*: Improved preservation by freeze–substitution. *J. Ultrastruct. Res.* **66,** 224–234.

Hughes, A. (1959). "A History of Cytology." Abelard-Schuman, London.

Johnson, B. F. (1967). Growth of the fission yeast, *S. pombe*, with late, eccentric, lytic fission in an unbalanced medium. *J. Bacteriol.* **94,** 192–195.

Johnson, B. F., and McDonald, I. J. (1983). Cell division: A separable cellular sub-cycle in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **129,** 3411–3419.

Johnson, B. F., Yoo, B. Y., and Calleja, G. B. (1973). Cell division in yeasts: Movement of organelles associated with cell plate growth of *S. pombe*. *J. Bacteriol.* **115,** 358–366.

Johnson, B. F., Yoo, B. Y., and Calleja, G. B. (1974). Cell division in yeasts. II. Template control of cell plate biogenesis in *Schizosaccharomyces pombe*. *In* "Cell Cycle Controls" (G. M. Padilla, I. L. Cameron, and A. Zimmerman, eds.), pp. 153–166. Academic Press, New York.

Johnson, B. F., Calleja, G. B., Boisclair, I., and Yoo, B. Y. (1979). Cell division in yeasts. III. The biased, asymmetric location of the septum in the fission yeast cell *S. pombe*. *Exp. Cell Res.* **123,** 253–259.

Johnson, B. F., Calleja, G. B., Yoo, B. Y., Zuker, M., and McDonald, I. (1982). Cell division: Key to cellular morphogenesis in the fission yeast, *Schizosaccharomyces*. *Int. Rev. Cytol.* **75,** 167–208.

Keddie, F. M., and Barajas, L. (1969). Three-dimensional reconstruction of *Pityrosporum* yeast cells based on serial section electron microscopy. *J. Ultrastruct. Res.* **29,** 260–275.

Kilmartin, J. V., and Adams, A. E. M. (1984). Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Sacch. cerevisiae*. *J. Cell Biol.* **98,** 922–933.

King, S. M., and Hyams, J. S. (1982). Interdependence of cell cycle events in *Schizosaccharomyces pombe*. Terminal phenotypes of *cdc* mutants arrested during DNA synthesis and cell division. *Protoplasma* **110,** 54–62.

Knaysi, G. (1940). A photomicrographic study of the rate of growth of some yeasts and bacteria. *J. Bacteriol.* **40,** 247–253.

Kopp, F. (1975). Electron microscopy of yeast. *Methods Cell Biol.* **11,** 23–44.

Lafontaine, J.-G., and Lord, A. (1973). An ultrastructural and radioautographic investigation of the nucleonemal component of plant interphase nucleoli. *J. Cell Sci.* **12,** 369–383.

McCully, E. K., and Bracker, C. E. (1972). Apical vesicles in growing bud cells of heterobasidiomycetous yeasts. *J. Bacteriol.* **109,** 922–926.

McCully, E. K., and Robinow, C. F. (1971). Mitosis in the fission yeast *S. pombe*: A comparative study with light and electron microscopy. *J. Cell Sci.* **9,** 475–507.

McCully, E. K., and Robinow, C. F. (1973). Mitosis in *Mucor hiemalis*: A comparative light and electron microscopical study. *Arch. Mikrobiol.* **94,** 133.

McDonald, I. J., Calleja, G. B., and Johnson, B. F. (1982). Conjugation in chemostat cultures of *S. pombe*. *J. Gen. Microbiol.* **128,** 1981–1987.

McGillivray, A. M., and Gow, N. A. R. (1986). Applied electrical fields polarize the growth of mycelial fungi. *J. Gen. Microbiol.* **132,** 2515–2525.

McKerracher, L. J., and Heath, I. D. (1986). Fungal nuclear behaviour analysed by ultraviolet microbeam irradiation. *Cell Motil. Cytoskeleton* **6,** 35–47.

Marks, J., and Hyams, J. S. (1985). Localization of F-actin through the cell division cycle of *S. pombe*. *Eur. J. Cell Biol.* **39,** 27–32.

Marks, J., Hagan, I. M., and Hyams, J. S. (1986). Growth polarity and cytokinesis in fission yeast: The role of the cytoskeleton. *J. Cell Sci. Suppl.* **5,** 229–241.

Marks, J., Hagan, I. M., and Hyams, J. S. (1987). Spatial association of F-actin with growth polarity and septation in the fission yeast *Schizosaccharomyces pombe*. *Spec. Publ. Soc. Gen. Microbiol.* **23,** 119–135.

Maupin-Szamier, P., and Pollard, T. D. (1978). Actin filament destruction by osmium tetroxide. *J. Cell Biol.* **77,** 837–852.

Mertins, P., and Gallwitz, D. (1987). A single intron-less actin gene in the fission yeast *Schizosaccharomyces pombe*: Nucleotide sequence and transcripts formed in homologous and heterologous yeasts. *Nucleic Acids Res.* **15,** 7369–7379.

Miller, J. J. (1984). *In vitro* experiments concerning the state of polyphosphate in the yeast vacuole. *Can. J. Microbiol.* **30,** 236–246.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exp. Cell Res.* **13,** 244–262.

Mitchison, J. M., and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **75,** 357–376.

Miyakawa, I., Aoi, H., Sando, N., and Kuroiwa, T. (1984). Fluorescence microscopic studies of mitochondrial nucleoids during meiosis and sporulation in the yeast *Sacch. cerevisiae*. *J. Cell Sci.* **66,** 21–38.

Miyata, M., Kanbe, T., and Tanaka, K. (1985). Morphological alterations of the fission yeast *S. pombe* in the presence of Aculeacin A: Spherical wall formation. *J. Gen. Microbiol.* **131,** 611–621.

Moens, P. B. (1971). Fine structure of ascospore development in the yeast *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **17,** 507–510.

Morris, N. R. (1980). Chromosome structure and the molecular biology of mitosis in eukaryotic micro-organisms. *Symp. Soc. Gen. Microbiol.* **30,** 41–75.

Murray, R. G. E. (1988). A structured life. *Annu. Rev. Microbiol.* **42,** 1–34.

Nadin-Davis, S. A., Nasim, A., and Beach, D. (1986). Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. *EMBO J.* **5,** 2963–2971.

Ng, R., and Abelson, J. (1980). Isolation of the gene for actin in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3912–3916.

Ng, R., Dandey, H., Larson, G., Rossi, J. J., and Abelson, J. (1985). A test for intron function in the yeast actin gene. *Nature (London)* **314,** 183–184.

Niwa, O., Matsumoto, T., and Yanagida, M. (1986). Construction of a minichromosome and its mitotic and meiotic behavior in fission yeast. *Mol. Gen. Genet.* **203,** 397–405.

Novick, P., and Botstein, D. (1985). Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell* **40,** 405–416.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. *Nature (London)* **256,** 547–551.

Oakley, B. R., and Morris, N. R. (1980). Nuclear movement is β-tubulin dependent in *Aspergillus nidulans*. *Cell* **19,** 255–262.

Okhura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T., and Yanagida, M. (1988). Cold-sensitive and caffeine-sensitive mutants of the *Schizosaccharomyces pombe dis* genes implicated in sister chromatid separation. *EMBO J.* **7,** 1465–1473.

Olson, L. W. (1974). Meiosis in the aquatic phycomycete *Allomyces macrogynus*. *C. R. Trav. Lab. Carlsberg* **40,** 113–124.

Olson, L. W., Edén, U., Egel-Mitami, M., and Egel, R. (1978). Asynaptic meiosis in fission yeast? *Hereditas* **89,** 189–199.

Osumi, M., and Sando, N. (1969). Division of yeast mitochondria in synchronous culture. *J. Electron. Microsc.* **18,** 47–56.

Oulevey, N., Deshusses, J., and Turian, G. (1970). Etude de la zone septale de *S. pombe* en division à ses étapes successives. *Protoplasma* **70,** 217–224.

Peterson, J., and Ris, H. (1976). Electron-microscopic study of the spindle and chromosome movement in the yeast *Sacch. cerevisiae*. *J. Cell Sci.* **22,** 219–242.

Pickett-Heaps, J. D., and Fowke, L. C. (1970). Mitosis, cytokinesis, and cell elongation in the desmid *Closterium littorale*. *J. Phycol.* **6,** 189–215.

Prescott, D. M. (1976). "Reproduction of Eukaryotic Cells," p. 9. Academic Press, New York.

Rappaport, R. (1986). Establishment of the mechanisms of cytokinesis in animal cells. *Int. Rev. Cytol.* **105,** 245–281.

Robinow, C. F. (1975). The preparation of yeasts for light microscopy. *Methods Cell Biol.* **11,** 1–22.

Robinow, C. F. (1977). The number of chromosomes in *S. pombe*: Light microscopy of stained preparations. *Genetics* **87,** 491–497.

Robinow, C. F. (1981a). The view through the microscope. *Proc. Int. Yeast Symp., 5th, 1980,* pp. 623–634.

Robinow, C. F. (1981b). Nuclear behaviour in conidial fungi. *In* "Biology of Conidial Fungi" (G. T. Cole and B. Kendrick, eds.), pp. 357–393. Academic Press, Orlando, Florida.

Robinow, C. F., and Bakerspigel, A. (1965). Somatic nuclei and forms of mitosis in fungi. *In* "The Fungi. An Advanced Treatise" (G. C. Ainsworth and A. S. Sussman, eds.), pp. 119–142. Academic Press, New York.

Robinow, C. F., and Marak, J. (1966). A fiber apparatus in the nucleus of the yeast cell. *J. Cell Biol.* **29,** 129–151.

Runeberg, P., Raudaskoski, M., and Virtanen, I. (1986). Cytoskeletal elements in the hyphae of the basidiomycete *Schizophyllum commune* visualised with indirect immunofluorescence and NBD-phallicidin. *Eur. J. Cell Biol.* **41,** 25–32.

Schopfer, W. H., Posternak, Th., and Wüstenfeld, D. (1962). Recherches sur le role du meso-inositol dans la biologie cellulaire de *Schizosaccharomyces pombe* Lindner. *Arch. Mikrobiol.* **44,** 113–151.

Schopfer, W. H., Wüstenfeld, D., and Turian, G. (1963). La division nucléaire chez *S. pombe*. *Arch. Mikrobiol.* **45,** 304–313.

Shortle, D., Haber, J. E., and Botstein, D. (1982). Lethal disruption of the yeast actin gene by integrative DNA transformation. *Science* **217,** 371–373.

Shortle, D., Novick, P., and Botstein, D. (1984). Construction and genetic characterization of temperature-sensitive alleles of the yeast actin gene. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4889–4893.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene *cdc2*$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45,** 261–268.

Sloof, W. Ch. (1970). The genus *Schizosaccharomyces* Lindner. *In* "The Yeasts, a Taxonomic Study" (J. Lodder, ed.), pp. 733–755. North-Holland, Amsterdam.

Smith, I. W., Wilkinson, J. F., and Duguid, J. B. (1954). Volutin production in *Aerobacter aerogenes* due to nutrient imbalance. *J. Bacteriol.* **68,** 450–463.

Stevens, B. (1977). Variation in number and volume of mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconstruction. *Biol. Cell.* **28,** 37–56.

Stevens, B. (1981). Mitochondrial structure. *In* "The Molecular Biology of the Yeast *Sacch. cerevisiae*. Life Cycle and Inheritance" (J. N. Strathern, E. W. Jones, and J. R. Broach, eds.), pp. 471–504. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Streiblová, E. (1984). The yeast cell wall—A marker system for cell cycle controls. *In* "The Microbial Cell Cycle" (P. Nurse and E. Streiblova, eds.), pp. 89–107. CRC Press, Boca Raton, Florida.

Streiblová, E., and Beran, K. (1963). Demonstration of yeast scars by fluorescence microscopy. *Exp. Cell Res.* **30,** 603–605

Streiblová, E., and Girbardt, M. (1980). Microfilaments and cytoplasmic microtubules in cell division cycle mutants of *S. pombe*. *Can. J. Microbiol.* **26,** 250–254.

Streiblová, E., Malek, I., and Beran, K. (1966). Structural changes in the cell wall of *S. pombe* during cell division. *J. Bacteriol.* **91,** 428–435.

Streiblová, E., Hasek, J., and Jelke, E. (1984). Septum patterns in *ts* mutants of *Schizosaccharomyces pombe* defective in genes *CDC3*, *CDC4*, *CDC8* and *CDC12*. *J. Cell Sci.* **69,** 47–65.

Takeda, T., and Yamamoto, M. (1987). Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3580–3584.

Tanaka, K., and Hirata, A. (1982). Ascospore development in the fission yeasts *S. pombe* and *S. japonicus*. *J. Cell Sci.* **56,** 263–279.

Tanaka, K., and Kanbe, T. (1986). Mitosis in the fission yeast *S. pombe* as revealed by freeze–substitution electron microscopy. *J. Cell Sci.* **80,** 253–268.

Tanaka, K., Kanbe, T., and Kuroiwa, T. (1985). Three-dimensional behaviour of mitochondria during cell division and germ tube formation in the dimorphic yeast *Candida albicans*. *J. Cell Sci.* **73,** 207–220.

Taylor, J. W., and Wells, K. (1979). The mitochondrion in mitotic and starved cells of *Bullera alba*. *Exp. Mycol.* **3,** 16–27.

Toda, T., Yamamoto, M., and Yanagida, M. (1981). Sequential alterations in the nuclear chromatin region during mitosis of the fission yeast *S. pombe*: Video fluorescence microscopy of synchronously growing wild-type and cold-sensitive *cdc* mutants by using a DNA-binding probe. *J. Cell Sci.* **52,** 271–287.

Toda, T., Umesono, K., and Hiraoka, A. (1983). Cold sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. *J. Mol. Biol.* **168,** 251–270.

Uemura, T., and Yanagida, M. (1984). Isolation of type I and II DNA topoisomerase mutants from fission yeast: Single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO J.* **3,** 1737–1744.

Uemura, T., and Yanagida, M. (1986). Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: Uncoordinated mitosis. *EMBO J.* **5,** 1003–1010.

Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., and Yanagida, M. (1987). DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell* **50,** 917–925.

Umesono, K., Hiraoka, Y., Toda, T., and Yanagida, M. (1983). Visualization of chromosomes in mitotically arrested cells of the fission yeast *S. pombe*. *Curr. Genet.* **7,** 123–128.

Vandre, D. D., Davis, F. M., Rao, P., and Borisy, G. G. (1986). Distribution of cytoskeletal proteins sharing a conserved phosphorylated epitope. *Eur. J. Cell Biol.* **41,** 72–81.

Walker, G. M. (1982). Cell cycle specificity of certain antimicrotubular drugs in *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **128,** 61–71.

Walker, G. M., Birch-Andersen, A., Hamburger, K., and Kramhoft, B. (1982). Magnesium-induced mitochondrial polymorphism and changes in respiratory metabolism in the fission yeast *S. pombe*. *Carlsberg Res. Commun.* **47,** 205–214.

Watts, F. Z., Miller, D. M., and Orr, E. (1985). Identification of myosin heavy chain in *Saccharomyces cerevisiae*. *Nature* (*London*) **316,** 83–85.

Watts, F. Z., Shiels, G., and Orr, E. (1987). The yeast *MYO1* gene encoding a myosin-like protein required for cell division. *EMBO J.* **6,** 3499–3505.

Wickerham, L. J., and Duprat, E. (1945). A remarkable fission yeast *S. versatilis* nov. spec. *J. Bacteriol.* **50,** 597–607.

Williamson, D. H. (1976). Packaging and recombination of mitochondrial DNA in vegetatively growing yeast cells. *In* "Genetics, Biogenesis and Bioenergetics of Mitochondria" (W. Bandlow *et al.*, eds.), pp. 117–123. De Gruyter, Berlin.

Williamson, D. H., and Fennell, D. J. (1975). The use of a fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. *Methods Cell Biol.* **12,** 335–351.

Yanagida, M., Hiraoka, Y., Uemura, T., Miyake, S., and Hirano, T. (1986). Control movements in mitosis in fission yeast. *In* "Yeast Cell Biology" (J. Hicks, ed.), pp. 279–297. Liss, New York.

Yoo, B. Y., Calleja, G. B., and Johnson, B. F. (1973). Ultrastructural changes of the fission yeast (*Schizosaccharomyces pombe*) during ascospore formation. *Arch. Microbiol.* **91,** 1–10.

Zickler, D. (1973). Evidence for the presence of DNA in the centrosomal plaques of *Ascobolus*. *Histochemie* **34,** 227–238.

Zickler, D., and Olson, L. W. (1975). The synaptonemal complex and the spindle plaque during meiosis in yeast. *Chromosoma* **50,** 1–23.

9

Morphogenesis of Fission Yeasts

BYRON F. JOHNSON,* MACHIKO MIYATA,† AND HISAO MIYATA‡

*Molecular Genetics Group
Division of Biological Sciences
National Research Council of Canada
Ottawa, Ontario, Canada K1A 0R6

†Gifu Pharmaceutical University
Gifu 502, Japan

‡Department of Biology
Faculty of Science
Nagoya University
Chikusa-Ku, Nagoya 464, Japan

MOLECULAR BIOLOGY OF THE
FISSION YEAST

I. INTRODUCTION

One of the strengths of the fission yeast system is the ease with which one can approach morphogenesis. The disarmingly simple cylindrical shape of the cell and its almost classic binary fission combine to attract the attention of those who would seek out the structure–function relationships that determine cell shape. Accordingly, right from Mitchison's classic paper (1957), those who have worked with fission yeast have had a secure intuitive feeling for the nature of its cell cycle. Such an intuitive feeling for the budding yeast cell cycle was at one time rare, although it is clear that Donald Williamson had it. With the advent of Hartwellian analysis biologists studying budding yeast began to develop a sense of the cell cycle. Clearly, that intuition arose secondarily.

It remains a truism that *Schizosaccharomyces pombe* is the second-best known yeast, both biochemically and genetically. Even so, enough now is known to enable one to elaborate hypotheses and then to devise the experiments to test them. On the other hand, sufficiently little is known so that even the most imaginative list of multiple hypotheses will sometimes be inadequate, and the organism will answer the experimenter with a surprise.

As the shape of a yeast cell is determined for the most part by its wall, experimental questions about morphogenesis become primarily questions about walls. It is worth keeping in mind that walls are elaborated outside the plasmalemma, hence morphogenesis occurs somewhat remote from the usual controls mediated by the nucleus. Questions about controls over morphogenesis tend to receive ambivalent answers. While detailing some answers about morphogenesis, we think it best to place the emphasis on unanswered questions. They abound, and they hold promise of many an experimental adventure in the future.

II. MORPHOMETRIC ANALYSIS

The dimensions of the fission yeast cell (*Schizosaccharomyces pombe*) approximate a cylinder whose diameter is about 3.5 μm and whose length increases from around 7 to 12–15 μm between divisions. Division at approximately the middle of the cylinder generates progeny cells ("sibs," "sisters," or "daughters" in a more common parlance) each having a new end and an old end. According to Mitchison's rule (see below, Section II,C), new extension is thought to be initiated at the older end.

A. Nature of Exponential Extension

Mitchison's classic paper (1957) on the interferometric measurement of dry mass of growing single fission yeast cells included an analysis of the patterns of extension of those cells. The length-increase curves "looked" exponential, and he came to accept them as such (Mitchison and Walker, 1959; Mitchison, 1970). Analysis of the mean length of newly inoculated populations of fission yeasts (Johnson, 1968a) suggested the same conclusion: extension proceeds at an exponential rate. It was also shown autoradiographically (Johnson, 1965a) that the rate of incorporation of ^{3}H from glucose into extending cell walls approximates exponentiality. Later, Streiblová and Wolf (1972) and H. Miyata *et al.* (1978) again drew the conclusion of exponential extension from measurements of the increase of length of individual cells. Exponential extension of fission yeast cells seemed to be accepted.

It was James *et al.* who first suggested that extension might be linear (1975). In spite of their sophisticated data analysis, there was no immediate rush by others to accept the notion of linear extension. They were quoted but, in the main, were ignored with respect to this point. More recently, Mitchison and Nurse (1985) have extensively examined the nature of extensile growth of the fission yeast, and they concluded that extension does proceed by linear steps, with each step followed by a step having higher extensile rate (Fig. 1A). Their data were derived from time-lapse photomicrography and from analysis of fluorescence micrographs. On the other hand, Tyson and Diekmann (1986) produced a curiously effective theory for extensile growth of the fission yeast based at least partly on the "exponential" data of H. Miyata *et al.* (1978). Finally, we too (H. Miyata *et al.*, 1988) have reexamined the growth of a variety of strains of fission yeasts, normal cells, cells of cell cycle mutants having temperature dependency, and cells whose growth was manipulated by the presence of hydroxyurea, with data being derived from time-lapse photomicrography. The assumption basic to both extensive studies (Mitchison and Nurse, 1985; H. Miyata *et al.*, 1988) is that something so fundamental as the nature of extension, that is, whether it is exponential or linear, is not apt to be converted from one to the other by the sorts of experimental manipulations applied. For instance, one mutant studied is a DNA-synthesis mutant, one whose ordinary mutational consequences, no matter how pleiotropic, are not likely to directly affect the nature of extension.

At any rate, despite the technical similarities between the two studies, the analyses were different and yielded interesting differences in interpretation. Mitchison and Nurse (1985) described extension of an "average"

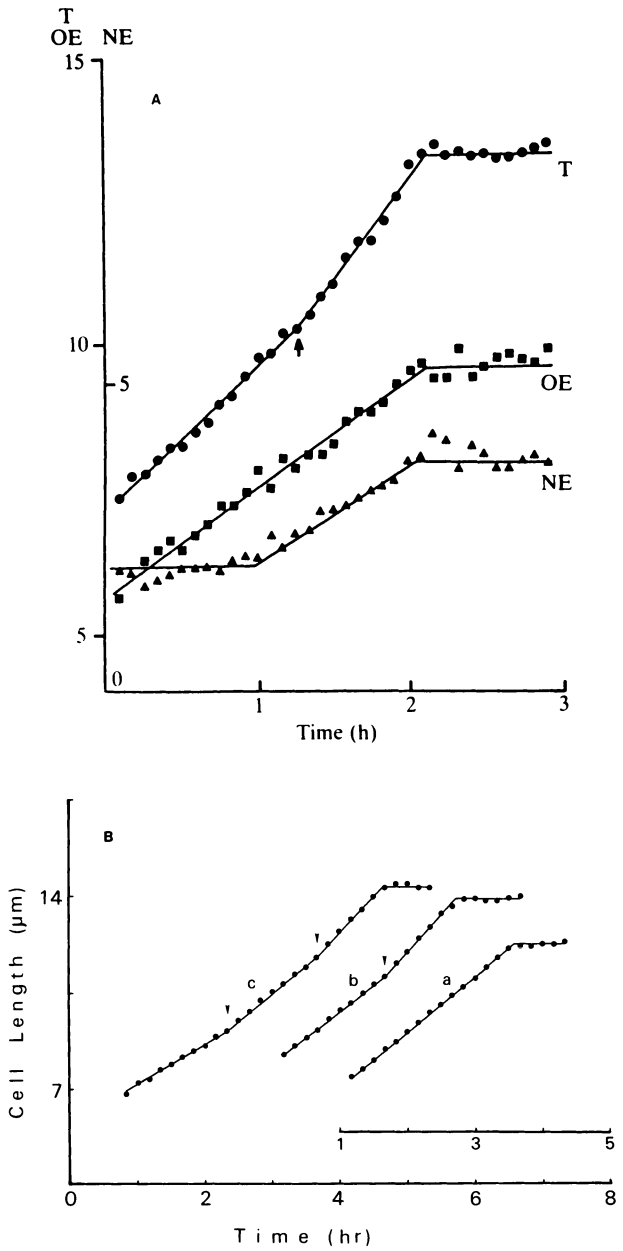


Fig. 1. (A) Growth in length of a single cell of *S. pombe* (strain 972 h^-) at 25°C during one cycle from cleavage to cleavage. The curves represent measurements of total length (T), length from fission scar to old end (OE), and length from fission scar to new end (NE). The arrow marks point of rate change in total length growth. [Adapted with permission from Mitchison and Nurse (1985). *J. Cell Sci.* **75,** 357–376.] (B) Growth in total length of three

cell by examining many time-lapse sequences and then averaging their data. H. Miyata *et al.* (1988) also examined many time-lapse sequences but, rather than averaging, classified their cells according to how many linear steps were found during the observed cell cycle (Fig. 1B). Both studies found linear steps.

Why had exponentiality of extension seemed so real for so long? The question of apparent exponentiality of a linear step system was addressed by H. Miyata *et al.* (1988). By classifying cells according to how many linear steps had characterized their growth during the cycle, and by noting the dispersion of the points between the steps, they readily concluded that any population of cells growing individually by linear steps but expressing as a population all of the various patterns of linear steps, and analyzed as a population, would genuinely seem to be extending exponentially. H. Miyata *et al.* (1988) therefore labeled extension as "pseudoexponential."

B. Complexity of Pseudoexponential Growth

The averaging mode of analysis used by Mitchison and Nurse (1985) generated emphasis on the point between the (typically two) linear steps and the point at which the new end first initiated its own extension. On average, the rate-change point (RCP, their terminology) was found to coincide with the moment of initiation of new-end growth (NETO, new end take-off, their terminology). As a consequence, these points quite naturally were deemed to be important and were discussed as such.

The classifying mode of analysis used by H. Miyata *et al.* (1988), by showing that some wild-type cells had no RCP [only a single linear step (James *et al.*, 1975)] whereas others had one and some had two, and by showing that only rarely did the RCP and NETO of an individual cell coincide, tended to reduce emphasis on the RCP and NETO. Nevertheless, the point at which an individual cell initiates growth at its new end is a significant point in the life cycle of the cell, and the mechanistic basis for that initiation and its placement within the cycle must be considered significant. H. Miyata *et al.* (1988) concluded that the new end initiated growth when the total growth rate arrived at a critical point.

---

cells of *S. pombe* (strain 972 h^-). Exponentially growing fission yeast cells were cultured in Edinburgh minimal medium (EMM3) at 25°C with shaking and were then transferred to EMM3–agar (2%) without temperature change and overlaid with a cover glass for microscopic observation. Cells a, b, and c have one, two, and three linear growth segments in their cycles; they are derived from divisions on the agar. The arrowheads mark points of rate change. The second abscissa relates to cell a. [H. Miyata (unpublished).]

Finally, the facts that some cells violate Mitchison's rule (see Section II,C) and initiate extensile growth at their new end (rather than the old end) right at the beginning of their cycle (H. Miyata *et al.*, 1986) and that some cells grow at both ends from the beginning of the cycle (Johnson, 1965a; H. Miyata *et al.*, 1986; H. Miyata, M. Miyata, and B. Johnson, unpublished) are also significant. Do these facts deny the NETO concept, or do they make NETO terribly important? Perhaps time and further analysis will allow further insight, or even a different insight. In any event, the RCP concept engenders no such ambivalence; the Edinburgh school has produced ample evidence for both its reality and its importance (see Chapter 6 by Mitchison, this volume).

C. Mitchison's Rule

Although Mitchison (1957) emphasized the interferometric and morphometric aspects of analysis he did note, incidentally, that newly divided cells frequently reinitiate extensile growth at the older of the two ends. This phenomenon has been referred to as Mitchison's rule (Calleja *et al.*, 1977). One frequently sees a picture of a fission yeast cell that has followed Mitchison's rule. For instance, cell II in Fig. 1 of Mitchison and Nurse (1985) is an obvious follower of the rule. Nevertheless, one sees enough cells with both ends apparently growing in synchrony to raise doubt about the generality of the rule.

The question was reexamined by H. Miyata *et al.* (1986), using time-lapse photomicrography to analyze growth over three cell cycles (two divisions). Cells plated on agar (according to H. Miyata and M. Miyata, 1981) extend and divide twice to form a linear array of four cells, two of which might be termed "inner" and the other two "outer" from their positions in the array (see Fig. 2 for two such linear arrays). They found (H. Miyata *et al.*, 1986) that outside cells always follow Mitchison's rule. Inside cells might be either monopolar or bipolar. The monopolar cells follow Mitchison's rule if their older end grew even slightly in the previous cycle but might violate Mitchison's rule if that older end did not extend in the previous cycle. Bipolar cells initiate new-end growth simultaneously with initiation of old-end growth. Division products of bipolar cells always follow Mitchison's rule. One may conclude that Mitchison's rule describes the most frequent pattern of cell extension, but that other patterns of extension occur at predictable frequencies (the outside–inside rule). The important conclusion is that sibs most often have different patterns of extension [and different proclivities for sexual interaction (H. Miyata and M. Miyata, 1981)].

To this point, one might consider these sibling differences to be either trivial ("Who cares which end grows?" or "Do you think the yeast cell

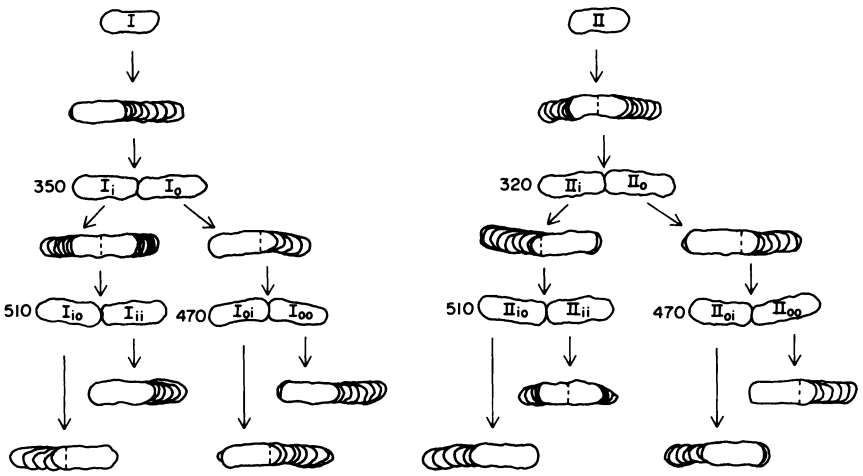


Fig. 2. Growth of cells which were followed by superimposing time-lapse photomicrographs every 20 min. In two cell lines, I cell and II cell, initial cells grew at the one end and at both ends, respectively. o, Outside cell; i, inside cell (see the text). Cells I_o, I_{oo}, I_{io}, I_{ii}, II_i, II_o, II_{oi}, II_{oo}, and II_{io} all follow Mitchison's rule. Cell I_{oi} violates Mitchison's rule. Cells I_i, II, and II_{ii} are all bipolar. By convention, I is stated to be monopolar and following Mitchison's rule. [Adapted with permission from H. Miyata *et al.* (1986). *Can. J. Microbiol.* **32,** 528–530.]

knows which half it is?") or serious ("Sexual interaction is serious business!"), but the bases for opinion are subjective. There is an objective basis for opinion. We have found (M. Miyata, H. Miyata, and B. Johnson, unpublished) that a variety of semilethal shocks (temperature shift, pH shift, and addition of acridine orange) kill fission yeast cells differentially. Raising the pH of the culture medium from 4.5 to 7.0 for 1.5–2 hr kills only a fraction of the cells, but, when recently divided pairs are examined (see Fig. 3), the sib with more scars ("older") is more apt to die than that with fewer.

When chemists and/or physicists begin to study biology, one of the tenets most difficult for them to abandon is the notion of universal identity (all carbon atoms, all phosphorus atoms are identical so all cells, organisms of a type, etc., must also be identical), the notion that nature is everything. However, the different patterns of extension summarized above show that nature versus nurture, the biologists' old conundrum, remains important. No two cells have precisely the same history, and no two have quite the same fates, as seen above.

How unusual is this phenomenon of sibling difference? Is it worrisome? Is it unique, or is there reason to believe that other systems might exhibit comparable sib differences, if looked for? The most obvious comparison

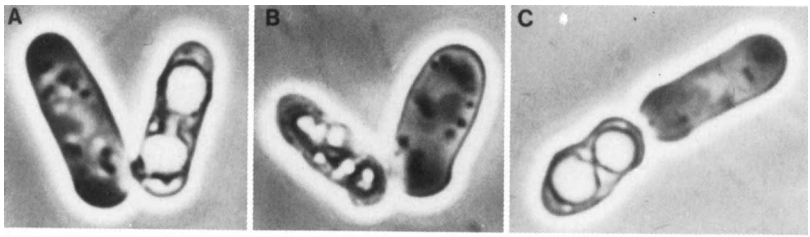


Fig. 3. (A–C) Asymmetric killing of recently divided fission yeast cells after exposure to a semilethal shock. The live and dead cells in each pair are easily recognized. [M. Miyata (unpublished).]

is with budding yeasts, where pronounced differences between mother and daughter cells have long been acknowledged. For instance, daughters usually are smaller than mothers (Johnson and Lu, 1975; Flegel, 1978) and, trivially, carry different scars from the cell division that separates them (Barton, 1950). Daughters are more sensitive to acriflavine (Ephrussi and Hottinguer, 1951) and are more apt to be *petite* in strains characterized by high-frequency spontaneous *petite* generation (Johnson *et al.*, 1978). Also, Hicks and Herskowitz (1976) have shown mother–daughter differences in mating-type gene switching. But, in this context, it is worth noting that only in budding yeasts are sibs so different from each other that they are commonly referred to as mother and daughter rather than as the sibs they are! Hence the question is not, are fission yeasts unique in having these sibling differences, but, rather, are yeasts unique in having these differences? Of course, the obvious answer is that all differentiation and development of higher organisms is based on the sibling products of cell division having different fates. So the phenomenon of sib differences in yeasts is not worrisome. If it seems unusual or unique, it is so because of the ease of identifying and tracing the differences, such as polarity of extension and sexual proclivity, and because of the ease with which the molecular basis of some of these obvious differences, such as mating-type gene switching, can be elucidated (Wilson and Herskowitz, 1986; Egel and Eie, 1987; see also Chapter 2 by Egel, this volume).

D. The "Classic" Cycle: A Second and Third Look

The fission yeast extends through the first three-quarters of its cycle and then has a constant-length stage for about one-quarter of the cycle (see Fig. 1; Mitchison, 1957). We have dealt above (Sections II,A and II,B) with the pseudoexponential or linear-step nature of the extension

portion. Mitosis and cell division occur during the constant-length portion.

After one invests the intense intellectual and physical effort required to gather the data in such a demanding, pioneering experiment as that of Mitchison (1957), one naturally hopes that his resultant curves not only will stand as classic but will be confirmed by any and all following experiments. The hope, then, is that no matter how the cell is cultured, it will grow just as it was first shown to grow: the original pattern is the pattern, and not just one pattern among many, not even just one pattern in a family of patterns. The discussion to this point suggests the fragility of such hopes, and, surely, one should expect that biological adaptability per se would be characterized by different growth responses to different cultural conditions.

The first serious reexamination of the pattern occurred in Mitchison's own laboratory, where Johnson (1968a) found that the mean length of the fission yeast cell (strain NCYC 132) declines through the log phase of a batch culture. The obvious implication was that the slope of the pattern must decline from cycle to cycle, given that the cycles were of constant length. Furthermore, it was found that the division index also declines [those data were scanty, but the decline has been confirmed (B. Johnson, unpublished)]. If the division index (percentage of cells in a recognizable stage of division) declines, then the constant-length stage of the cell cycle (1) must be associated with something other than cell division or (2) must itself be shortening, from cycle to cycle, through the log phase. The second alternative was held to be the more likely (Johnson, 1968a), allowing the consequent conclusion that the ratio of extension portion to constant-length portion, 3 : 1 in the analysis of Mitchison (1957; confirmed by Streiblová and Wolf, 1972; and again by H. Miyata, M. Miyata, and B. Johnson, unpublished), must decline in a batch culture commensurate with the decline in division index. Thus, for two reasons the slope of the extensive portion of the cell cycle is predicted to decline through the log phase. Although no one has experimentally confirmed the prediction of this second look, it seems reasonable to believe that the original (Mitchison, 1957) cell cycle pattern is but one of a family of similar curves.

A more direct look, the third look, came from the laboratory of Herbert Kubitschek. His interest was stimulated by the discovery (Kubitschek and Ward, 1985) that the buoyant density of fission yeast cells is constant, independent of either growth rate or cell size, thus independent of cell age, that is, progress through the cell cycle. This result was contrary to the conclusion of Mitchison (1957) who had derived cell density as the ratio of dry mass to cell volume and had shown that it varies in a regular and rationalizable way.

If the conundrum was obvious, its solution was not. But where two sets of conclusions from two good laboratories are incompatible, it seems more likely that some basic assumption is wrong rather than that someone has not gathered the data correctly. Of course, Kubitschek did not question his own assumptions but was concerned with the osmolality of the agar medium in which cells had been suspended by Mitchison. Its osmolality had been high, perhaps inordinately high. Could it have influenced growth conditions in such a way that the cell densities were affected?

The effect of osmolality on growth of the fission yeast was examined (Kubitschek and Clay, 1986), and, in the course of examination, a different pattern of growth, referred to as "linear" by Kubitschek and Clay, was discovered. "Linear" in this circumstance does not mean "linear growth as opposed to exponential growth" (see Section II,B) but rather, "linear growth without the constant-volume stage." Because they set out to look for an effect of higher versus lower osmolality on the growth patterns, it was natural that they should interpret the data in that context. However, there are some problems whose answers are not immediately obvious. In the first place, they used three plating media: (1) yeast extract–peptone–dextrose (YEPD) with 4% agar, osmolality 550 mmol/kg; (2) Edinburgh minimal medium (EMM) with 3% agar, osmolality 400 mmol/kg; and (3) YEPD with 1.6% agar, osmolality 420 mmol/kg.

The growth of 60 cells was monitored, 18 on medium (1), 24 on medium (2), and 18 on medium (3), and the growth pattern of each cell was eventually tabulated as "linear" or "biphasic," the latter meaning the classic pattern of Mitchison (1957). All ($\frac{18}{18}$) of the cells growing on medium (1), the medium with the highest osmolality (550), were biphasic. Lowering the osmolality generated some linear patterns, but not in proportion with the decrease in osmolality: $\frac{11}{18}$ (61%) were linear on medium (3) (osmolality, 420), but only $\frac{6}{24}$ (25%) were linear on medium (2) with the lowest osmolality (400 mmol/kg). Note that if osmolality were the whole story, there would have been more linear patterns, rather than fewer, on medium (2). Kubitschek and Clay (1986) did not discuss the anomaly; it is as though they blended the two results and made comparisons only between experiments with none linear and experiments with some linear. The samples were small but the labor intense. Mitchison and Nurse reported on 53 cells, and Streiblová and Wolf on 75; hence perhaps Kubitschek and Clay cannot be faulted for conclusions based on the study of 60 cells.

There was another interesting anomaly. Kubitschek and Clay (1986) found some alternation of generations in expression of the linear-growth pattern. They discussed in terms of mothers and daughters the transition from biphasic to linear, 5 examples; linear to biphasic, 5; and unchanged, 18. This phenomenon of switching back and forth is reminiscent of the outside–inside rule (H. Miyata *et al.*, 1986) discussed in Section II,C.

However, the mother–daughter terminology is ambiguous, and one cannot decide what pattern of switching is being described. For instance, most biologists would discuss cell division in terms of a mother cell generating two daughter cells. If that convention applies, then five biphasic cells generated ten linear cells (sisters were identical even if different from their mothers!), and the converse, with 18 generating 18 pairs of daughters the same as themselves. On the other hand, if Kubitschek and Clay were applying the budding yeast convention (criticized above, see Section II,C), then the progeny of five biphasic cells comprised five biphasics and five linears, etc., and we are left to wonder which one was mother in the first place. Because of these ambiguities, we cannot immediately decide what has happened, nor can we decide if comparison with the outside–inside rule is apt. For the moment, we will accept the interpretation of Kubitschek and Clay (1986), that higher osmolality directs the biphasic (classic Mitchison) pattern and that lower osmolality directs some complex interchange between biphasic and linear patterns.

[A personal communication from H. Kubitschek suggests that perturbation of the organism if more apt (than osmolality) to cause the switch from linear to biphasic. What causes the switch from biphasic to linear is less obvious. Why all of the cells cultured at high osmolality should always have been perturbed is not obvious either.]

This approach and these results are intriguing. One assumes that Kubitschek and Clay (1986) did these experiments with the expectation that they would shed light on why the densities measured in their laboratory were invariant and why the densities measured in Edinburgh varied. They made a new, unexpected, and possibly important observation, but did not return to the original question: did the heightened osmolality in Mitchison's experiments lead to the varied densities? One would expect that, having discovered such an interesting effect related to the osmolality of the medium, they would immediately have grown fission yeasts on a high-osmolality liquid medium and then tested to see if the buoyant density might now be variable. We await the results of such an experiment. [H. Kubitschek (personal communication) now believes that cells grown biphasically might have variable density.]

Kubitschek and Clay (1986) have shown us 4 of their 17 linear curves (their Fig. 3). We assume that these four show the least biphasic character, but each of the four shows a last point at or near the same value as the previous point; they seem biphasic too, just less so than those described as biphasic. Finally, Kubitschek and Clay (1986) measured cell diameters, but we delay discussion of those data until Section II,E.

We conclude that the classic cellular growth curve of Mitchison (1957) is the typical curve in a family of curves, most or all of which bear obvious resemblence to the type curve. If we note that the volume data (Mitchi-

son, 1957) were gathered as length data (and merely converted arithmetically to volume), then we can say with confidence that even the 17 length curves (Kubitschek and Clay, 1986) depicting linear growth belong in the same family of curves. The proportion of extensile stage to constant-length stage may vary as a function of osmolality [does this explain the observations of Johnson (1968a) as well?] and other factors not yet assessed, but, in the main, the cell grows much as it was first shown to grow. The second and third looks have had more of a confirmatory nature than a contrasting nature. What will the fourth look show?

E. Cylindrical Diameter: Assumptions and Realities

We mentioned in Section II,D that the volume curves of Mitchison (1957) were collected as length data and converted to volumes arithmetically. It was assumed that the cell "grows only in length, so the increase in volume of a growing cell is directly proportional to the increase in length." We have mentioned elsewhere (Johnson and Lu, 1975) the apparent reasonableness of this assumption for those experiments, even though showing that the cylindrical diameter of a fission yeast cell increases as a linear function of the number of fission scars.

By analogy with the late cell cycle increase in volume of the budding yeast (Johnson, 1965b), and by inspection, we (Johnson and Lu, 1975) decided that the increase in cylindrical diameter of the fission yeast cell occurs late in the cell cycle; this is essentially another assumption. To establish the timing of the increase with precision did not seem to merit the work required. However, this complacency was shattered by a report (Kubitschek and Ward, 1985) of constant cell density in the fission yeast, so different from the curves of Mitchison (1957) showing variable density. Could a late cell cycle increase in cylindrical diameter account for the difference? In other words, was the problem based on Mitchison's assumption (1957) of constant diameter? Much of the variability (Mitchison, 1957) was based on that assumption of constant diameter, hence constant volume through the constant-length stage. New experiments were clearly needed. These were provided by Kubitschek and Clay (1986) and by our laboratory (B. Johnson, M. Miyata, and H. Miyata, unpublished).

Kutbitschek and Clay (1986) found two patterns of volume increase of fission yeast cells, the biphasic and the linear, as described above. Each seemed to have its own characteristic behavior of the diameter. According to them, the biphasic cells had a diameter that was constant or near-constant ["cell widths were constant throughout the cell cycle or quickly became so after minor adjustments early in the cycle" (Kubitschek and

Clay, 1986)] through most of the cell cycle, whereas the linearly growing cells increased constantly. The finding of an apparently constant cylindrical diameter in the biphasic cells did not perturb Kubitschek and Clay because they believed that the biphasic cells were more or less atypical. However, one of us (H. Miyata) has measured approximately 400 cells through their cell cycle and has always found the typical Mitchison curve [biphasic in the terminology of Kubitschek and Clay (1986)]. Furthermore, we have examined the length distributions of batch and chemostat cultures at a variety of dilution rates and in most cases found distributions appropriate for the Mitchisonian curve (H. Miyata, M. Miyata, and B. Johnson, unpublished). We do not believe that these biphasic cells are atypical. Hence we believe that their cylindrical diameter bears closer examination.

Kubitschek and Clay (1986) show data for the growth in width of four cells. Cell A showed no increase; B, almost 6%; C, almost 5%; and D, almost 3%. Those three cells shown to increase in width probably were increasing at the time of the first measurement, hence the estimates of increase must be considered to perhaps err on the small side, thus be minimal. Any rise in the estimates puts them in the same neighborhood as 10% (established by Johnson and Lu, 1975); the differences between these estimates may be small enough to neglect. However, neither this early cell cycle increase of the biphasic cells nor the through the cell cycle increase of the linear cells can explain the difference in the density measurement. We too have examined the cylindrical diameter through the cell cycle by a different method and have concluded that the increase is more or less constant through the cycle (B. Johnson, M. Miyata, and H. Miyata, unpublished). We (Johnson and Lu, 1975) have shown that the increase is probably not accretive in the fission yeast, and it is probably not in the budding yeast. The increase in diameter is a morphogenetic reality, one that proceeds by unknown, undetermined mechanisms.

The immediate interest in the width increases was stimulated by the difference between Mitchison's variable densities (1957) and Kubitschek and Ward's constant densities (1985). For the moment, we accept (H. E. Kubitschek, personal communication) that the biphasic (classic Mitchison) cells might have variable density, but would like to see the point demonstrated experimentally.

It is obvious that some of the early, apparently reasonable assumptions about the cylindrical diameter of the fission yeast cell [constant (Mitchison, 1957); increases, that increase probably late in the cell cycle (Johnson and Lu, 1975)] have been wrong. The consequences of those erroneous assumptions have not been dire because the adjustments to our understanding or morphogenesis have not been great. It is worth noting

that those who make their experimental way in science by testing critically the assumptions of their forebears must also make assumptions. These too will be tested if they become crucial. Nothing in science is carved in stone.

III. MECHANISM OF EXTENSILE GROWTH

No one knows with confidence how yeast cells extend their walls. Their walls are similar in principle to those of plant cells: both have structural polysaccharide molecules embedded in a matrix, much like reinforcing rods in concrete. However, while the dominant structural polysaccharide in plant cell walls is cellulose, a straight-chain, β-1,4-linked glucan, the dominant structural polysaccharide in yeast cell walls is a branched β-1,3- and β-1,6-linked glucan.

The branching nature of the structural polysaccharides probably precludes any notion of biosynthesis via a classic "softening" mechanism in which the matrix is hydrolyzed to weakness and that weakness allows a sliding response of the structural molecules to the turgor pressure. Such a paradigm as this was and basically still is accepted by most physiologists who study plant cells and the growth of their walls (Morré and Eisinger, 1968; Green, 1969; Ray, 1969; see Masuda and Yamamoto, 1985, for a recent review). One must assume that the same paradigm was in the minds of contemporary fungal physiologists (Aronson, 1965; Robertson, 1965; Park and Robinson, 1966) who considered the problem of apical growth of hyphae and discussed that growth using the "softening" terminology of plant physiologists.

In a seminal paper published in 1965, Megnet showed that when fission yeast cells are cultured in the presence of 2-deoxyglucose (2DG), they lyse. Furthermore, he showed that they lyse primarily at the tips of the cells shown by autoradiography (Johnson, 1965a) to be the sites of extension-associated glucan synthesis. Later it was shown (Johnson and Rupert, 1967; Johnson, 1968b,c) that the rate of killing by 2DG-induced lysis occurs strictly in proportion to the mean length of the fission yeast cell population. Because of the pseudoexponential nature of extension (Section II,B), this meant that, on average, the longer the cell the faster it would be extending. The implication was that the rate of initiation of lysis was a function of the rate of cellular extension. Furthermore, because 2DG is so innocuous a compound that it did not seem logical that it might be causing lysis directly (like lysozyme on gram-positive bacteria), an indirect cause for the lysis was sought. In addition, the tough nature of the branched glucan structural polysaccharide in the wall seemed to demand the participation of a glucanase for the lysis. Altogether, it seemed (John-

son, 1968b) that the 2DG-induced lysis must be happening because the normal mechanism for extension was being confounded by the assimilated 2DG. But as the older paradigm made little sense in these circumstances, a new paradigm was generated (Johnson, 1968b).

According to the new paradigm, the structural glucan of the yeast cell wall (and hyphal walls of fungi, of course) grows by covalent insertion of glucose or oligoglucan into cuts made endohydrolytically in the preexisting glucan molecules. Turgor pressure stretches the weakened wall, making space for the insertion of glucose or oligoglucan. [We are not concerned here with the details of 2DG experiments, except to note that the 2DG-activated endohydrolases remain active and continue to degrade the glucan, even degrading glucan that had been present before addition of the 2DG (Johnson, 1968c).] It is relevant that Barras (1969, 1972) was able to show that the cell walls of the fission yeast contain the imputed glucan endohydrolase, an observation confirmed by Fleet and Phaff (1975).

The new paradigm has had two more models applied. In their application, Bartnicki-Garcia and Lippman (1972) and Gooday (1977) emphasized growth of hyphae and were more or less explicit that chitin was the relevant structural molecule. The model of Bartnicki-Garcia and Lippman (1972) encompassed a "delicate balance" between biosynthesis and autolysis. Although this notion of balance has become more or less accepted by many reviewers, it is one of the reasons that Wessels (1986) prefers the older paradigm. We too believe that nature abhors such a delicate balance, and that whenever possible, synthetic capacity must far exceed autolytic capacity in such a growing system. An excess of biosynthetic capacity removes some of the potential for suicide otherwise intrinsic to the paradigm. While the newer paradigm, as originally proposed for yeasts (Johnson, 1968b) and later applied to molds (Bartnicki-Garcia and Lippman, 1972), was based on observations of experimentally induced suicidal lysis of growing cells, there seems little point in making the potential for suicide a predominant element. Accordingly, the notion of a delicate balance is retained as intrinsic to the specific model of Barnicki-Garcia and Lippman under the paradigm, but we consider the notion to be irrelevant to the paradigm.

Wessels (1986; Wessels and Sietsma, 1981) also believes that lytic enzymes are needed only to initiate growth that later becomes apical (and once apical, can continue without lytic enzymes). From that particular conjectural framework, one can make some predictions about what 2DG should do when added to various fission yeast systems. Thus, there should be a burst of lysis as the cells initiate growth at inoculation, a lysis-free period as the cells continue to grow without benefit of the lytic enzymes, and then another burst of lysis at the beginning of the second cycle when new ends initiate or old ends reinitiate extension. A secondary

prediction is that the shortest cells would lyse, and the longest would be immune to lysis until division or immediately thereafter. The facts are quite contrary to prediction: on average, the shorter the cell population, the less sensitive to 2DG (Johnson and Rupert, 1967), and when a population of log-phase lysing cells is measured, it is the longer cells that are lysed (Johnson *et al.*, 1974a). An assumption basic to these predictions is that 2DG-induced lysis bears some direct relationship to growth and activation of lytic enzymes, hardly an onerous assumption, but untested.

Another lytic agent of interest is aculeacin A (Acu), known to inhibit β-glucan synthesis in fission yeast cell walls (M. Miyata *et al.*, 1980, 1985). Three lytic effects of Acu are of interest. (1) Lysis at the tips of growing fission yeast cells looked about the same whether 2DG or Acu was the lytic agent. (2) Two different patterns of lysis at the septum occurred, depending on which lytic agent was applied. Whether these differences are ascribable to differential penetration of the two lytic agents or to dramatically different modes of inducing lysis remains to be determined. (3) Lysis induced by Acu is a function of cell length with the longer cells (hence faster growing) being the more susceptible (see Table I), just as was shown for 2DG-induced lysis (Johnson *et al.*, 1974a) discussed above. For the moment we do not understand how these results bear on either current paradigm, but we believe that any final theory of wall extension must take them into account, hence we include them here.

It is worth noting that Wessels and Sietsma (1981; Wessels, 1986) produce significant arguments in favor of the older paradigm, but no one has

TABLE I

Length Dependence of Aculeacin A-Induced Lysis of Fission Yeast Cells[a]

Cell density (cells/ml)	Initial mean cell length (μm)	Control, final mean cell length (μm)	Plus Acu (1 μg/ml), % lysis
5.0×10^7	11.7	12.3	7
1.6×10^7	13.0	19.0	10
1.0×10^6	13.2	23.0	73

[a]Logarithmic-phase cells of *S. pombe* (strain *cdc10-129*) incubated at 25°C for various times were transferred to fresh EMM2 at 35°C and incubated further for 6 hr. All cell divisions stopped after 3 hr; at that time, counts of initial cell density, measurements of initial cell length, and additions of aculeacin A were done. At the sixth hour, measurements of final mean cell length and tabulations of lysed cells were done. $N = 100$. [From M. Miyata *et al.* (1985).]

performed a critical experiment that can convincingly exclude once and for all one or the other paradigm. A careful analysis, perhaps by some autoradiographic means, of the incorporation of new glucose into the middle or onto the ends of preexisting molecules, needs to be performed.

Finally, it has been convenient to idealize the wall here, treating it as being more or less uniform through its entire thickness. That was a modeling convenience, however, and should not blind one to the fact that the wall is more complex than that. Selective hydrolysis by different enzymes shows (M. Miyata *et al.*, 1985) that the structural elements in the inner regions of the wall are probably α-glucan, with β-glucans predominant as structural elements in the outer regions of the wall. For the moment, we do not see those observations as being contradictory to the notion (discussed in detail by Robinow and Johnson, 1989) that the walls of yeasts are not intrinsically organized in layers.

IV. CELL DIVISION: THE OTHER DETERMINANT OF MORPHOGENESIS

If a cell resembles a sausage, and grows mostly at its ends and divides at or near its middle, then all cells in a population of such sausages should look much like one another. How closely they seem to resemble each other is a function of the perceptiveness of the beholder: friends of identical twins see the differences, casual acquaintances see only the overwhelming similarities. Hence, perception is enhanced by extended acquaintance. The problem for fission yeasts is posed (Fig. 4). Are these cells all identical or do they differ? Examined closely, each of the 10 cells in Fig. 4 can be seen to have its own character. Those characters are mostly differences in cell length, a function of passage through the cell cycle, and abrupt differences in diameter at the fission scars (Johnson and Lu, 1975), a function of cell division. Only 2 of the 10 cells have precisely the same distribution of fission scars; others with identical numbers of scars have different dispositions of cylindrical wall among them. Where these sausages seem to differ, most of the difference is division related.

Cell division occurs in two phases, the elaboration of the septum and fission of the septum. Overall, the process has been reviewed in some depth and relatively recently (Streiblová, 1981; Nurse, 1981; Johnson *et al.*, 1982; Robinow and Johnson, 1989). Accordingly, a framework adequate for discussion and recent developments only are presented.

A. Symmetry and Asymmetry

We shall see below that the elaboration of the septum proceeds with simple symmetry. As a consequence for the new ends formed at the site of

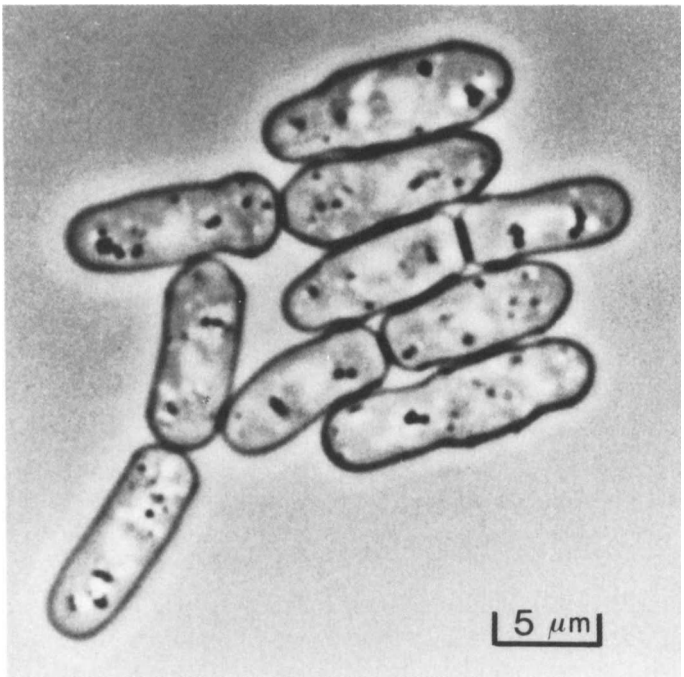


Fig. 4. Phase-contrast photomicrograph of living fission yeast cells (strain NCYC 132) taken from a log-phase culture grown in 2% Malt Extract Broth (Oxoid, Basingstoke, England). The refractive index of the medium was increased by adding Ficoll to 25%. Several cells are dividing. One cell has finished mitosis, but shows no definitive signs of septation. The differences among the cells are small, but sufficient to allow each of the 10 its own identity—each has had a unique morphogenesis. Bar, 5 μm. [Reprinted with permission from Johnson *et al.* (1982). *Int. Rev. Cytol.* **75,** 167–208.]

division, each consists of a scar and a half-septum and each is the perfect mirror image of the other. While the discussion of placement of the septum necessarily emphasizes those conditions that predicate asymmetry of volume of the sibs, we emphasize that those conditions are extraordinary, and that the usual conditions of culturing do lead to symmetry, that is, equal volumes of the sibs resulting from a division.

These symmetries lead to a powerful advantage the fission yeast cell has over the budding yeast cell. The experimenter on cell division of fission yeasts knows that the cells have these symmetries whereas the experimenter on cell division of budding yeasts hopes that the asymmetries will not confuse the results.

1. Septum and Its Elaboration

The septum of the dividing fission yeast cell is elaborated symmetrically with respect to the sibs it will generate. That is, there is no discernible "sidedness" about it: an imaginary probe passing through the developing septum from sib A to sib B, parallel to the cell's long axis, would pass through the same layers arranged in the same ways and thicknesses as it would if it were to pass in the opposite direction. This is true at any stage of cell division. In contrast, the septum of the budding yeast *Saccharomyces* is quite asymmetric and has a real sidedness: these asymmetries give rise to the differences in bud scar on the mother and birth scar on the daughter (Barton, 1950) noted in Section II,C.

The septum of the fission yeast is elaborated in two phases, with the first phase consisting of the centripetal growth (Johnson *et al.*, 1973, 1974b) of a β-glucan structure (Horisberger and Rouvet-Vauthey, 1985), the primary septum (Johnson *et al.*, 1977). This primary septum is initiated as a thin ring circumscribing the inner surface of the old cylindrical wall at about the midpoint. Its centripetal growth bisects the cell with an electron-transparent, Calcofluor-positive (hence, very bright in the fluorescence microscope) disk. The second phase, elaboration of the secondary septa, is initiated before the final centripetal closure of the primary septum is completed. The secondary septa have electron density comparable to that of the old cylindrical wall and do not react with Calcofluor. The secondary septa begin apposed to the primary septum, and they, too, grow centripetally from their origins as rings on the old cylindrical wall. The mature septum is a three-layered object then, with the secondary septa separated by the electron-transparent primary septum.

Fission, the second phase of cell division, proceeds first by endohydrolytic erosion of the old cylindrical wall precisely in line with the primary septum. Ordinarily, the primary septum is hydrolyzed immediately following the cylindrical wall erosion. This leaves the secondary septa as the new ends of the just-generated sibs. These new ends assume a hemispherical shape under turgor pressure. We noted above that the secondary septa do not react with Calcofluor, so the resultant new ends appear dark in the fluorescence microscope.

The fission sequence is not always followed in the above order, and sometimes one can recognize, in the fluorescence microscope, the primary septum eroding while the old cell wall remains unhydrolyzed (B. F. Johnson, unpublished). We interpret this to mean that the enzyme system responsible for hydrolyzing the primary septum is different from that which erodes the old cell wall. It also means that the usual erosive sequence is not obligatorily ordered.

2. *Placement of Septum*

We have discussed in Section II,C how more than one-half of the cells in a population follow Mitchison's rule, hence extend mostly at their older end. When such cells have extended to division size, they have a scarred "fat" end and an unscarred "thin" end, the older end (see Fig. 4). The scarred region is fat as a consequence of the nonaccretional, stretching growth discussed in Section II,E (Johnson and Lu, 1975). Logically, a cell having such a shape cannot simultaneously divide symmetrically with respect to both length and volume.

The logic has been examined and reexamined, with interesting consequences. First, it was shown that cells of strain NCYC 132 growing in batch culture divide symmetrically with respect to volume and asymmetrically with respect to length: there is a long sib and a short sib produced at most cell divisions (Johnson *et al.*, 1979), but their volumes are ostensibly equal. In retrospect, this was the "logical" answer, and it was found to apply in the circumstance of nonstressed growth (e.g., Malt Extract Broth, log phase, optimum temperature).

One of the interesting ways to stress growth is with a chemostat. Vraná (1983a,b) has shown that with excess substrate, cells of strain 972 h^- divide as discussed above, logically and symmetrically with respect to volume. However, Vraná found that as the system was stressed by lowering the dilution rate *(D),* the septum was placed ever more asymmetrically. Thus division produced sibs asymmetric with respect to both volume and length. Volume ratios of 4 : 3 were seen at $D = 0.03$/hr. The most obvious conclusion is that logic means little in this game. However, it was not at all obvious in 1983 why the septum was being so asymmetrically placed in these slowly growing fission yeast cells.

The problem of volume asymmetry was readdressed by M. Miyata *et al.* (1986a). Vraná (1983a) had noted qualitatively that the volume asymmetry was associated with changed proportions of the fission yeast cell—so changed that Vraná calculated volumes of the slowly growing cells as though they were ellipsoids of revolution rather than as round-ended cylinders! It had been noted (M. Miyata *et al.*, 1980, 1985) that fission yeast cells grown in the presence of an antifungal antibiotic, aculeacin A, develop round-bottomed flask (RBF)-like shapes, thus are even more aberrant in form than Vraná's ellipsoids of revolution. After these RBF-like cells were transferred to medium without aculeacin A and were allowed to divide, they divided at the boundary plane between the spheroidal and the cylindrical regions (Fig. 5) (M. Miyata *et al.*, 1986a). The mean volume ratio was 1.94. From extensive quantitative comparison, they concluded that the more the morphology deviated from the cylindrical form, the greater was the degree of volume asymmetry.

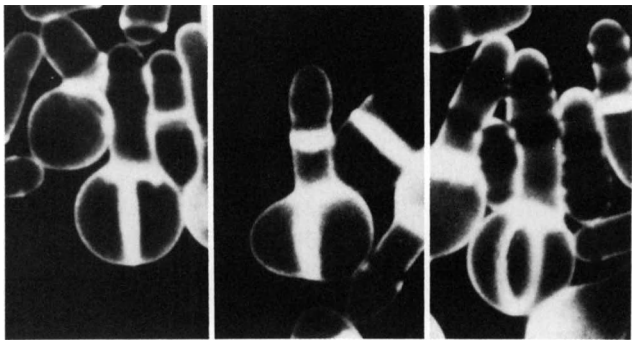


Fig. 5. Cell division in an RBF-like fission yeast cell treated with aculeacin A and then returned to normal medium. Note that the second septum intersects the first in each example. [M. Miyata (unpublished).]

3. Orientation of Septum

One can examine many dividing fission yeast cells before seeing a septum whose orientation demands attention. Whenever long cylinders become two short cylinders so uniformly, one rarely wonders what rules might apply. Is the septum laid down at right angles to the old cylindrical wall? Is it perpendicular to the long axis of the dividing cell? Can it originate on cylindrical wall derived from the secondary septum of an earlier division? Are old fission scars ever bisected? Bisected fission scars on germinating spores were discussed earlier (Johnson *et al.*, 1982) as rare violations of some rule forbidding new septa from forming on the remnants of old septa. In hindsight, the rule merely reflects the regularity imposed by the usual cylindrical shape. When growth circumstances generate an unusual shape, one begins to see unusual orientations of the septum. More than one-half ($\frac{250}{470}$) the spheroidal cells produced by division of the RBF-like cells (Section IV,A,2; M. Miyata *et al.*, 1986b) had septa perpendicular to the previous septum (see Fig. 5). This was such a large sample that the authors readily concluded that the perpendicular septum was entirely within the rules, that is, entirely normal considering the shape of the cell. In addition, cell length to septum diameter ratios were calculated for a variety of regular, hence identifiable, noncylindrical fission yeast cells (580 in total); those ratios equalled or exceeded unity in every case. This allowed the deduction that dividing fission yeast cells with unusual shape identify by some means their longer axis and then place their new septum transverse to that longer axis (M. Miyata *et al.*, 1986a). The obvious final conclusion is that fission yeast cells with normal proportions orient their septum in the same way.

B. Biochemistry of Cell Division

Cell cycle control over division of fission yeast cells has been carefully worked out in the laboratory of Mitchison and was reviewed in some detail (Nurse, 1981). A speculative model for the mechanism of cell division per se has been published (Johnson *et al.*, 1977), but little more can be added. The model has not been tested in any way.

Physiological conditions can impede the fission process while allowing growth to occur (Johnson and McDonald, 1983). The consequence when those conditions pertain is a multiseptate and branched form of the fission yeast, a form that comes to dominate in chemostat culture. Mutants having much the same phenotype may be found (B. Johnson, B. Yoo, and I. McDonald, unpublished) and were discussed earlier (Johnson *et al.*, 1982). The mutant gene segregates normally with the wild-type gene (B. Johnson, I. McDonald, J. Erratt, and A. Nasim, unpublished). The gene must encode a glucanase, probably an endoglucanase, but it cannot be one of the glucanases involved with extension, because extension proceeds normally in the mutant.

The observation noted in Section IV,A,1 that the primary septum can sometimes be seen to break down before the old cylindrical wall has eroded suggests very strongly that the primary septum is removed via an enzymatic reaction rather than that it merely "falls away and leaves a dark division scar" (Mitchison and Nurse, 1985). At the moment, there seems little reason for implicating a physical mechanism for fission, quite contrary to the situation for *Saccharomyces*. Barton (1950) showed by light microscopy that the *Saccharomyces* birth scar is much larger than the bud scar, and Gay and Martin have shown the same with an elegant electron micrograph (1971, Fig. 15 therein). It seems obvious that in the budding yeast, there is ample opportunity for a tearing separation, perhaps even a shearing, when the one side stretches more than the other at scission.

C. Fission Scars

1. Nature and Origin

All yeasts bear scars as trophies of past divisional events. Some authors refer to these as division scars, but fission of the fission yeast cell is rather specific, even the generic name was so derived (Lindner, 1893). Accordingly, we prefer to refer to the scars as fission scars. The fission scar terminology is peculiarly appropriate, we think, because the first vestiges of the fission scar appear as a consequence of fission itself, the autolytic cutting of the old cylindrical wall (Section IV,A,1).

Cell division in the fission yeast has been examined, mostly retrospectively, via a classical analysis of its product, the fission scar, using electron microscopy and the then new technique of fluorescence microscopy of primuline-stained yeast cells (Streiblová *et al.*, 1966). The thin sections of the wall (see Fig. 6) show, among other things, the following: (1) the layering of the wall at the scar; (2) the raised edge of the scar; (3) the fuscannel, an electron-dense ring externally apposed to the cylindrical wall, proximal to its raised edge (sparsely discussed by Streiblová *et al.*, 1966; named and its ontogeny discussed in detail by Johnson *et al.*, 1973); (4) the scar plug, the new end of the cell derived from the secondary septum; (5) the plug wall band, a region of secondary septum-derived wall remaining recognizable after outgrowth of the scar plug; (6) the fact that when the cylindrical diameter of the cell increases (Section II,E), the old scars increase even more, always becoming the broadest region of the cell. Scarcely more is known today about the nature of the scar itself. The layering (1) occurs because the secondary septa grow to underlie a portion of the old cylindrical wall. The raised edge (2) occurs at fission with the endohydrolysis of the old cylindrical wall. Its outward curvature suggests that the outside of the wall is under more tension than the inside.

The scar is the weakest portion of the wall, perhaps even weaker than the extending end. This was illustrated by Mitchison (1970, Fig. 2 therein), who prepared cell wall "ghosts" by smashing cells in a Mickle disintegrator. It was found that most of the breaks were at or proximal to a fission scar. The scars are also the portion of the wall most susceptible to attack by alkali (Johnson *et al.*, 1982, Fig. 12 therein; M. Miyata *et al.*, 1985, Fig. 4b therein).

2. *Frequency of Fission Scars*

a. Tabulations. Any sort of tabulation of fission scars is apt to be fraught with error. Some forms of microscopy do not allow easy visualization of the scars, some do not allow discrimination between adjoining scars, and the sectioning artifacts generated during preparation for electron microscopy might yield low estimates. Fortunately, there is a ready check on overall accuracy: each division generates one new cell and two new scars. Hence there should be a mean number of 2 scars/cell in any sizeable population [this relationship was derived mathematically by Calleja *et al.* (1980) and by Hamada (1982), but it is no more true than when obtained simply, as above]. No significant tabulations have appeared since the subject was last reviewed (Johnson *et al.*, 1982).

b. Theoretical Approaches. In 1980, Calleja *et al.* presented several analyses of fission scars as permanent records. Their fundamental as-

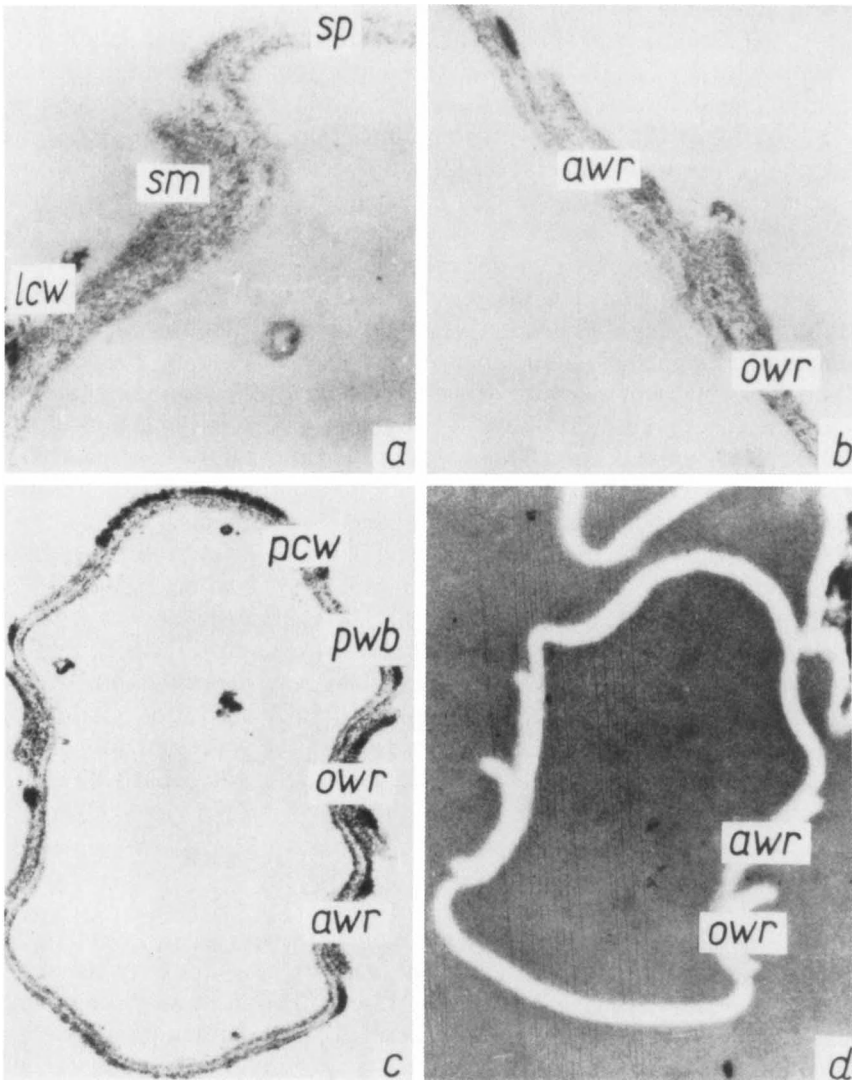


Fig. 6. Ultrathin sections through a cell wall of *S. pombe*. (a) Longitudinal section through a division scar. lcw, Lateral cell wall; sm, scar margin; sp, scar plug. The boundary between the scar margin and the plug is well marked. Fixed with $KMnO_4$. ×38,720. (b) Section through a part of the lateral wall. owr, Original wall ring partially visible; awr, additional wall ring. Fixed with $KMnO_4$. ×38,720. (c) Longitudinal section through an isolated wall of a three-scar cell. pcw, Polar cell wall; pwb, plug wall band; owr, original wall ring; awr, additional wall ring. Fixed with $KMnO_4$. ×19,360. (d) Longitudinal section through an isolated cell wall of a three-scar cell. awr, Additional wall ring; owr, original wall ring. Fixed with OsO_4. ×19,360. [Reprinted with permission from Streiblová *et al.* (1966). *J. Bacteriol.* **91,** 428–435.]

sumption was that the fission rate of a cell is independent of the number of scars borne by the cell. That analysis of probabilities has been expanded by Hamada (1982) to cope with the situation in which the fission rate of a cell is scar-class dependent. However, Hamada felt that the data (598 cells tabulated) accumulated by Calleja *et al.* (1980) were inadequate to test his conclusion, and no one has since ventured to generate a larger sample. Unfortunately, Hamada did not indicate how large a sample might satisfy the criteria.

c. Future of Scars Analysis. Although the theoretical approaches to date have been mostly satisfying, there is little reason to believe that they are the last word. No one has taken into account the topographical distribution of scars on the cells, and, likewise, no one has considered the consequences of Mitchison's rule and its violations (Section II,C). We note that scars are possibly trivial, but they are the subject of a considerable literature with respect to budding yeasts and the modest literature for fission yeast discussed above. Trivial perhaps, but they fascinate.

3. Growth of Scars

Fission yeast scars grow, presumably in tandem with the cylindrical growth of the cell. A glance at Fig. 6 readily convinces one that the scars grow even more rapidly than the rest of the wall, for their breadth is greatest. We have noted that growth of the cylindrical diameter is nonaccretional (Section II,E). However, no one has even speculated as to how it might be happening.

V. SEXUAL MORPHOGENESIS

Sexual morphogenesis of fission yeasts was last reviewed in 1981 by Calleja *et al.* with natural emphasis on the cell wall. Since then, two papers (Ashton and Moens, 1982; Tanaka and Hirata, 1982) bearing many excellent electron micrographs depicting the nucleus and cytoplasm during conjugation (see Chapter 8 by Robinow and Hyams, this volume) have appeared, but they tell us little about overall morphogenesis.

A. Conjugation and Forespore Membrane

Ashton and Moens (1982) showed that sibling conjugants of *Schizosaccharomyces octosporus* have remnants of the septum distorting their conjugation tube, hence they may be recognized cytologically *ex post facto*.

The existence of sib conjugation demands homothally and a mating-type gene half-switch (Johnson *et al.*, 1984) associated with the last mitosis. Whether the half-switch occurs before or after mitosis may be irrelevant. However, the appearance of these sib conjugants is consistent with notions of the postmitotic nuclei having different mating-type genes expressed and these nuclei having committed themselves to conjugation even before cell division has been completed (Johnson *et al.*, 1984). These studies of Ashton and Moens (1982) end just before they should have been seeing details of the forespore membrane. Determining the origin of the forespore membrane predominated in a study by Tanaka and Hirata (1982). Whereas earlier workers on *S. pombe* (Yoo *et al.*, 1973) had decided on a nuclear membrane origin, Tanaka and Hirata, using phosphotungstic acid–chromic acid staining, determined that the forespore membrane was derived from fusion of vesicles of endoplasmic reticulum origin. The nature of the forespore membrane is important in this context because of its intimate role in the morphogenesis of the ascospore wall. We tentatively accept Tanaka and Hirata's (1982) story, believing that their techniques specific for membranes should have yielded better insight than Yoo *et al.* (1973) whose techniques were more appropriate for wall morphogenesis. It would be useful, however, to see confirmation of Tanaka and Hirata's (1982) conclusions before final acceptance.

Tanaka and Hirata made no mention of a biologically interesting fact apparent in their micrographs, namely, that the spores in a single ascus mature asynchronously, as discussed earlier by Yoo *et al.* (1973). One supposes that this morphogenetic asynchrony is a consequence of some biological drive to obtain at least one spore mature enough to survive even though some cataclysmic event might destroy the remaining presumptive spores before they themselves matured and thereby attained the capacity for longevity we associate with spores. No one has speculated about controls to establish asynchrony.

B. Pheromone-Induced Morphogenesis

Is there evidence for or against the existence of sexual pheromones in *Schizosaccharomyces?* Evidence has long been sought yet rarely found. Accordingly, we discuss the successes and the failures in an attempt to establish conclusions based on both. We deem the abundance of negative results as being instructive. Until recently, the evidence seemed markedly scanty, consisting of two results in one paper. Friedmann and Egel (1978) removed h^+ cells and h^- cells from the media in which they had been grown, switched the media for a time, mixed the cells, and then assayed

for a pheromone effect by measuring the time at which zygotes were seen. They found that "zygote formation started about 20 min earlier as compared to the controls (mixed without previous [medium] exchange)." A difference of 20 min over an assay period of 180 min [the 3 hr from the time of nitrogen depletion until the curve presenting the percentage of zygotes (Friedmann and Egel, 1978) rises appreciably] is not noteworthy in our opinion. We feel that if the authors had wished to make a significant point of this accelerated zygote formation, they would have presented an analysis by paired observations, etc. We treat this evidence for pheromones as lightly as did the original authors.

The other result of Friedmann and Egel (1978) is not to be so lightly dismissed. They examined the banding patterns in gels of DNA-binding proteins extracted from h^+ cells and h^- cells whose media had been switched, as above, but which were not placed in direct contact with cells having the obverse mating type. Their DNA-binding proteins isolated from both cultures "revealed a pattern identical to that of h^{90} spo-phase." This remains the only "early" evidence for the existence of sexual pheromones in *S. pombe* (Friedmann and Egel, 1978; "early" in this context means a consequence that can be noted soon after the switch). It might be the only early evidence, but it is good evidence. Furthermore, there must be an h^+ pheromone and an h^- pheromone, because both types of cells responded to medium conditioned by growth of the obverse type: the h^+ exchange gel and the h^- exchange gel are both obviously dissimilar to the control h^+ gel, and both are obviously similar to the h^{90} spo gel in spite of heavy-handed photographic reproduction by the publisher.

Other older results are all negative, and they are either briefly mentioned in passing (Egel, 1971; Calleja *et al.*, 1977) or not at all (T. W. James, personal communication; B. Johnson, unpublished; H. Miyata, unpublished). James looked for evidence of G_1 arrest, and Johnson and Miyata at different times have looked for morphogenetic evidence of a pheromonal action. In none of these experiments could evidence for a sexual pheromone be found.

In light of the above, it was very exciting when Yamamoto's group did produce morphogenetic evidence for a sexual pheromone (Fukui *et al.*, 1986). They mixed h^+ *ras1*$^+$ cells with h^- *ras1*$^-$ cells and placed them together on sporulation agar. The presence of the *ras1*$^-$ gene prevents conjugation. When the mixture of cells was removed from the agar for microscopic examination, 32 unusually elongated cells were all identified as having h^+ mating type, and 21 short or spherical cells from the same mixture were all found to have h^- mating type. By itself, the experiment does not rule out a role for direct cell-to-cell contact, as the authors acknowledge. They then placed an individual h^+ cell on sporulation agar,

surrounded by but not in direct contact with h^- *ras1*$^-$ cells. All cells in the field attained the typical *ras1*$^-$ short, round phenotype except for the individual h^+ cell, still visibly isolated from the h^- *ras1*$^-$ cells, and now nearly 20 μm long. Various controls showed that only h^+ *ras1*$^+$ cells displayed elongation. A diffusible factor seems to be the answer. We owe this unusual observation to an unusual laboratory—wherein molecular biologists actually look at the cells they study.

More recently (1987), and also much less recently (1950), Leupold has addressed the problem. Leupold (1987) showed that haploid h^- cells secrete an "M-factor" and that haploid h^+ cells secrete a "P-factor" (obviously better terms than h^- pheromone and h^+ pheromone as used above). These factors induce obvious conjugation-tube formation in cells having the obverse mating type; detection is not readily made using haploid testers, but diploids homozygous for the mating-type locus, h^+/h^+ and h^-/h^-, constitute useful testers that respond to M-factor and P-factor, respectively.

The experiments of Friedmann and Egel (1978), Fukui *et al.* (1986), and Leupold (1987) yield convincing evidence that fission yeast cells having obverse mating types may communicate via diffusible factors. We discuss these diffusible factors under the heading of pheromones, as that is probably what they are. Nevertheless, we feel that the last point is not yet established by these experiments. For instance, Friedmann and Egel (1978) have not shown that their DNA-binding proteins really are involved with mating or preparation for mating; furthermore, the function of no single one of their DNA-binding proteins is known. Mere correlation is not good enough. However, the changes in DNA-binding proteins they show to occur do take place within a reasonable time course: they are early. All is consistent with a pheromone interpretation, it is just that insufficient evidence is available.

On the other hand, there are two problems with Yamamoto's evidence in spite of their ingenious derivation. The first is that the elongated cells do not look preconjugal. They may be preconjugal, and perhaps there is some way of establishing that fact, but it should be done. The larger problem is with the time course of events: they are not early, in the sense outlined above. Conjugation of homothallic h^{90} begins at 3 hr after nitrogen depletion in Egel's laboratory (Friedmann and Egel, 1978), of homothallic NCYC 132 at 2 hr in our laboratory (Calleja *et al.*, 1981), and of the mixed heterothallic Leupold strains 972 h^- and 975 h^+ at about 1 hr in Egel's laboratory (Egel, 1971) and at about 7 hr in our laboratory (Johnson *et al.*, 1987). Seen in these terms, preconjugal morphogenesis should not be requiring 3 days, 2 days, or the 1 day of some of the experiments shown (Fukui *et al.*, 1986) or perhaps the several hours in Leupold (1987, Fig. 1 therein). Perhaps the basic events take less time and are only

scored later. Whatever, they are "late," and the apparent time course prevents for now critical acceptance of the results as evidence for pheromone-induced morphogenesis.

There is a very old experiment (Leupold, 1950) with convincing evidence. [It is not obvious why everyone interested in the subject has missed this old result. Leupold himself has been characteristically modest about it until his recent paper (1987).] Leupold (1950) allowed mixed h^+ and h^- cells to grow on agar and followed their stationary-phase behavior by a time-lapse approach. At 2 hr, 7 min, with only one dividing cell in the field, hence the group has just now become stationary phase, an h^+ type cell could be seen to be growing a narrow preconjugation tube toward its potential h^- mate. Conjugation between these two cells was scored at 2 hr, 37 min. The resultant zygote later produced four ascospores.

Assembling these results, we readily conclude that both h^+ and h^- cells secrete factors that serve as sexual pheromones. These have been named P-factor and M-factor (Leupold, 1987). The timing of the response to the M-factor in the 1950 experiment was quite adequate. This was unlikely to be true for any of the other experiments using sexual morphogenesis as a parameter, and one wonders why. To be functional, a pheromone must be secreted in reasonable abundance, it must be reasonably stable, and the target cell must be sensitively responsive.

Haploid target cells (h^+ and h^-) have been shown by Leupold to be markedly less sensitive in their response to obverse mating factors than are the diploids (h^+/h^+ and h^-/h^-). So target cell insensitivity has been one of the problems. It is also possible that the target cells were not examined soon enough, but Leupold's (1987) 18 hr h^-/h^- cells were barely deformed (their last residual divisions occurred sometime during the previous 6 hr).

The abundance of a factor and its stability are obviously interdependent in their effect. Only one conditioned-medium experiment has worked, that of Friedmann and Egel (1978), whereas all others (Egel, 1971; Calleja *et al.*, 1977; T. W. James, personal communication; H. Miyata, unpublished) have failed. One suspects that stability of the factors has been more important than abundance of secretion in these experiments. On the other hand, only one mutual plating experiment has worked (Leupold, 1950), and several others (Fukui *et al.*, 1986; Leupold, 1987; B. Johnson, unpublished; H. Miyata, unpublished) may be deemed as failures by virtue of either no effect or overdelayed effect. Secretion must be scanty in these experiments, and it is obvious that not every cell on the agar was secreting factor. We shall continue to wonder about the failed experiments until more information, from more successful experiments, is available. However, one conclusion is evident: fission yeast cells secrete mating factors and respond to mating factors. The only logical alternative to

pheromones, communication mediated exclusively through physical contact between potential maters, is not now worth considering as a requirement for mating, even though the distance between cells may be exceedingly small.

VI. PROGRESS AND PROSPECTS

If morphogenesis of the fission yeast cell is a combination of extensile growth, cell division, nonaccretional growth in diameter, and apparently little else, then one might consider that one knows much about how a fission yeast cell attains and retains its sausagelike appearance, etc. The sausage shape is a natural consequence of these processes: morphogenesis is solved!

There is one major caveat. It is, simply, that basically the same mechanisms apply to morphogenesis of the budding yeast; however, we understand neither how its form is generated, nor how its morphogenesis is different from that of the fission yeast. If we do not understand how modest modifications of these basic morphogenetic activities lead to the typical budding yeast shape, then it is likely that we understand less about fission yeast morphogenesis than we wish to admit.

It is not just that the intuitive feeling for the budding yeast cell cycle is weaker (see Section I), although that weakness will contribute to our sense of ignorance, nor that the shape is so much more complex. The basic problem is that the strength of the intuitive feeling for the fission yeast cell, as discussed above (see Section I), actually deludes us, seduces us to believe that we understand more than we really do. And it is only when faced with the reality that more or less the same mechanisms can produce such a different cell, such a different life habit, that we must sense that we probably are just as ignorant about fission yeast morphogenesis, and worse, that we are easily disabused of that sense of ignorance.

A brief history of the study of morphogenesis shows that the basics were learned early on by the study of normal, wild-type cells. The early studies of cell division of Robinow (1946) by light microscopy and of extensile growth of Mitchison (1957) come easily to mind. Immunofluorescence analysis (May, 1962) and autoradiography (Johnson, 1965a) put tip growth on a quantitative basis. We have already discussed in adequate detail the later studies of Streiblová *et al.* (1966) and of Johnson *et al.* (1973) of cell division by electron microscopy. Working from such an apparently solid foundation, Mitchison's group moved on to the study

of conditional mutants affecting cell size and structure (the famous *wee*). Somewhat later, drug-mediated morphogenetic changes were analyzed. The recent papers by Mitchison and Nurse (1985) and H. Miyata *et al.* (1988) describe analyses of extension based on studies of all of these, the normal cells, the conditional lethal mutants, and hydroxyurea-modified extension patterns. A recent paper by May and Mitchison (1986) introduces a novel analysis of "tiger tails," fission yeast cells brightly banded by lectin-bound fluorescent dyes to illustrate regional growth. The recent spate of papers from the laboratories of Mitchison and Kubitschek as well as our own suggests that the end is not yet in sight, that there is more to be learned about fission yeast morphogenesis by analysis in new ways.

However, the fact that we have not yet been able to transform notions of fission yeast morphogenesis into explanations for the differences in budding yeast morphogenesis suggests that perhaps it is time for a more deliberately comparative approach. The study of differences in detail just might help to illuminate morphogenesis of the fission yeast cell too.

It is always worth asking if there might be some remote but general application of results from our parochial experiments; if there are broader lessons to be learned; if one is learning facts basic to a simple system that might themselves be appropriate to a more complex system but be difficult to uncover there. Specifically, is it possible that the study of morphogenesis of a sausage like the fission yeast cell can lead in any way to a better understanding of the morphogenesis of multicellular organisms with their complex tissues, organs, etc.? We think the possibility is real.

In an erudite discussion of morphogenesis of metazoans (specifically, insects), Larsen and McLaughlin (1987) expand an earlier discussion by Stent (1982) and comment:

> We observe only that knowledge of the molecular biology of gene products alone will not render morphogenesis intelligible. In any system undergoing morphogenesis, we need to know which of the cell parameters outlined earlier are affected by genes, since changes in cell behaviour generate new tissue shapes and hence new environments for continuing morphogenesis. Implicit in this approach is the *non-existence* of a genetic programme for morphogenesis which determines the time and place of gene activity. [Reproduced from E. Larsen and H. M. G. McLaughlin (1987). The morphogenetic alphabet: Lessons for simple-minded genes. *BioEssays* **7**, 130–132, by copyright permission of Cambridge Univ. Press, London and New York.]

Even if one can reasonably conclude, as they do, that there is no genetic program for morphogenesis of an animal, it does not necessarily follow that the same is true at the cellular level. Indeed, Larsen and McLaughlin (1987) made the point that

> Attempts to understand the structural development of organs and tissues through a consideration merely of the molecular composition of relevant genes and their

products could be compared to attempts at understanding Mendel's rules through an analysis of the structure and sequence of DNA. In order to understand the material basis of Mendel's rules it is necessary to examine an intermediate level of organization, the chromosome. Just as chromosomal mechanics during meiosis are necessary to explain Mendelian genetics, it is necessary to comprehend *cellular*-level behaviour in order to elucidate the mechanisms by which genes influence shape. Gene–cell interaction is the intermediate level of organization which must become the focus of studies on gene action during morphogenesis. [Reproduced from E. Larsen and H. M. G. McLaughlin (1987). The morphogenetic alphabet: Lessons for simple-minded genes. *BioEssays* **7,** 130–132, by copyright permission of Cambridge Univ. Press, London and New York.]

In this context, what does the study of yeast morphogenesis have to offer? Simply, we have morphogenesis genes (Johnson *et al.*, 1982). We do not know in intimate detail how they function, how a gene product from wild-type genes [alleles of, say, mutant *cdc12* of *Schizosaccharomyces* (Streiblová and Girbardt, 1980) or of *cdc24* of *Saccharomyces* (Sloat and Pringle, 1978)], probably protein, can direct the localization of other molecules to form a β-glucan primary septum in dividing fission yeast cells or a chitin ring in predivisional budding yeast cells. But we know that mutants of these genes *fail* in the morphogenetic step. If there is any hope of understanding the generation of a particular cell shape in a developing animal or plant, it lies in the study of single cells having their own particular shape. We think the study of morphogenesis of yeast cells has much to offer in this context.

ACKNOWLEDGMENTS

Our thoughts on morphogenesis have been developed through years of interaction with Gode B. Calleja, Bong Y. Yoo, Teena Walker, and Ian J. McDonald, among others. This is NRCC publication number 29193.

REFERENCES

Aronson, J. M. (1965). The cell wall. *In* "The Fungi" (G. C. Ainsworth and A. S. Sussman, eds.), Vol. 1, pp. 49–76. Academic Press, New York.

Ashton, M.-L., and Moens, P. B. (1982). Light and electron microscopy of conjugation in the yeast, *Schizosaccharomyces octosporus*. *Can. J. Microbiol.* **28,** 1059–1077.

Barras, D. R. (1969). An endo-β-1,3-glucanase from *Schizosaccharomyces pombe*. *Antonie van Leeuwenhoek* **35** (Suppl.), 17–18.

Barras, D. R. (1972). A β-glucan endo-hydrolase from *Schizosaccharomyces pombe* and its role in cell wall growth. *Antonie van Leeuwenhoek* **38,** 65–80.

Bartnicki-Garcia, S., and Lippman, E. (1972). The bursting tendency of hyphal tips of fungi:

Presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. *J. Gen. Microbiol.* **73,** 487–500.

Barton, A. A. (1950). Some aspects of cell division in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **4,** 84–87.

Calleja, G. B., Yoo, B. Y., and Johnson, B. F. (1977). Fusion and erosion of cell walls during conjugation in the fission yeast (*Schizosaccharomyces pombe*). *J. Cell Sci.* **25,** 139–155.

Calleja, G. B., Zuker, M., and Johnson, B. F. (1980). Analyses of fission scars as permanent records of cell division in *Schizosaccharomyces pombe*. *J. Theor. Biol.* **84,** 523–544.

Calleja, G. B., Johnson, B. F., and Yoo, B. Y. (1981). The cell wall as sex organelle in fission yeast. *In* "Sexual Interaction in Eukaryotic Microbes" (D. H. O'Day and P. A. Horgen, eds.), pp. 225–259. Academic Press, New York.

Egel, R. (1971). Physiological aspects of conjugation in fission yeast. *Planta* **98,** 89–96.

Egel, R., and Eie, B. (1987). Cell lineage asymmetry in *Schizosaccharomyces pombe:* Unilateral transmission of a high-frequency state for mating-type switching in diploid pedigrees. *Curr. Genet.* **12,** 429–433.

Ephrussi, B., and Hottinguer, H. (1951). Cytoplasmic constituents of heredity. On an unstable cell state in yeast. *Cold Spring Harbor Symp. Quant. Biol.* **16,** 75–85.

Fleet, G. H., and Phaff, H. J. (1975). Glucanases in *Schizosaccharomyces*. Isolation and properties of an exo-β-glucanase from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. *versatilis*. *Biochim. Biophys. Acta* **410,** 318–332.

Flegel, T. W. (1978). Difference in generation times for mother and daughter cells in yeasts. *Can. J. Microbiol.* **24,** 827–833.

Friedmann, K. L., and Egel, R. (1978). Protein patterns during sporulation in fission yeast. *Z. Naturforsch.* **33c,** 84–91.

Fukui, Y., Kaziro, Y., and Yamamoto, M. (1986). Mating pheromone-like diffusible factor released by *Schizosaccharomyces pombe*. *EMBO J.* **5,** 1991–1993.

Gay, J. L., and Martin, M. (1971). An electron microscopic study of bud development in *Saccharomycodes ludwigii* and *Saccharomyces cerevisiae*. *Arch. Microbiol.* **78,** 145–157.

Gooday, G. W. (1977). Biosynthesis of the fungal wall—Mechanisms and implications. *J. Gen. Microbiol.* **99,** 1–11.

Green, P. B. (1969). Cell morphogenesis. *Annu. Rev. Plant Physiol.* **20,** 365–394.

Hamada, T. (1982). Stationary scar-class structure of populations of *Schizosaccharomyces pombe*. *J. Theor. Biol.* **99,** 835–838.

Hicks, J. B., and Herskowitz, I. (1976). Interconversion of yeast mating types. I. Direct observations of the action of the homothallism (HO) gene. *Genetics* **83,** 245–258.

Horisberger, M., and Rouvet-Vauthey, M. (1985). Cell wall architecture of the fission yeast *Schizosaccharomyces pombe*. *Experientia* **41,** 748–750.

James, T. W., Hemond, P., Czer, G., and Bohman, R. (1975). Parametric analysis of volume distributions of *Schizosaccharomyces pombe* and other cells. *Exp. Cell Res.* **94,** 267–276.

Johnson, B. F. (1965a). Autoradiographic analysis of regional cell wall growth of yeasts. *Schizosaccharomyces pombe*. *Exp. Cell Res.* **39,** 613–624.

Johnson, B. F. (1965b). Morphometric analysis of yeast cells. Adult cell volume of *Saccharomyces cerevisiae*. *Exp. Cell Res.* **39,** 577–583.

Johnson, B. F. (1968a). Morphometric analysis of yeast cells. II. Cell size of *Schizosaccharomyces pombe* during the growth cycle. *Exp. Cell Res.* **49,** 59–68.

Johnson, B. F. (1968b). Lysis of yeast cell walls induced by 2-deoxyglucose at their sites of glucan synthesis. *J. Bacteriol.* **95,** 1169–1172.

Johnson, B. F. (1968c). Dissolution of yeast glucan induced by 2-deoxyglucose. *Exp. Cell Res.* **50,** 692–694.

Johnson, B. F., and Lu, C. (1975). Morphometric analysis of yeast cells. IV. Increase of the cylindrical diameter of *Schizosaccharomyces pombe* during the cell cycle. *Exp. Cell Res.* **95,** 154–158.

Johnson, B. F., and McDonald, I. J. (1983). Cell division: A separable cellular sub-cycle in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **129,** 3411–3419.

Johnson, B. F., and Rupert, C. M. (1967). Cellular growth rates of the fission yeast, *Schizosaccharomyces pombe*, and variable sensitivity to 2-deoxyglucose. *Exp. Cell Res.* **48,** 618–620.

Johnson, B. F., Yoo, B. Y., and Calleja, G. B. (1973). Cell division in yeasts: Movement of organelles associated with cell plate growth of *Schizosaccharomyces pombe*. *J. Bacteriol.* **115,** 358–366.

Johnson, B. F., Lu, C., and Brandwein, S. (1974a). Morphometric analysis of yeast cells. III. Size distribution of 2-deoxyglucose-induced lysing *Schizosaccharomyces pombe* cells and their sites of lysis. *Can. J. Genet. Cytol.* **16,** 593–598.

Johnson, B. F., Yoo, B. Y., and Calleja, G. B. (1974b). Cell division in yeasts. II. Template control of cell plate biogenesis in *Schizosaccharomyces pombe*. *In* "Cell Cycle Controls" (G. M. Padilla, I. L. Cameron, and A. Zimmerman, eds.), pp. 153–166. Academic Press, New York.

Johnson, B. F., Calleja, G. B., and Yoo, B. Y. (1977). A model for controlled antolysis during differential morphogenesis of fission yeast. *In* "Eucaryotic Microbes as Model Developmental Systems" (D. H. O'Day and P. A. Horgen, eds.), pp. 212–229. Dekker, New York.

Johnson, B. F., James, A. P., Gridgeman, N. T., Lusena, C. V., and Lee, E.-H. (1978). The effect of different cell cycles in yeast on expression of the cytoplasmic *petite* mutation. *In* "Cell Cycle Regulation" (J. R. Jeter, Jr., I. L. Cameron, G. M. Padilla, and A. M. Zimmerman, eds.), pp. 203–219. Academic Press, New York.

Johnson, B. F., Calleja, G. B., Boisclair, I., and Yoo, B. Y. (1979). Cell division in yeasts. II. The biased, asymmetric location of the septum in the fission yeast cell, *Schizosaccharomyces pombe*. *Exp. Cell Res.* **123,** 253–259.

Johnson, B. F., Calleja, G. B., Yoo, B. Y., Zuker, M., and McDonald, I. J. (1982). Cell division: Key to cellular morphogenesis in the fission yeast, *Schizosaccharomyces*. *Int. Rev. Cytol.* **75,** 167–208.

Johnson, B. F., Calleja, G. B., and Zuker, M. (1984). Mating-type gene switching in a homothallic fission yeast. *J. Theor. Biol.* **110,** 299–312.

Johnson, B. F., Walker, T., Miyata, M., Miyata, H., and Calleja, G. B. (1987). Sexual coflocculation and asexual self-flocculation of heterothallic fission-yeast cells (*Schizosaccharomyces pombe*). *Can. J. Microbiol.* **33,** 684–688.

Kubitschek, H. E., and Clay, K. B. (1986). A second growth state for *Schizosaccharomyces pombe*. *Exp. Cell Res.* **165,** 243–254.

Kubitschek, H. E., and Ward, R. A. (1985). Buoyant density constancy of *Schizosaccharomyces pombe* cells. *J. Bacteriol.* **162,** 902–904.

Larsen, E., and McLaughlin, H. M. G. (1987). The morphogenetic alphabet: Lessons for simple-minded genes. *BioEssays* **7,** 130–132.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterothallie bei *Schizosaccharomyces pombe*. *C. R. Lab. Carlsberg Ser. Physiol.* **24,** 381–480.

Leupold, U. (1987). Sex appeal in fission yeast. *Curr. Genet.* **12,** 543–545.

Lindner, P. (1893). *Schizosaccharomyces pombe* n. sp., ein neuer Gährungserreger. *Wochenschr. Brau.* **10,** 1298–1300.

Masuda, Y., and Yamamoto, R. (1985). Cell-wall changes during auxin-induced cell extension. Mechanical properties and constituent polysaccharides of the cell wall. *In* "Biochemistry of Plant Cell Walls" (C. T. Brett and J. R. Hillman, eds.), pp. 269–300. Cambridge Univ. Press, London and New York.

May, J. W. (1962). Sites of cell-wall extension demonstrated by the use of fluorescent antibody. *Exp. Cell Res.* **27,** 170–172.

May, J. W., and Mitchison, J. M. (1986). Length growth in fission yeast cells measured by two novel techniques. *Nature (London)* **322,** 752–754.

Megnet, R. (1965). Effect of 2-deoxyglucose on *Schizosaccharomyces pombe*. *J. Bacteriol.* **90,** 1032–1035.

Mitchison, J. M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. *Exp. Cell Res.* **13,** 244–262.

Mitchison, J. M. (1970). Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* **4,** 131–165.

Mitchison, J. M., and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **75,** 357–376.

Mitchison, J. M., and Walker, P. M. B. (1959). RNA synthesis during the cell life cycle of a fission yeast *Schizosaccharomyces pombe*. *Exp. Cell Res.* **16,** 49–58.

Miyata, H., and Miyata, M. (1981). Mode of conjugation in homothallic cells of *Schizosaccharomyces pombe*. *J. Gen. Appl. Microbiol.* **27,** 365–371.

Miyata, H., Miyata, M., and Ito, M. (1978). The cell cycle in the fission yeast, *Schizosaccharomyces pombe*. I. Relationship between cell size and cycle time. *Cell Struct. Funct.* **3,** 39–46.

Miyata, H., Miyata, M., and Johnson, B. F. (1986). Patterns of extension growth of the fission yeast, *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **32,** 528–530.

Miyata, H., Miyata, M., and Johnson, B. F. (1988). Pseudo-exponential growth in length of the fission yeast, *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **34,** 1338–1343.

Miyata, M., Kitamura, J., and Miyata, H. (1980). Lysis of growing fission-yeast cells induced by aculeacin A, a new antifungal antibiotic. *Arch. Microbiol.* **127,** 11–16.

Miyata, M., Kanbe, T., and Tanaka, K. (1985). Morphological alterations of the fission yeast *Schizosaccharomyces pombe* in the presence of aculeacin A: Spherical wall formation. *J. Gen. Microbiol.* **131,** 611–621.

Miyata, M., Miyata, H., and Johnson, B. F. (1986a). Asymmetric location of the septum in morphologically altered cells of the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **132,** 883–891.

Miyata, M., Miyata, H., and Johnson, B. F. (1986b). Establishment of septum orientation in a morphologically altered fission yeast, *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **132,** 2535–2540.

Morré, D. J., and Eisinger, W. R. (1968). Cell wall extensibility: Its control by auxin and relationship to cell elongation. *In* "Biochemistry and Physiology of Plant Growth Substances" (F. Wightman and G. Setterfield, eds.), pp. 625–645. Runge Press, Ottawa.

Nurse, P. (1981). Genetic analysis of the cell cycle. *Symp. Soc. Gen. Microbiol.* **31,** 291–315.

Park, D., and Robinson, P. M. (1966). Aspects of hyphal morphogenesis in fungi. *In* "Trends in Plant Morphogenesis" (E. G. Cutter, ed.), pp. 27–44. Longmans, London.

Ray, P. M. (1969). The action of auxin and cell enlargement in plants. *Dev. Biol. Suppl.* **3,** 172–205.

Robertson, N. F. (1965). The mechanism of cellular extension and branching. *In* "The

Fungi" (G. C. Ainsworth and A. S. Sussman, eds.), Vol. 1, pp. 613–623. Academic Press, New York.

Robinow, C. F. (1946). Addendum to R. J. Dubos, "The Bacterial Cell," pp. 355–377. Harvard Univ. Press, Cambridge, Massachusetts.

Robinow, C. F., and Johnson, B. F. (1989). Yeast cytology. *In* "The Yeasts" (A. H. Rose and J. S. Harrison, eds.), Vol. 4. Academic Press, San Diego, California.

Sloat, B. F., and Pringle, J. R. (1978). Mutant of yeast defective in cellular morphogenesis. *Science* **200,** 1171–1173.

Stent, G. S. (1982). The cellular basis of morphogenetic change group report. *In* "Evolution and Development" (Dahlem Konferenzen) (J. T. Bonner, ed.), pp. 110–114. Springer-Verlag, Berlin.

Streiblová, E. (1981). Fission. *In* "Yeast Cell Envelopes: Biochemistry, Biophysics, and Ultrastructure" (W. N. Arnold, ed.), Vol. 2, pp. 79–92. CRC Press, Boca Raton, Florida.

Streiblová, E., and Girbardt, M. (1980). Microfilaments and cytoplasmic microtubules in cell division cycle mutants of *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **26,** 250–254.

Streiblová, E., and Wolf, A. (1972). Cell wall growth during the cell cycle of *Schizosaccharomyces pombe*. *Z. Allg. Mikrobiol.* **12,** 673–684.

Streiblová, E., Málek, I., and Beran, K. (1966). Structural changes in the cell wall of *Schizosaccharomyces pombe* during cell division. *J. Bacteriol.* **91,** 428–435.

Tanaka, K., and Hirata, A. (1982). Ascospore development in the fission yeasts *Schizosaccharomyces pombe* and *S. japonicus*. *J. Cell. Sci.* **56,** 263–279.

Tyson, J. J., and Diekmann, O. (1986). Sloppy size control of the cell division cycle. *J. Theor. Biol.* **118,** 405–426.

Vraná, D. (1983a). Morphological properties of *Schizosaccharomyces pombe* in a continuous culture. *Folia Microbiol.* **28,** 414–419.

Vraná, D. (1983b). The fission yeast *Schizosaccharomyces pombe* in continuous culture. *Biotech. Bioeng.* **25,** 1989–1994.

Wessels, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. *Int. Rev. Cytol.* **104,** 37–79.

Wessels, J. G. H., and Sietsma, J. H. (1981). Fungal cell walls: A survey. *Encycl. Plant Physiol. New Ser.* **13B,** 383–394.

Wilson, K. L., and Herskowitz, I. (1986). Sequences upstream of the STE6 gene required for its expression and regulation by the mating type locus in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 2536–2540.

Yoo, B. Y., Calleja, G. B., and Johnson, B. F. (1973). Ultrastructural changes of the fission yeast (*Schizosaccharomyces pombe*) during ascospore formation. *Arch. Microbiol.* **91,** 1–10.

10

Continuous Culture and Intermediary Carbon Metabolism

I. J. MCDONALD* AND C. S. TSAI†

*Division of Biological Sciences
National Research Council of Canada
Ottawa, Ontario, Canada K1A 0R6

†Department of Chemistry
Carleton University
Ottawa, Ontario, Canada K1S 5B6

I. INTRODUCTION

This chapter is divided into two essentially separate sections. Section II discusses the use of chemostat cultures for studies on the effects of various growth conditions on the morphology, physiology, and genetics of fission yeasts. Section III covers carbon metabolism of *Schizosaccharomyces pombe* compared with that of *Saccharomyces cerevisiae*, and be-

MOLECULAR BIOLOGY OF THE
FISSION YEAST

cause chemostat cultures have been used in some of these studies, discussion of such results is included in Section III.

II. CONTINUOUS CULTURE

Studies of the metabolic activities of microorganisms frequently overlook the possible effect on the results of the environmental conditions which may be profoundly altered by but cannot be strictly controlled by the cells during their growth. Thus, in order to survive in their natural environments, microbial cells adjust physiologically to wide variations in growth conditions and, as a consequence, are able to alter structurally and functionally to changes in temperature, pH, nutrient supply, concentration of metabolic products, dissolved oxygen ([DO]), etc. Traditionally in the laboratory, cultures have been grown as batch cultures, and metabolic changes have been measured in response to changes in media and conditions of culturing. In such closed systems, however, as the culture proceeds through the growth curve the growth environment changes progressively; cell numbers increase, nutrients become less available, and products increase in number and concentration. Thus, during incubation of batch cultures, the growth rate of cells varies; cells divide slowly at the start, quickly achieve an exponential rate, then gradually slow down until maximum stationary phase is reached. The net result is that metabolic activities recorded during batch growth represent the sum of a combination of metabolic adjustments by the cells, and, as a consequence, it is difficult, if not impossible, to analyze precisely cause and effect. Thus, the technique becomes unsuitable where it is desirable to control environmental conditions for studies of growth and metabolism. In such situations, continuous culture techniques can offer many advantages over batch cultures.

Continuous culture techniques allow microorganisms to be grown in a time-independent dimension. In such open cultures (as opposed to closed batch cultures) the effects of individual environmental factors on the physiology and metabolism of an organism can be examined independently. Basically, the investigator (rather than the organism) controls conditions of growth by maintaining continuous cultures under steady state, that is, where factors such as temperature, pH, [DO], the concentration of cells and of nutrients, and, as a direct consequence, the growth rate of cells and the concentration of metabolic products are maintained at constant levels until they are deliberately changed. By the use of a continuous-culture technique it is possible, therefore, to examine the effect of a

change of a single environmental condition on the physiology, metabolism, cell morphology, enzyme activity, etc., of a culture.

There are three types of continuous cultures depending on the method used to maintain steady state; they are turbidostats, chemostats, and continuous-phased cultures, which are described fully in other publications (Calcott, 1981; Dawson, 1965, 1972; Evans *et al.*, 1970). Of these, the chemostat is probably the most versatile. It can be used in a great number of ways to study microbial physiology (Herbert *et al.*, 1956; Neijssel and Tempest, 1979; Tempest, 1970). In a chemostat, steady-state conditions are maintained by the continuous addition of medium that has one nutrient present in growth-limiting quantities and by the simultaneous removal of an equal volume of culture. The culture volume and the cell density are thereby kept constant. The specific growth rate (μ) of cells in a chemostat is controlled by the rate of medium addition (since $\mu = D$ (hr^{-1}) = F (ml hr^{-1})/V (ml), where D is the dilution rate, F the medium flow rate, and V the culture volume) and, therefore, can be controlled by the researcher. (Note that mass doubling time, t_d, of cells equals ln 2/D.) The effects of changes in growth rate on cell morphology, physiology, and metabolism can then be evaluated. Similarly, when cells are maintained at a constant growth rate, specific effects caused by changes in the nutrient that is limiting growth, in the temperature of growth, in the degree of oxygenation, or in the pH level can be independently ascertained. For these reasons, the chemostat has been used extensively for studies with bacteria, yeasts, and molds, and results obtained with chemostat cultures of *Schizosaccharomyces* spp. are discussed herein.

A. Mutation

One of the first uses of the chemostat was to study mutations in bacteria (Novick and Szilard, 1950), and the technique has been used to study spontaneous mutations of *S. pombe* to trichodermin resistance (McAthey and Kilbey, 1976, 1977, 1978). When *S. pombe his2-245* h^- was grown in chemostat cultures (McAthey and Kilbey, 1977) under glucose limitation the number of tdr^R mutants per 10^6 cells per week increased as the growth rate increased (i.e., as the number of generations through which the culture was maintained increased) (Fig. 1), whereas under histidine limitation the number of tdr^R mutants remained constant (Fig. 2). From these data the authors concluded, "Under glucose-limitation, mutation accumulation is directly proportional to the rate of cell division, while under histidine-limitation, accumulation is proportional to chronological time."

Because of the mode of operation of a chemostat culture, spontaneous

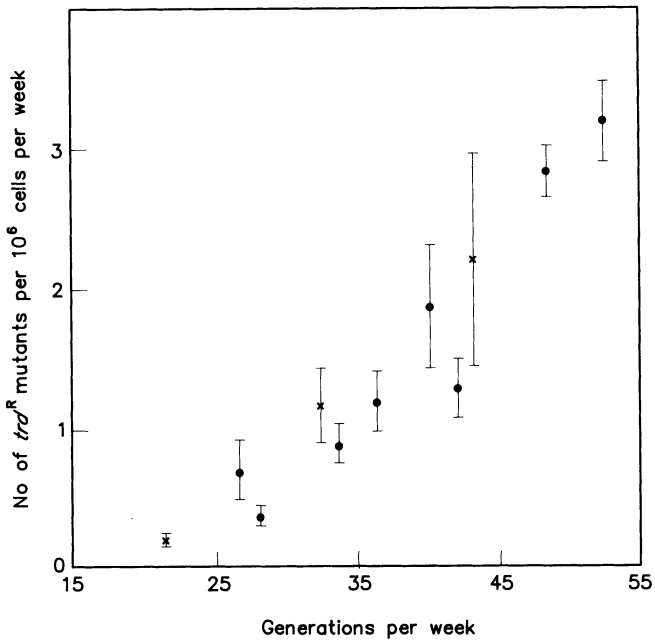


Fig. 1. Kinetics of accumulation of spontaneous *trd*^{R} mutants in continuous culture of *S. pombe* where growth is limited by the supply of glucose. Each point represents data obtained from between one and four separate chemostat experiments. In individual experiments, the least-squares linear regression lines were calculated and fitted in the standard manner. Error bars represent the standard errors of these regression lines. x, Wild-type 975 h^+; ●, *his2-245* h^-. [Reproduced with permission from McAthey and Kilbey (1977).]

mutants produced at a constant rate per generation, growing at the same rate as the wild-type cells, and not subjected to inhibition or stimulation must accumulate at a linear rate. The numbers of mutants accumulating will be directly related to the number of generations through which the culture has passed. Thus the data (Fig. 1) for glucose-limited chemostat cultures of *S. pombe* (975 h^+ and *his2-245* h^-) appear to be consistent with the production of spontaneous mutants at a constant rate per generation and with their subsequent growth at the same rate as the wild-type cells.

The data (Fig. 2) for histidine-limited cultures of *S. pombe* (McAthey and Kilbey, 1977) show a constant number of mutants per 10^6 cells per week at each growth rate tested. From these data the authors concluded that the mutation rate per generation decreases as the growth rate is increased. However, these data (Fig. 2) suggest to the reviewer the possibility that the histidine-limited cultures were in steady state with a con-

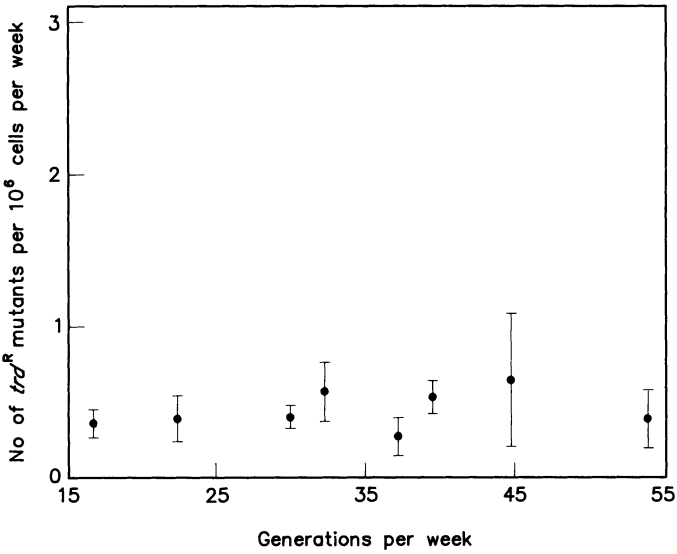


Fig. 2. Kinetics of accumulation of spontaneous trd^R mutants in continuous culture of *S. pombe* where growth is limited by the supply of histidine. Data analysis and symbols are as described in Fig. 1 legend. [Reproduced with permission from McAthey and Kilbey (1977).]

stant ratio of mutant (trd^R) to wild-type (trd^S) cells at each growth rate tested. If this is so, then possible explanations are that under histidine-limitation (1) mutation did not occur, (2) mutants were produced but did not grow, (3) mutants were produced but grew more slowly than the wild-type cells, or (4) mutants were produced and grew but did not express themselves. Each of these situations would result in a steady state with a constant ratio of wild-type to mutant cells.

It should be emphasized here, however, that P. McAthey (personal communication) has stated that irrespective of the strain of *S. pombe* used or of the growth limitation imposed, a linear increase in mutant numbers with time was observed and with histidine limitation the slopes remained more or less constant at each imposed growth rate. Under such circumstances, knowing the levels of trd^R mutants present in histidine-limited cultures after the same number of generations would be a valuable contribution to an understanding of the results. Such data would establish the existence, or nonexistence, of the same steady-state condition at each dilution rate under histidine limitation.

When compared with data for histidine limitation, data for lysine limitation (McAthey and Kilbey, 1978) show much less constant ratios of mutant to wild-type cells at each growth rate tested (i.e., the number of

mutants varied between a low of ~1.5 and a high of ~3.0/10^6 cells at 45 and 26 generations/week, with the numbers varying up and down inconsistently at the other seven growth rates tested). Thus, the slopes of mutant generation with lysine limitation vary considerably at each growth rate. Here again a comparison of the number of mutants present after the same number of generations at each growth rate would contribute considerably to an assessment of the effect of lysine limitation on mutation in *S. pombe lys1-131 h*$^-$ (McAthey and Kilbey, 1978).

B. Cell Morphology and Cell Division

Cell morphology and cell division have been studied using chemostat cultures of *S. pombe*. Several papers have reported on the relationship of cell size to the growth rate of cultures. In glucose-limited cultures, Kothari *et al.* (1972) found that cell size decreased as growth rates were increased from a t_d of approximately 13.8 to about 3.5 hr, then increased as growth rates were increased further to a t_d of approximately 1.5 hr. At low growth rates, the mean cell size, as noted by the authors, probably was influenced by the presence of numerous large round cells noticeably different in shape from the rods with hemispherical ends found in batch cultures. Cell sizes may have been influenced as well by the cultural conditions since the authors noted that *S. pombe* cultures were under "severe product inhibition by ethanol," namely, approximately 0.1%. [However, see discussion below regarding ethanol metabolism (Tsai *et al.*, 1987; McDonald *et al.*, 1987) and conjugation (McDonald *et al.*, 1982).]

In a study of nitrogen-limited chemostat cultures of *S. pombe*, Fantes and Nurse (1977) found that the mean doubling volume of cells, measured as protein content, increased as the dilution rate increased from a D of around 0.1 to about 0.28 hr^{-1} (growth rates increased from $t_d \sim 7$ to $t_d \sim$ 1.5 hr). Similar results were obtained when cells were grown in batch cultures with poor nitrogen, phosphorous, or carbon sources. Effects of nutritional shifts up and down (i.e., to effect changes in growth rates) in batch cultures on the relationship of cell length to cell division were examined. That there is a cell size requirement for entry into cell division is a conclusion by the authors. The trend for mean protein content per cell to decrease as the growth rate was increased was also found when the frequency function for protein was measured by the use of flow micrometry (protein stained with fluorescein isothiocyanate) in glucose-limited chemostat cultures of *S. pombe* (Agar and Bailey, 1981a).

Frequency functions for RNA and DNA as well as for protein were

recorded by Agar and Bailey (1981a). Their results indicated that at high growth rates DNA synthesis was complete near cell separation whereas at low growth rates it was completed in some cells before and in other cells after cell separation. These authors also used data from glucose-limited chemostat cultures of *S. pombe* in analyses of single-cell protein synthesis kinetics (Agar and Bailey, 1981b). They concluded that utilization of frequency function data is more sensitive for determining single-cell kinetics than is monitoring metabolic dynamics of a single cell or synchronous culture analysis.

The size of cells of *S. pombe* at cell division has been measured in cells from glucose-limited chemostat cultures (Vraná, 1983a,b; Barford, 1985a). Lengths, widths, and volumes of cells grown at different dilution rates were examined (Vraná, 1983a,b). Cell division resulted in two cells of different length, width, and volume (Vraná, 1983a,b). At low dilution rate ($D \sim 0.03$ hr^{-1}) the longer sib accounted for approximately 60% and the shorter sib for around 40% of the total volume of the two progeny. At high dilution rate ($D \sim 0.2$ hr^{-1}) both sibs accounted for approximately 50% of the total volume. Johnson *et al.* (1979) had shown previously that dividing cells of *S. pombe* from batch cultures possessed asymmetrically located septa but had concluded that the volumes of the two cells were the same. More recently, the location of septa and cell morphologies were examined when cells of *S. pombe*, which had been altered morphologically into round-bottomed flask (RBF)-like cells by growth in the presence of the antibiotic aculeacin, were grown in antibiotic-free batch culture (Miyata *et al.*, 1986). The RBF-like cells divided to give one cylindrical and one spherical sib, and these in turn divided to give two cylindrical and two spherical sibs, respectively. From these results the authors concluded that cell morphology directly influences septum positioning, a conclusion supported by the finding that volume ratios of short to long sibs from cells grown at low and high dilution rates, calculated from data in Vraná (1983a), agreed with ratios from RBF-like and cylindrical cells (Miyata *et al.*, 1986).

Carbon-limited chemostat cultures also were used to study the effect of growth rate of *S. pombe* on the cell length at the time of cell division (Barford, 1985a). As the specific growth rate increased, a smaller portion of the cell cycle was required for total length increase of cells. The results question a previous proposal derived from batch culture data, which stated that cells grow in length only during the first 75% of the cell cycle (Mitchison, 1970).

Chemostat results, therefore, indicate that cell morphology is influenced by growth rate. Using *S. pombe* grown at constant rate in glucose-limited chemostat cultures, Johnson and McDonald (1983) examined cell

morphologies under different conditions of [DO], pH, and temperature. A chemostat culture operating at $D = 0.6\ hr^{-1}$ ($t_d \sim 70$ min), pH 5.5, 32°C, and [DO] around 35% contained cells with sausagelike morphology (Fig. 3a) as usually seen in batch culture. However, when this culture was adjusted to pH 3.75 and to [DO] around 20%, elongated, multiseptate, and hyphal cells began to appear (Fig. 3b), and the division index (DI, the proportion of septate cells) increased. When the culture conditions were again changed by increasing the [DO] to approximately 60% the number of hyphal cells and the DI declined rapidly. As a result of the examination of these and other changes in growth conditions on cell division and cell morphology, the authors suggested that nuclear processes were coupled more tightly with cell extension than with cell division. The observations were explained on the basis of the possible existence of three potentially separable subcycles (G, growth; D, division; and N, nuclear, as shown by Nurse *et al.*, 1976) running in parallel in the cell cycle of the fission yeast

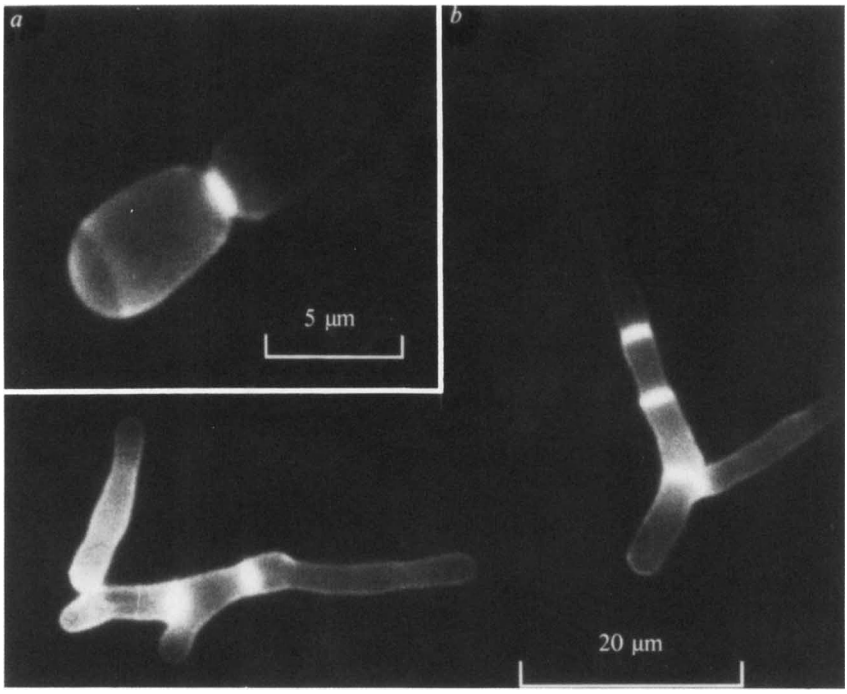


Fig. 3. Photomicrographs of *S. pombe* cells stained with Calcofluor White M2R New. The brightly stained regions are septa. (*a*) A uniseptate during fission and (*b*) multiseptates with branches. [Reproduced with permission from Johnson and McDonald (1983).]

rather than two (G, growth; DD, DNA–division) as proposed by Mitchison (1971).

Branched cells as described above have also been found in ammonia- and magnesium-limited chemostat cultures (I. McDonald, unpublished data) as well as in batch cultures (Mitchison, 1970; Lindner, 1893). In chemostat culture, the branching hyphae are physiologically induced, and the phenomenon can be readily reversed by changes in cultural conditions. Such hyphae have been termed ephemeral (Johnson *et al.*, 1982). However, some branched forms of *S. pombe* isolated from chemostat cultures can be maintained without reverting. Branched cultures transferred monthly on yeast extract–peptone–dextrose agar slants for about 2 years retained about 10% of the total population as branched forms (I. McDonald, unpublished data).

During studies on the effects of environmental conditions on cell division of *S. pombe* in glucose-limited chemostat cultures, it was found that conjugants and asci were produced (McDonald *et al.*, 1982). Examination of these chemostat cultures showed that conjugation occurred when growth was limited by glucose but not by nitrogen and that conjugation was favored by low growth rates and low [DO]. Data from batch cultures in synthetic medium [Edinburgh minimal medium (EMM2), Mitchison, 1970] showed that while ethanol (1%) did not interfere with growth of *S. pombe* 968^{h90}, it reduced conjugation by approximately 50% (McDonald *et al.*, 1982).

Because growth of *S. pombe* can be affected by the degree of aeration of a culture, studies on stirring rates, which can affect the degree of aeration, are important. Vraná and Kalasová (1982) studied the effect of stirring frequencies on the cell concentration in chemostat cultures (glucose limited?) of *S. pombe*. At $D = 0.05\ hr^{-1}$, the highest frequency of stirring that could be used without some washout was 11.9 Hz. Coagulation and sedimentation occurred at 10.2 Hz, cells were slowly lost over a 7-day period at 13.6 Hz, and rapid washout occurred at 17.0 Hz (Vraná and Kalasová, 1982). The paper does not, however, report on *S. pombe* cell morphologies at different stirring frequencies.

Thus, studies of chemostat cultures of *S. pombe* have resulted in some interesting if sometimes apparently contradictory results concerning cell morphology and cell division. It is clear that environmental conditions during growth affect the ultimate result, and some of the apparent contradictions are probably due to differences in the actual growth conditions employed (e.g., differences in basal media, temperatures of growth, pH of growth, and [DO]). The results obtained do, however, indicate that the chemostat can be a powerful tool for studies in morphogenesis and of the cell cycle.

C. Metabolism

Chemostat cultures have been used extensively for studies on the metabolic activities of many microorganisms including species of fission yeast. Nitrogen metabolism of *Schizosaccharomyces* spp. has been studied by the use of chemostat cultures limited by ammonia, glucose, phosphorus, or glutamate nitrogen (Brown *et al.*, 1973; Johnson and Brown, 1974; Van Andel and Brown, 1977). The activities of ammonia-assimilating systems have been assessed and compared with those of other yeasts and bacteria. In ammonia-limited chemostat cultures of *S. pombe* (NCYC 535) and *Schizosaccharomyces malidevorans* (NCYC 683), ammonia assimilation was found to proceed via the glutamine synthetase/glutamate synthase (GS/GOGAT) system first described in *Aerobacter aerogenes* by Tempest *et al.* (1970). This system, which is important in the assimilation of ammonia at low concentrations, was not detected in either *Schizosaccharomyces versatalis* (NCYC 419) or *Schizosaccharomyces octosporus* (NCYC 131) (Johnson and Brown, 1974) and was not detected in other yeasts, for example, *Candida* spp., *Saccharomyces* spp., *Pichia fermentans*, or *Brettanomyces lambia* (Burn *et al.*, 1974). That the genus *Schizosaccharomyces* is heterogeneous with respect to ammonia assimilation was the conclusion of the authors (Johnson and Brown, 1974).

The carbon metabolism of *S. pombe* has also been studied in chemostat cultures. Brändli (1980) studied the regulation of malate dehydrogenase in carbon-limited chemostat cultures. During this investigation, copper and iron deficiencies, which were not detectable in batch cultures, were discovered in Wickerham's (1951) medium. In a revised synthetic medium (D_w) formulated to alleviate the copper and iron deficiencies, growth was not inhibited until the dilution rate reached 0.14 hr^{-1}, whereas in Wickerham medium inhibition of growth began at $D = 0.05$ hr^{-1} (Brändli, 1980; Fiechter *et al.*, 1981). Fermentation and respiration were also studied in chemostat cultures of *S. pombe* (Barford, 1985b; McDonald *et al.*, 1987; Tsai *et al.*, 1987), and these articles are discussed later in this chapter.

III. CARBON METABOLISM OF *SCHIZOSACCHAROMYCES POMBE* VERSUS *SACCHAROMYCES CEREVISIAE*

Both *S. pombe* and *Sacch. cerevisiae* utilize sugars for growth and energy metabolism. D-Glucose, which is the commonest source of energy, is catabolized respiratively to carbon dioxide and water:

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

or fermentatively to carbon dioxide and ethanol:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Respiration and fermentation differ in the ultimate fate of pyruvate formed from glycolytic catabolism of D-glucose.

Pure fermentative glucose metabolism occurs only under anaerobic conditions. The anaerobic fermentation via glycolysis yields 2 mol each of ATP and ethanol per mole of D-glucose consumed. Assuming the free energy for hydrolysis of ATP is 29.3 kJ mol^{-1}, only 58.6 kJ of 2.87×10^3 kJ potentially available energy is utilized in the fermentative process. This inefficient utilization of energy results in minimal biomass formation.

Respirative glucose catabolism occurs in aerobic continuous cultures at low dilution rates. The energy yield of oxidative respiration via glycolysis, the tricarboxylic acid cycle, and the electron transfer chain is 38 mol ATP. This corresponds to an energy yield efficiency of 38.8% resulting in the maximal mass formation for the respirative process (Fig. 4) (McDonald *et al.*, 1987).

At high dilution rates in aerobic continuous cultures, both respirative and fermentative pathways are active and growth is respirofermentative (Käppeli *et al.*, 1985). Aerobic production of ethanol is accompanied by respirative oxidation of D-glucose with reduced biomass formation. Activities of respiratory enzymes are lower in cells exhibiting respirofermentative metabolism of D-glucose as compared with cells growing respiratively (Fiechter *et al.*, 1981).

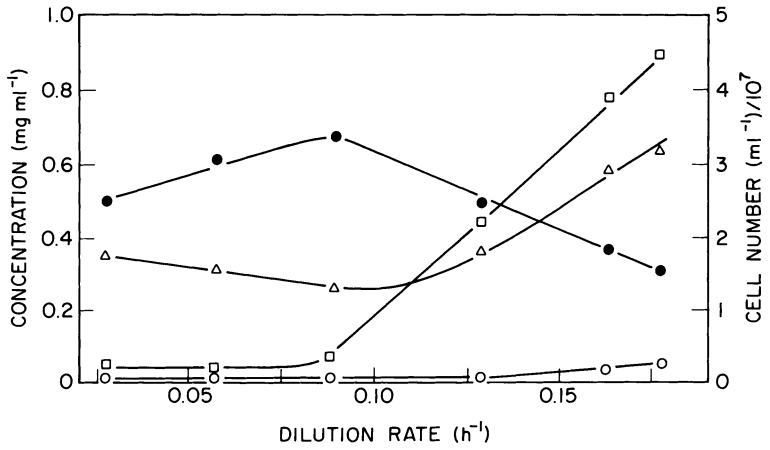


Fig. 4. Chemostat culture of *S. pombe* with 2.0 mg ml^{-1} D-glucose and 1.0 mg ml^{-1} acetate as the carbon source maintained at 30°C, pH 5.0. Steady-state concentrations of cells (●), D-glucose (○), ethanol (□), and acetate (△) are indicated. [Reproduced with permission from McDonald *et al.* (1987).]

Carbon metabolism in *Sacch. cerevisiae* has been extensively investigated (Sols *et al.*, 1971; Barnett, 1976). Most of the enzymes involved in glucose metabolism from the budding yeast have been purified or characterized. The same cannot be said, however, for the enzymes from *S. pombe*. Equivalent studies for the fission yeast are lacking. Therefore, the carbon metabolism of *S. pombe* is largely inferred from studies of *Sacch. cerevisiae* and their differences are discussed. The regulatory mechanisms of glucose metabolism in yeasts have been discussed earlier (Fiechter *et al.*, 1981). The following part of this chapter deals with biochemical studies on the mechanism and related aspects of carbon metabolism.

A. Intermediary Carbon Metabolism

The uptake of D-glucose by yeasts requires catalyzed transport because the plasma membrane of the yeast cell forms a limiting barrier (Cirillo, 1961). In *Sacch. cerevisiae*, the process is mediated by both high- and low-affinity transport systems (Bisson and Fraenkel, 1983; Bisson *et al.*, 1987). While the low-affinity transport system appears to operate by constitutively facilitated diffusion, the high-affinity transport system is subject to catabolic inactivation (Busturia and Lagunas, 1986). After entering the cell, D-glucose is catabolized mainly via glycolysis which is undoubtedly the most ubiquitous pathway of carbon metabolism. Glycolysis phosphorylates 1 mol D-glucose twice and splits it into 2 mol triose phosphate which is oxidized to pyruvate. All intermediates in the glycolytic pathway are phosphorylated derivatives having either six or three carbons (Axelrod, 1967).

The high yield of glycolytic enzymes in *Sacch. cerevisiae* (Scopers, 1973) facilitates their purification and characterization (see Table I). Partially purified enzymes have been successfully reconstituted as an ethanol fermentation system (Welch and Scopes, 1985). The identical pathway catalyzed by the same enzymes is operative in *S. pombe* although enzyme structures may differ.

Of the ten reaction steps constituting the glycolytic pathway, five reactions are catalyzed by phosphotransferases. Hexokinase (HK), 6-phosphofructokinase (PFK), phosphoglycerate kinase (PGK), and pyruvate kinase (PK) catalyze intermolecular transphosphorylations between ATP and ROH where ROH is D-glucose, D-fructose 6-phosphate (F6P), 3-phosphoglycerate (3PGA), and pyruvate, respectively.

Two types of enzymes are capable of phosphorylating D-glucose: a constitutive glucose kinase and an adaptive HK whose two isozymes, PI and PII, vary with the carbon source of the growth medium (Muratsubaki

TABLE I

Yeast Glycolytic Enzymes[a]

Enzyme	Systematic name	Reaction	Cofactor	Number of subunits	Reference (*Methods in Enzymology*)
HK	ATP:hexose 6-phospho-transferase (EC 2.7.1.1)	Hexose + ATP → hexose 6P + ADP	Mg^{2+}	2	**42,** 6–20 (1975)
PGI	D-Glucose-6-phosphate ketol-isomerase (EC 5.3.1.9)	G6P → FGP	None	2	**41,** 57–61 (1975)
PFK	ATP:F6P 1-phosphotransferase (EC 2.7.1.11)	F6P + ATP → FBP + ADP	Mg^{2+}	4	**90,** 49–60 (1982)
Aldolase	Fructose-1,6-bisphosphate D-glyceralde-3P-lyase (EC 4.1.2.13)	FBP → DHAP + Ga3P	None	2	**9,** 480–486 (1966)
TPI	D-Glyceraldehyde-3P ketol-isomerase (EC 5.3.1.1)	DHAP → Ga3P	None	2	**41,** 434–438 (1975)
TDH	D-Glyceraldehyde-3P:NAD^+ oxidoreductase (phosphorylating) (EC 1.2.1.12)	Ga3P + NAD^+ + P_i → 1,3-PGA + NADH	Zn^{2+}	4	**89,** 326–335 (1982)
PGK	ATP:3PGA-1-phosphotransferase (EC 2.7.2.3)	1,3-PGA + ADP → 3PGA + ATP	Mg^{2+}	1	**90,** 115–120 (1982)
PGM	2,3BisPGA:2PGA phosphotransferase (EC 5.4.2.1)	3PGA → 2PGA	2,3-PGA	4	**42,** 435–450 (1975)
ENO	2PGA hydro-lyase (EC 4.2.1.11)	2PGA → PEP + H_2O	M^{2+}	2	**9,** 670–679 (1966)
PK	ATP:pyruvate O^2-phosphotransferase (EC 2.7.1.40)	PEP + ADP → pyruvate + ATP	K^+ Mg^{2+}	4	**42,** 176–182 (1975)

[a]The enzymes are listed according to their sequential order in the glycolytic pathway. Abbreviations are defined in the text except FBP (D-fructose 1,6-bisphosphate), Ga3P (glyceraldehyde 3-phosphate), and DHAP (dihydroxyacetone phosphate). References for the purification and characterization of *Sacch. cerevisiae* enzymes (except TPI, from *Saccharomyces carlesbergensis*) are taken from *Methods in Enzymology*.

and Katsume, 1979). Yeast HK exists as either a monomer or a dimer in response to allosteric effectors (Derechin *et al.*, 1972; Shill *et al.*, 1974). Most strikingly, the dimeric enzyme exhibits nonsymmetric interaction of subunits (Steitz *et al.*, 1976). Another allosteric enzyme, yeast PFK, is an octamer consisting of distinct regulatory (α) and catalytic (β) subunits, $\alpha_4\beta_4$ (Plietz *et al.*, 1978). The substrate F6P is homotropic. ATP is a negative effector while NH_4^+, AMP, and D-fructose 2,6-bisphosphate are positive effectors. Conformation changes in PFK that accompany the binding of allosteric effectors, F6P and ATP, have been demonstrated (Laurent *et al.*, 1984). Phosphoglycerate kinase and PK catalyze the two ATP-forming reactions in the glycolytic pathway. The primary sequences of yeast PGK (Hitzeman *et al.*, 1982) and PK (Burke *et al.*, 1983) genes have been elucidated. The cloned PK gene is regulated by D-glucose (Burke *et al.*, 1983). Crystallographic studies of yeast PGK (Bryant *et al.*, 1974) and HK (Steitz *et al.*, 1976) indicate a central pleated β sheet flanked by α helices as the conserved nucleotide binding site for kinases.

The fifth phosphotransferase, phosphoglycerate mutase (PGM), mediates the interconversion between 3PGA and 2-phosphoglycerate (2PGA), requiring 2,3-bisphosphoglycerate (2,3-PGA) as a cofactor. However, PGM enzymes from *S. pombe* and from *Sacch. cerevisiae* differ in that the fission yeast enzyme is a monomer with a molecular weight of 23K (Price *et al.*, 1985) whereas the budding yeast PGM is a tetramer with a subunit weight of 28K (Fothergill and Harkins, 1982). Chemical modifications suggest that PGM from *S. pombe* is similar to that from the budding yeast in possessing one or more essential histidine residues (Price *et al.*, 1985). The three-dimensional structure of the tetrameric PGM has been determined (Campbell *et al.*, 1974).

Two isomerases, phosphoglucose isomerase (PGI, glucose-6-phosphate ketol-isomerase) and triose-phosphate isomerase (TPI), facilitate aldose–ketose interconversion via an enediol intermediate. The X-ray crystallographic structure of yeast TPI (Alber *et al.*, 1981) and the nucleotide sequence of its gene (Alber and Kawasaki, 1982) have been determined.

One reaction each is catalyzed by lyase, oxidoreductase, and hydrolase. Aldolase cleaves the C—C bond of D-fructose 1,6-bisphosphate to yield triose phosphates. Glyceraldehyde-3-phosphate dehydrogenase (TDH for triose-phosphate dehydrogenase) catalyzes a hydride transfer from C-1 of glyceraldehyde 3-phosphate to NAD^+ with a concomitant phosphorylation to form 1,3-bisphosphoglycerate. The nucleotide sequence of the TDH gene from *Sacch. cerevisiae* has been determined (Holland and Holland, 1979). Three structural genes code for catalytically active TDH (McAlister and Holland, 1985). The relative proportions of

expression of each gene are not significantly affected by the carbon source of the growth medium.

Enolase (ENO) effects the dehydration of 2PGA to PEP. Two yeast ENO genes encode for the enzymes which show 95% homology in their amino acid sequences (Holland *et al.*, 1981). One ENO gene is constitutively expressed while the other is inductively expressed in the presence of D-glucose (McAlister and Holland, 1982).

An alternative pathway to glycolysis as a means of oxidizing D-glucose is the pentose phosphate pathway, which is characterized by the overall reaction:

$$6\ \text{D-Glucose 6-phosphate} + 12\ NADP^+ + 6\ H_2O \rightarrow$$
$$5\ \text{hexose 6-phosphate} + 12\ NADPH + 12\ H^+ + 6\ CO_2 + H_3PO_4$$

Energy is channeled to the production of NADPH necessary for reductive syntheses, and in *Sacch. cerevisiae* the pentose phosphate pathway accounts for the production of almost all the cellular requirements for NADPH (Lagunas and Gancedo, 1973). The pathway consists of an initial oxidative decarboxylation of D-glucose 6-phosphate (G6P) and a series of interrelated reactions that interconvert three-, four-, five-, six-, and seven-carbon sugars. The presence of the pentose phosphate pathway is implicated in *S. pombe* by its ability to ferment D-xylulose to ethanol (Wang *et al.*, 1980; Ueng *et al.*, 1981). Unlike the case for *Sacch. cerevisiae* which also reduces xylulose to xylitol, glycitol production in the xylulose fermentation by *S. pombe* is minimal. Ethanol does not seem to affect the rate of xylulose uptake but inhibits its own production (Roman *et al.*, 1984).

Glucose-6-phosphate dehydrogenase mediates the hydride transfer from C-1 of G6P to $NADP^+$ yielding 6-phosphogluconolactone which is hydrolyzed to 6-phosphogluconate by gluconolactonase (Table II). 6-Phosphogluconate dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconate to D-ribulose 5-phosphate presumably via a 2-oxo acid intermediate. Two enzymes, phosphopentose isomerase (ribose-5-phosphate ketol-isomerase) which isomerizes the carbonyl group of pentoses and phosphopentose-3-epimerase which inverts the configuration of the 3-hydroxyl group, interconvert ketopentose phosphate and aldopentose phosphate. The remaining interconverting steps, which function to rearrange the carbon skeleton of six pentoses to five hexoses, are catalyzed by transketolase and transaldolase.

Besides D-glucose, yeasts may utilize glycerol as a carbon and energy source under aerobiosis. Utilization of glycerol by *Sacch. cerevisiae* involves a glycerol kinase and a glycerol-3-phosphate dehydrogenase which

TABLE II

Yeast Enzymes of the Pentose Phosphate Pathway[a]

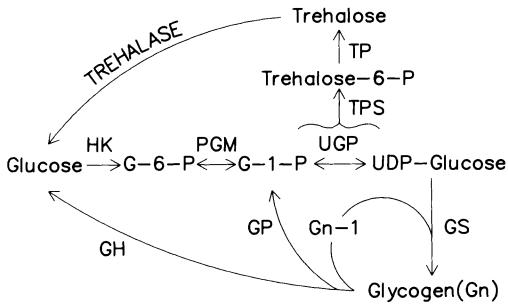
Enzyme	Systematic name	Reaction	Reference (*Methods in Enzymology*)
Glucose-6-phosphate dehydrogenase	G6P:$NADP^+$ 1-oxidoreductase (EC 1.1.1.49)	G6P + $NADP^+$ → glucono-1,5-lactone 6P + NADH	**9,** 116–125 (1966)[b]
Gluconolactonase	D-Glucono-1,5-lactone lactonohydrolase (EC 3.1.1.17)	Glucono-1,5-lactone 6P + H_2O → 6P-gluconate	
6-Phosphogluconate dehydrogenase	6P-Gluconate:$NADP^+$ 2-oxidoreductase (EC 1.1.1.44)	6P-Gluconate + $NADP^+$ → Ru5P + CO_2 + NADPH	**41,** 237–240 (1975)[c]
Phosphopentose isomerase	D-Ribose-5P ketol-isomerase (EC 5.3.1.6)	Ru5P → R5P	**41,** 427–429 (1975)[c]
Phosphopentose-3-epimerase	D-Ribulose-5P:3-epimerase (EC 5.1.3.1)	Ru5P → Xu5P	**9,** 605–608 (1966)[b]
Transketolase	Sedoheptulose-7P:glyceralde-3P glycolaldehyde transferase (EC 2.2.1.1)	R5P + Xu5P → Su7P + Ga3P	**90,** 209–223 (1982)[b]
Transaldolase	Sedoheptulose-7P:glyceralde-3P glyceronetransferase (EC 2.2.1.2)	Su7P + Ga3P → E4P + F6P	**42,** 290–297 (1975)[c]
Triose-phosphate isomerase	D-Glyceralde-3P ketol-isomerase (EC 5.3.1.1)	Ga3P → DHAP	**41,** 434–438 (1975)[d]

[a]References for the purification and characterization of enzymes are taken from *Methods in Enzymology*.
[b]*Saccharomyces cerevisiae*.
[c]*Candida utilis*.
[d]*Saccharomyces carlesbergensis*.

convert glycerol to dihydroxyacetone phosphate (DHAP) via glycerol 3-phosphate (Sprague and Cronan, 1977). The fission yeast *S. pombe* lacks glycerol kinase and, therefore, utilizes glycerol through a different pathway. Glycerol is first dehydrogenated to dihydroxyacetone which is phosphorylated to DHAP by dihydroxyacetone kinase (Gancedo *et al.*, 1986; Vasiliadis *et al.*, 1987). Glycerol dehydrogenase from *S. pombe* reversibly and specifically oxidizes C-2 of glycerol to produce dihydroxyacetone. A number of *vic*-diols are also susceptible to oxidation (Marshall *et al.*, 1985). The fission yeast dihydroxyacetone kinase is a tetramer and highly specific for dihydroxyacetone as the phosphoryl group acceptor (Marshall *et al.*, 1986).

In response to nutrient limitation, D-glucose is converted to glycogen and trehalose as reserve carbohydrates in yeasts (Kuenzi and Fiechter, 1972; Lillie and Pringle, 1980) (Scheme 1). Biosynthesis of glycogen is catalyzed by hexokinase (HK), phosphoglucomutase (PGM), UDPglucose pyrophosphorylase (UGP), and glycogen synthase (GS) (Manners, 1971). The phosphorylation–dephosphorylation interconverts two forms of yeast GS which differ in the their sensitivity to G6P and adenine nucleotide regulations (Rothman-Denes and Cabib, 1971; Huang and Cabib, 1974). The regeneration of D-glucose from glycogen is mediated by glycogen phosphorylase (GP) (Fosset *et al.*, 1971), which is activated by phosphorylation (Wingender-Drissden and Becker, 1983), and glycogen hydrolase (GH) (Colonna and Magee, 1978).

Biosynthesis of trehalose is catalyzed by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphatase (TP) (Panek, 1985). Trehalase effects the hydrolysis of trehalose to D-glucose. Yeasts produce two types of trehalases, an acidic pH-optimal, heat-stable nonregulatory enzyme and a neutral pH-optimal regulatory enzyme activated by cAMP-dependent phosphorylation (Thevelein, 1984). Both trehalases are found in



Scheme 1.

Sacch. cerevisiae while only the nonregulatory trehalase in *S. pombe* is reported to increase in its activity during sporulation (Inoue and Shimoda, 1981).

B. Ultimate Carbon Catabolism

All yeast cells are capable of catabolically utilizing D-glucose respiratively and fermentatively (Fiechter *et al.*, 1981; Tsai *et al.*, 1987). The respirative and fermentative pathways share identical intermediary metabolic steps as far as the formation of pyruvate; however, the ultimate fates of pyruvate differ. In the respirative pathway, pyruvate is converted to acetyl-CoA which is oxidized to carbon dioxide and water by the tricarboxylic acid (TCA) cycle. The entry into the TCA cycle via acetyl-CoA is mediated by the pyruvate dehydrogenase complex (PDC) (Reed and Cox, 1966).

Pyruvate dehydrogenase complex has been purified from budding yeast (Kresze and Ronft, 1981a). Most of the structural, kinetic, and regulatory properties of yeast PDC (Kresze and Ronft, 1981b) are very similar to those reported for mammalian PDC. The yeast complex consists of pyruvate dehydrogenase (PDH) which catalyzes the decarboxylation of pyruvate via thiamin pyrophosphate (TPP), lipoate acetyltransferase (LAT) which facilitates the transfer of acetyl group of the decarboxylated pyruvate to coenzyme A (CoA) via protein-bound lipoamide ($LipS_2$), and lipoamide dehydrogenase (LDH) which regenerates $LipS_2$ from $Lip(SH)_2$. Lipoamide dehydrogenase is a flavoenzyme containing a redox-active sulfide at the catalytic site. The gene encoding yeast LDH has been cloned (Roy and Dawes 1987).

The TCA cycle as a catabolic pathway oxidizes carbon atoms of acetyl-CoA completely to carbon dioxide (see Table III):

$$\text{Acetyl-CoA} + \text{ADP} + H_3PO_4 + H_2O + 3\ \text{NAD}^+ + \text{FAD} \rightarrow$$
$$2\ CO_2 + \text{ATP} + 3\ \text{NADH} + 3\ H^+ + \text{FAD} \cdot H_2 + \text{CoA}$$

Four oxidoreductases which provide the oxidative activity as well as two hydrolases, one each of lyase and ligase, constitute the cycle. Some of these TCA cycle enzymes have been demonstrated in *S. pombe* (Poole and Lloyd, 1973; Flury *et al.*, 1974; C. S. Tsai, unpublished observation). All eight enzymes from various sources exhibit nearly absolute specificities for their substrates and products.

In the aldol condensation of acetyl-CoA with oxaloacetate catalyzed by citrate synthase, the acetyl moiety of acetyl-CoA attacks the *si* face of the oxo group of oxaloacetate. The condensation is inhibited by ATP. Aconitate hydratase catalyzes the stereoselective interconversion among ci-

TABLE III

Enzymes of the Tricarboxylic Acid Cycle[a]

Enzyme	Systematic name	Reaction	Cofactor	ΔG^{0} at pH 7 (kJ mol^{-1})
Citrate synthase	Citrate oxaloacetate-lyase (EC 4.1.3.7)	Acetyl-CoA + oxaloacetate + $H_2O \rightarrow$ citrate + CoA	Mg^{2+}	−38.0
Aconitase	Citrate(isocitrate) hydro-lyase (EC 4.2.1.3)	Citrate → *cis*-aconitate → isocitrate	Fe^{2+}	+6.65
Isocitrate dehydrogenase	Isocitrate:NAD^+ oxidoreductase (decarboxylating) (EC 1.1.1.41)	Isocitrate + $NAD^+ \rightarrow$ 2-oxoglutarate + NAD + CO_2	Mn^{2+}	−7.11
2-Oxoglutarate dehydrogenase complex	2-Oxoglutarate:CoA oxidoreductase (decarboxylating and succinylating) (EC 1.2.4.2)	2-Oxoglutarate + NAD^+ + CoA → succinyl-CoA + CO_2 + NADH	Thiamin pp lipoamide	−36.9
Succinyl-CoA synthetase	Succinate:CoA ligase (GDP-forming) (EC 6.2.1.4)	Succinyl-CoA + GDP + $P_i \rightarrow$ succinate + GTP + CoA	Mg^{2+}	−3.35
Succinate dehydrogenase	Succinate:(acceptor) oxidoreductase (EC 1.3.99.1)	Succinate + acceptor → fumarate + reduced acceptor	FAD, Fe^{2+}	0
Fumarase	L-Malate hydro-lyase (EC 4.2.1.2)	Fumarate + $H_2O \rightarrow$ L-malate	None	−3.68
Malate dehydrogenase	L-Malate:NAD^+ oxidoreductase (EC 1.1.1.37)	L-Malate + $NAD^+ \rightarrow$ oxaloacetate + NADH	None	−28.0

[a]The enzymes are listed according to their sequential order in the TCA cycle from the entry of acetyl-CoA.

trate, *cis*-aconitate, and (*2R,3S*)-isocitrate which is oxidized to 2-oxoglutarate by isocitrate dehydrogenase. Of NAD^+- and $NADP^+$-linked isocitrate dehydrogenases, the NAD^+-dependent enzyme participates in the TCA cycle. Yeast isocitrate dehydrogenase is an allosteric enzyme which is activated by AMP and inhibited by citrate (Sols *et al.*, 1971). Fission yeast cells grown in a chemostat culture containing D-glucose and acetate exhibit higher isocitrate dehydrogenase activities than cells grown in a D-glucose-limited chemostat culture (C. S. Tsai and Mitton, unpublished observation). 2-Oxoglutarate undergoes oxidative decarboxylation to form succinyl-CoA by the action of the 2-oxoglutarate dehydrogenase complex in a sequence of reactions analogous to PDC catalysis. Succinyl-CoA is cleaved to succinate with formation of ATP by succinyl-CoA synthetase. The trans dehydrogenation of succinate by succinate dehydrogenase yields fumarate. A trans addition of water to fumarate by fumarate hydratase forms L-malate [(*2S*)-malate] which is oxidized to oxaloacetate by L-malate dehydrogenase (MDH). Succinate dehydrogenase, fumarate hydratase, and MDH show overall exponential increases in their activities by rising to two peaks per cell cycle in synchronous cultures of *S. pombe* (Poole and Lloyd, 1973). The high L-malate oxidizing activity of *S. pombe* has been investigated for a possible application in wine deacidification (Snow and Gallander, 1979). Two MDH isozymes which differ in their isoelectric points are elaborated in response to media D-glucose concentration in *S. pombe* (Flury *et al.*, 1974).

The TCA cycle is an amphibolic pathway which functions in both the catabolic generation of ATP and the anabolic synthesis of biomolecular precursors. The glyoxalate cycle serves as a bypass from the TCA cycle to replenish C_4 intermediates for yeasts capable of anaplerotic utilization of C_2 compounds (Kornberg and Elsden, 1961). Two enzymes, isocitrate lyase which cleaves isocitrate to glyoxalate and succinate and malate synthase which synthesizes L-malate from glyoxalate and acetyl-CoA, provide the bypass. These enzymes are induced by C_2 compounds (Gonzalez, 1977) and begin to appear in budding yeast cells only after D-glucose has been consumed (Haarasilta and Oura, 1975). Succinate and oxaloacetate are feedback inhibitors of isocitrate lyase, which is subject to a reversible catabolic inactivation by D-glucose (Lopez-Boado *et al.*, 1987). In addition, oxaloacetate inhibits malate synthase. The two glyoxalate cycle enzymes have not been demonstrated in *S. pombe* (Fiechter *et al.*, 1981; C. S. Tsai and Mitton, unpublished observation) although the fission yeast is capable of utilizing acetate and ethanol in the presence of D-glucose (Tsai *et al.*, 1987).

The coupled transfer of displaced electrons by the TCA cycle in the form of NADH via the mitochondrial respiratory chain to molecular oxy-

gen regenerates NAD^+ and yields ATP. Some respiratory cytochromes and enzymes have been demonstrated in *S. pombe* (Heslot *et al.*, 1970; Poole and Lloyd, 1973). Phosphorylation occurs at two sites with the consequent synthesis of 2 mol ATP per mole of NADH oxidized in *S. pombe* and *Sacch. cerevisiae* (Heslot *et al.*, 1970; Sols *et al.*, 1971; Mackler and Haynes, 1973).

In alcohol fermentation, pyruvate is converted to ethanol via acetaldehyde. Pyruvate decarboxylase mediates the formation of acetaldehyde by an analogous mechanism catalyzed by the PDH component of PDC. Alcohol dehydrogenase (ADH) catalyzes the hydride transfer from NADH to acetaldehyde to form ethanol. The dehydrogenase exists as three distinct isozymes, one mitochondrial and two cytosolic forms, in *Sacch. cerevisiae* (Lutstorf and Megnet, 1968). The cytosolic isozyme, ADH-I (high K_m for ethanol) is constitutive and primarily responsible for the production of ethanol (Wills, 1976). Low-K_m ADH-II is gluconeogenic and repressed by D-glucose (Lutstorf and Megnet, 1968). The mitochondrial isozyme, mADH, found in aerobically grown yeast cells accounts for the ability of budding yeast mitochondria to utilize ethanol as a substrate (Wenger and Bernofsky, 1971). By contrast, *S. pombe* contains only one ADH (Megnet, 1967). The nucleotide sequences for genes encoding the budding yeast ADH-I (Bennetzen and Hall, 1982), ADH-II (Russell *et al.*, 1983), and the fission yeast ADH (Russell and Hall, 1983) have been determined. The amino acid sequences of these yeast ADH have extensive similarity (51%), and, in particular, the sequences around the catalytic site are highly conserved.

C. Regulation of Carbon Metabolism

D-Glucose is metabolized by *S. pombe* fermentatively and respiratively. The pure fermentative glucose catabolism occurs only under anaerobic conditions. Ethanol production is accompanied by inefficient energy production. The respirative glucose catabolism is active in aerobic continuous cultures at low dilution rates (McDonald *et al.*, 1987). Since D-glucose is completely oxidized to CO_2 and H_2O, the energy production is efficient. At high dilution rates in aerobic continuous cultures both respirative and fermentative pathways are possible, and the growth is respirofermentative (Käppeli, 1986; McDonald *et al.*, 1987). Aerobic ethanol production is accompanied by respirative oxidation of D-glucose. Activities of respiratory enzymes are lower in cells exhibiting respirofermentative glucose metabolism as compared to cells growing respiratively (Käppeli and Sonnleitner, 1986). Since pyruvate is the common interme-

diate channeled through the respirative and fermentative pathways via pyruvate decarboxylase and the PDC, the concentration of pyruvate and relative activities of the two enzymes are likely determinants of the relative preference for the fermentative and respirative pathways.

To adjust the rate of D-glucose utilization to the metabolic requirements of yeast cells, the fermentative and respirative pathways are mutually regulatory. Thus, a repression of glycolysis by oxygen is termed the Pasteur effect or oxygen effect, whereas a repression of respiration by D-glucose is known as the Crabtree effect or *glucose effect* (Fiechter *et al.*, 1981). These represent the repression of one energy metabolism by another.

The universal energy currency in biological systems is ATP, which is interconverted with ADP or AMP between energy-yielding catabolic and energy-consuming anabolic pathways. Enzymes that either regenerate or utilize ATP are likely susceptible to allosteric regulation by ATP, ADP, or AMP of the adenylate pool, defined as the energy charge, (ATP + $\frac{1}{2}$ADP)/(ATP + ADP + AMP) (Atkinson, 1968, 1969). In this regard, NAD^+-dependent isocitrate dehydrogenase is activated by AMP. Similarly, PFK is subject to allosteric regulation, with ATP and citrate acting as inhibitors while ADP and AMP function as activators. Since respiration is linked to the TCA cycle and phosphorylation, a resulting increase in citrate and ATP concentrations represses the rate of glycolysis by inhibiting PFK. This provides a tenable explanation for the Pasteur effect (Sols *et al.*, 1971). However, the Pasteur effect is almost unnoticeable in growing yeast cells. It is observed in yeast cells only under special experimental conditions such as growth in the presence of a low concentration of D-glucose in chemostat culture or in the resting state (Lagunas, 1979). At the onset of the Pasteur effect, the reduced rate of fermentation presumably is due to an inactivation in the sugar transport systems in resting cells or a limitation of the D-glucose concentration in chemostat cultures (Lagunas, 1986).

Schizosaccharomyces pombe is sensitive to D-glucose and as such the fission yeast is subject to the imposition of and release from D-glucose repression (McDonald *et al.*, 1987). In growing *S. pombe* cells, D-glucose decreases the activity of catabolite-sensitive enzymes (Schlanderer and Dellweg, 1974). Activities of several TCA cycle and respiratory enzymes increase with D-glucose derepression (Poole and Lloyd, 1973). The repressed fission yeast cells contain one MDH isozyme with an isoelectric point of 6.4 whereas the fully derepressed cells elaborate another MDH isozyme with an isoelectric point of 5.4 (Flury *et al.*, 1974).

Although the glucose effect in yeasts refers to the catabolite repression or inactivation of certain enzymes and the facilitated ethanol fermentation

by D-glucose, the two processes do not necessarily originate from an identical mechanism. Catabolite repression or inactivation is the reduction in the synthesis or activities of key enzymes in the repressed pathways by excess D-glucose (Paigen and Williams, 1970). In *Sacch. cerevisiae*, an excess of D-glucose elicits catabolite repression/inactivation by repressing certain enzymes of gluconeogenesis (Haarasila and Oura, 1975; Gancedo and Schwerzmann, 1976; Mazon *et al.*, 1982; Herrero *et al.*, 1985), the TCA cycle (Polakis and Bartley, 1965; Ferguson *et al.*, 1967; Fiechter *et al.*, 1981), the electron-transfer chain (Fiechter *et al.*, 1981; Herrero *et al.*, 1985), the glyoxalate bypass (Witt *et al.*, 1966; Lopez-Boado *et al.*, 1987), and the sugar transport system (Busturia and Lagunas, 1986). Cyclic adenosine 3′,5′-monophosphate (cAMP) is implicated particularly in the catabolic repression–inactivation of gluconeogenic enzymes (Lopez-Boado *et al.*, 1987; Francois *et al.*, 1987). However, there is no compelling evidence for the involvement of cAMP in mediating yeast catabolite repression in general (Matsumoto *et al.*, 1983; Eraso and Gancedo, 1984). Since 2-deoxyglucose and glucosamine, which are phosphorylated by ATP but not catabolized by yeasts, also exhibit the repressing effect (Witt *et al.*, 1966), the glucose repression is likely caused by D-glucose itself or by the energy charge-related process(es). Genetic studies of *Sacch. cerevisiae* reveal that the regulatory domain of a hexokinase isozyme, HK PII, is responsible for expressing the glucose repression (Entian and Fröhlich, 1984; Niederacher and Entian, 1987).

Aerobically, ethanol fermentation competes with oxidative respiration for the common intermediate, pyruvate. Since PDC exhibits a higher affinity for pyruvate than pyruvate decarboxylase (Kresze and Ronft, 1981a; Banuelos and Gancedo, 1978), respiration is favored at low glucose concentration because of a low yield in pyruvate. On the other hand, since activities of the TCA cycle enzymes (Lowenstein, 1967) and respiration (Lagunas, 1979) are relatively low in yeast cells, the rate of respiration may reach the maximum rate (capacity) at high D-glucose concentrations (Barford and Hall, 1979; Rieger *et al.*, 1983). This leads to an overflow of pyruvate as the glycolytic flux exceeds the maximum possible respiratory flux, thus resulting in ethanol production (Käppeli, 1986; Käppeli and Sonnleitner, 1986). A less favored fermentative path operates to handle the excess carbon flow when the respiratory path has been saturated. Thus, yeast cells grow respiratively in aerobic glucose-limited continuous cultures at low dilution rates because D-glucose is supplied at levels below that supporting the maximum rate of respiration. In aerobic continuous cultures at high dilution rates, the rate of D-glucose supply exceeds the maximum rate of glucose consumption by respiration; therefore, yeast cells grow respirofermentatively (Barford, 1985b; Sonnleitner

and Käppeli, 1986). The excess D-glucose causes the ethanol accumulation by virtue of oversaturating the respiratory capacity and the catabolite repression–inactivation by mechanisms yet to be fully elucidated.

REFERENCES

Agar, D. W., and Bailey, J. E. (1981a). Continuous cultivation of fission yeast: Classical and flow microfluorimetry observations. *Biotechnol. Bioeng.* **23,** 2217–2229.

Agar, D. W., and Bailey, J. E. (1981b). Continuous cultivation of fission yeast: Analysis of single cell protein synthesis kinetics. *Biotechnol. Bioeng.* **23,** 2315–2331.

Alber, T., and Kawasaki, G. (1982). Nucleotide sequence of the triose phosphate isomerase gene of *Saccharomyces cerevisiae*. *J. Mol. Appl. Genet.* **1,** 419–434.

Alber, T., Banner, D. W., Bloomer, A. S., Petska, G. A., Phillip, D., Rivers, P. S., and Wilson, I. A. (1981). On the three-dimensional structure and catalytic mechanism of triose phosphate isomerase. *Philos. Trans. R. Soc. London B* **293,** 159–171.

Atkinson, D. E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifier. *Biochemistry* **7,** 4030–4034.

Atkinson, D. E. (1969). Regulation of enzyme function. *Annu. Rev. Microbiol.* **23,** 47–68.

Axelrod, B. (1967). Glycolysis. *In* "Metabolic Pathways" (D. M. Greenberg, ed.), 3rd ed., Vol. 1, pp. 112–145. Academic Press, New York.

Banuelos, M., and Gancedo, C. (1978). *In situ* study of the glycolytic pathway in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **117,** 197–201.

Barford, J. P. (1985a). Analysis of the variation of cell length during the cell cycle of *Schizosaccharomyces pombe* using an asynchronous chemostat culture. *J. Gen. Appl. Microbiol.* **31,** 87–92.

Barford, J. P. (1985b). Control of fermentation and respiration in *Schizosaccharomyces pombe*. *J. Ferment. Technol.* **63,** 555–558.

Barford, J. P., and Hall, R. J. (1979). An examination of the Crabtree effect in *Saccharomyces cerevisiae:* The role of respiratory adaptation. *J. Gen. Microbiol.* **114,** 267–275.

Barnett, J. A. (1976). The utilization of sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **32,** 125–234.

Bennetzen, J. L., and Hall, B. D. (1982). The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. *J. Biol. Chem.* **257,** 3018–3025.

Bisson, L. F., and Fraenkel, D. G. (1983). Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **30,** 1730–1734.

Bisson, L. F., Neigeborn, L., Carlson, M., and Fraenkel, D. G. (1987). The *SNF3* gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* **169,** 1656–1662.

Brändli, E. (1980). Untersuchungen zur Regulation and Funktion der Malat-Dehydrogenase in *Schizosaccharomyces pombe*. Thesis, Eidgenössiche Technische Hochschule, Zurich, No. 6549.

Brown, C. M., Burn, V. J., and Johnson, B. (1973). Presence of glutamate synthetase in fission yeasts and its possible role in ammonia assimilation. *Nature (London) New Biol.* **246,** 115–116.

Bryant, T. N., Watson, H. C., and Wendell, P. L. (1974). Structure of yeast phosphoglycerate kinase. *Nature (London)* **247,** 14–17.

Burke, R. L., Tekamp-Olson, P., and Najarian, R. (1983). The isolation, characterization

and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **258,** 2193–2201.

Burn, V. J., Turner, P. R., and Brown, C. M. (1974). Aspects of inorganic nitrogen assimilation in yeasts. *Antonie van Leeuwenhoek* **40,** 93–102.

Busturia, A., and Lagunas, R. (1986). Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **132,** 379–385.

Calcott, P. H. (1981). *In* "Continuous Culture of Cells" (P. H. Calcott, ed.), Vol. 1, pp. 13–26. CRC Press, Boca Raton, Florida.

Campbell, J. W., Watson, H. C., and Hodgson, G. I. (1974). Structure of yeast phosphoglycerate mutase. *Nature (London)* **250,** 301–303.

Cirillo, V. P. (1961). Sugar transport in microorganisms. *Annu. Rev. Microbiol.* **15,** 197–218.

Colonna, W. J., and Magee, P. T. (1978). Glycogenolytic enzymes in sporulating yeast. *J. Bacteriol.* **134,** 844–853.

Dawson, P. S. S. (1965). Continuous phased growth with a modified chemostat. *Can. J. Microbiol.* **11,** 893–903.

Dawson, P. S. S. (1972). Continuously synchronized growth. *J. Appl. Chem. Biotechnol.* **22,** 79–103.

Derechin, M., Rustum, Y. M., and Barnard, E. A. (1972). Dissociation of yeast hexokinase under the influence of substrates. *Biochemistry* **11,** 1793–1797.

Entian, K. D., and Fröhlich, K. U. (1984). *Saccharomyces cerevisiae* mutants provide evidence of hexokinase PII as a bifunctional enzyme with catalytic and regulatory domains for triggering carbon catabolite repression. *J. Bacteriol.* **158,** 29–35.

Eraso, P., and Gancedo, J. M. (1984). Catabolite repression in yeasts is not associated with low levels of cAMP. *Eur. J. Biochem.* **141,** 195–198.

Evans, C. G. T., Herbert, D., and Tempest, D. W. (1970). The continuous cultivation of microorganisms. 2. Construction of a chemostat. *Methods Microbiol.* **2,** 277–327.

Fantes, P., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. *Exp. Cell Res.* **107,** 377–386.

Ferguson, J. J., Jr., Ball, M., and Holzer, H. (1967). Yeast malate dehydrogenase: Enzyme inactivation in catabolite repression. *Eur. J. Biochem.* **1,** 21–25.

Fiechter, A., Fuhrman, G. F., and Käppeli, O. (1981). Regulation of glucose metabolism in growing yeast cells. *Adv. Microbiol. Physiol.* **22,** 123–183.

Flury, U., Heer, B., and Fiechter, A. (1974). Isozyme pattern of malate dehydrogenase during respiratory derepression in *Schizosaccharomyces pombe*. *Arch. Microbiol.* **97,** 141–148.

Fosset, M., Muir, L. W., Nielsen, L. D., and Fischer, E. F. (1971). Purification and properties of yeast glycogen phosphorylase *a* and *b*. Biochemistry **10,** 4105–4113.

Fothergill, L. A., and Harkins, R. N. (1982). The amino acid sequence of yeast phosphoglycerate mutase. *Proc. R. Soc. London B* **125,** 19–44.

Francois, J., Eraso, P., and Gancedo, C. (1987). Changes in the concentration of cAMP, fructose 2,6-bisphosphate and related metabolites and enzymes in *Saccharomyces cerevisiae* during growth on glucose. *Eur. J. Biochem.* **164,** 369–373.

Gancedo, C., and Schwerzmann, K. (1976). Inactivation by glucose of phosphoenol-pyruvate carboxykinase from *Saccharomyces cerevisiae*. *Arch. Microbiol.* **109,** 221–225.

Gancedo, C., Llobell, A., Ribas, J.-C., and Luchi, F. (1986). Isolation and characterization of mutants from *Schizosaccharomyces pombe* defective in glycerol metabolism. *Eur. J. Biochem.* **159,** 171–174.

Gonzalez, E. (1977). Two-carbon assimilative capacity and the induction of isocitrate lyase in *Saccharomyces cerevisiae*. *J. Bacteriol.* **129,** 1343–1348.

Haarasilta, S., and Oura, E. (1975). On the activity and regulation of anaplerotic and gluco-

neogenetic enzymes during the growth process of bakers' yeast. The biphasic growth. *Eur. J. Biochem.* **52,** 1–7.

Herbert, D., Ellsworth, R., and Telling, R. C. (1956). The continuous culture of bacteria; a theoretical and experimental study. *J. Gen. Microbiol.* **14,** 601–622.

Herrero, P., Fernandez, R., and Moreno, F. (1985). Differential sensitivities to glucose and galactose repression of gluconeogenic and respiratory enzymes from *Saccharomyces cerevisiae*. *Arch. Microbiol.* **143,** 216–219.

Heslot, H., Goffeau, A., and Louis, C. (1970). Respiratory metabolism of a petit negative yeast *Schizosaccharomyces pombe* 972 h^-. *J. Bacteriol.* **104,** 473–481.

Hitzeman, R. A., Hagie, F. E., Hayflick, J. S., Chen, C. Y., Seeburg, P. H., and Derynck, R. (1982). The primary structure of the *Saccharomyces cerevisiae* gene for 3-phosphoglycerate kinase. *Nucleic Acids Res.* **10,** 7791–7808.

Holland, J. P., and Holland, M. J. (1979). The primary structure of a glyceraldehyde-3-phosphate dehydrogenase gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **254,** 9839–9845.

Holland, M. J., Holland, J. P., Thill, G. P., and Jackson, K. A. (1981). The primary structures of two yeast enolase genes. *J. Biol. Chem.* **256,** 1385–1395.

Huang, K. P., and Cabib, E. (1974). Yeast glycogen synthetase in the glucose 6-phosphate dependent form. *J. Biol. Chem.* **249,** 3852–3857.

Hunsley, J. R., and Suelter, C. H. (1969). Yeast pyruvate kinase II. Kinetic properties. *J. Biol. Chem.* **244,** 4819–4822.

Inoue, H., and Shimoda, C. (1981). Changes in trehalose content and trehalase activity during spore germination in fission yeast, *Schizosaccharomyces pombe*. *Arch. Microbiol.* **129,** 19–22.

Johnson, B., and Brown, C. M. (1974). The enzymes of ammonia assimilation in *Schizosaccharomyces* spp. and in *Saccharomyces ludwigii*. *J. Gen. Microbiol.* **85,** 169–172.

Johnson, B. F., and McDonald, I. J. (1983). Cell division: A separable cellular sub-cycle in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **129,** 3411–3419.

Johnson, B. F., Calleja, G. B., Boisclair, I., and Yoo, B. Y. (1979). Cell division in yeasts. III. The biased, asymmetric location of the septum in the fission yeast cell *Schizosaccharomyces pombe*. *Exp. Cell Res.* **123,** 253–259.

Johnson, B. F., Calleja, G. B., Yoo, B. Y., Zuker, M., and McDonald, I. J. (1982). Cell division: Key to cellular morphogenesis in the fission yeast, *Schizosaccharomyces*. *Int. Rev. Cytol.* **75,** 167–208.

Käppeli, O. (1986). Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv. Microbiol. Physiol.* **28,** 181–209.

Käppeli, O., and Sonnleitner, B. (1986). Regulation of sugar metabolism in *Saccharomyces*-type yeast: Experimental and conceptual considerations. *CRC Crit. Rev. Biotechnol.* **4,** 299–325.

Käppeli, O., Arreguin, M., and Rieger, M. (1985). The respirative breakdown of glucose by *Saccharomyces cerevisiae:* An assessment of a physiological state. *J. Gen. Microbiol.* **131,** 1411–1416.

Kornberg, H. L., and Elsden, S. R. (1961). The metabolism of 2-carbon compounds by microorganisms. *Adv. Enzymol.* **23,** 401–470.

Kothari, I. R., Martin, G. C., Reilly, P. J., Martin, P. J., and Eakman, J. M. (1972). Estimation of parameters in population models for *Schizosaccharomyces pombe* from chemostat data. *Biotechnol. Bioeng.* **14,** 915–938.

Kresze, G. B., and Ronft, H. (1981a). Pyruvate dehydrogenase complex from bakers' yeast, 1. Purification and some kinetic and regulatory properties. *Eur. J. Biochem.* **119,** 573–579.

Kresze, G. B., and Ronft, H. (1981b). Pyruvate dehydrogenase complex from bakers' yeast,

2. Molecular structure, dissociation and implication for the origin of mitochondria. *Eur. J. Biochem.* **119,** 581–587.

Kuenzi, M. T., and Fiechter, A. (1972). Regulation of carbohydrate composition of *Saccharomyces cerevisiae* under growth limitation. *Arch. Microbiol.* **84,** 254–265.

Lagunas, R. (1979). Energetic irrelevance of aerobiosis for *S. cerevisiae* growing on sugars. *Mol. Cell. Biochem.* **27,** 139–146.

Lagunas, R. (1986). Misconceptions about the energy metabolism of *Saccharomyces cerevisiae*. *Yeast* **2,** 221–228.

Lagunas, R., and Gancedo, C. (1973). Reduced pyridine nucleotide balance in glucose-growing *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **37,** 90–94.

Laurent, M., Tijane, M. N., Roucous, C., Seydoux, F. J., and Tardieu, A. (1984). Solution X-ray scattering studies of the yeast phosphofructokinase allosteric transition. *J. Biol. Chem.* **259,** 3124–3126.

Lillie, S. H., and Pringle, J. R. (1980). Reserve carbohydrate metabolism in *Saccharomyces cerevisiae:* Responses to nutrient limitation. *J. Bacteriol.* **143,** 1384–1394.

Lindner, P. (1893). *Schizosaccharomyces pombe* n. sp., ein neuer Gährungserreger. *Wochenschr. Brau.* **10,** 1298–1300.

Lopez-Boado, Y. S., Herrero, P., Gascon, S., and Moreno, F. (1987). Catabolite inactivation of isocitrate lyase from *Saccharomyces cerevisiae*. *Arch. Microbiol.* **147,** 231–234.

Lowenstein, J. M. (1967). The tricarboxylic acid cycle. *In* "Metabolic Pathways" (D. M. Greenberg, ed.), 3rd ed., Vol. 1, pp. 146–270. Academic Press, New York.

Lutstorf, U., and Megnet, R. (1968). Multiple forms of alcohol dehydrogenase in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **126,** 933–944.

McAlister, L., and Holland, M. J. (1982). Target deletion of yeast enolase structural gene. Identification and isolation of yeast enolase isozymes. *J. Biol. Chem.* **257,** 7181–7188.

McAlister, L., and Holland, M. J. (1985). Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. *J. Biol. Chem.* **260,** 15019–15029.

McAthey, P., and Kilbey, B. J. (1976). *Trichodermin;* a selective agent for screening forward mutations in continuous cultures of *Schizosaccharomyces pombe*. *Biol. Zentralbl.* **95,** 415–421.

McAthey, P., and Kilbey, B. J. (1977). Mutations in continuous cultures of *Schizosaccharomyces pombe*. I. Dependence of the kinetics of mutation accumulation upon the growth-limiting nutrilite. *Mutat. Res.* **44,** 227–234.

McAthey, P., and Kilbey, B. J. (1978). Mutation in continuous cultures of *Schizosaccharomyces pombe*. II. Effect of amino acid starvation on mutational response and DNA concentration. *Mutat. Res.* **50,** 175–180.

McDonald, I. J., Calleja, G. B., and Johnson, B. F. (1982). Conjugation in chemostat cultures of *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **128,** 1981–1987.

McDonald, I. J., Walker, T., Johnson, B. F., Aveledo, A. J., and Tsai, C. S. (1987). Effects of ethanol and acetate on glucose-limited chemostat cultures of *Schizosaccharomyces pombe*, a fission yeast. *Can. J. Microbiol.* **33,** 598–601.

Mackler, B., and Haynes, D. (1973). Studies on oxidative phosphorylation in *Saccharomyces cerevisiae* and *Saccharomyces carlesbergensis*. *Biochim. Biophys. Acta* **292,** 88–91.

Manners, D. J. (1971). The structure and biosynthesis of storage carbohydrates in yeast. *In* "The Yeasts" (A. H. Rose and J. S. Harrison, eds.), Vol. 2, pp. 419–439. Academic Press, New York.

Marshall, J. H., May, J. W., and Sloan, J. (1985). Purification and properties of glycerol:NAD^+ 2-oxidoreductase (glycerol dehydrogenase) from *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **131,** 1581–1588.

Marshall, J. H., May, J. W., Sloan, J., and Vasiliadis, G. E. (1986). Purification and properties of dihydroxyacetone kinase from *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **132,** 2611–2614.

Matsumoto, K., Uno, I., Ishikawa, T., and Oshima, Y. (1983). Cyclic AMP may not be involved in catabolite repression in *Saccharomyces cerevisiae:* Evidence from mutants unable to synthesize it. *J. Bacteriol.* **156,** 898–900.

Mazon, M. J., Gancedo, J. M., and Gancedo, C. (1982). Phosphorylation and inactivation of yeast fructose bisphosphatase *in vivo* by glucose and by proton ionophores. A possible role for cAMP. *Eur. J. Biochem.* **127,** 605–608.

Megnet, R. (1967). Mutants partially deficient in alcohol dehydrogenase in *Schizosaccharomyces pombe*. *Arch. Biochem. Biophys.* **121,** 194–201.

Mitchison, J. M. (1970). Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* **4,** 131–165.

Mitchison, J. M. (1971). "The Biology of the Cell Cycle." Cambridge Univ. Press, London and New York.

Miyata, M., Miyata, H., and Johnson, B. F. (1986). Asymmetric location of the septum in morphologically altered cells of the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **132,** 883–891.

Muratsubaki, H., and Katsume, T. (1979). Distribution of hexokinase isozymes depending on a carbon source in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **86,** 1030–1036.

Neijssel, O. M., and Tempest, D. W. (1979). The physiology of metabolic over-production. *Symp. Soc. Gen. Microbiol. 29th,* pp. 53–82.

Niederacher, D., and Entian, K. D. (1987). Isolation and characterization of the regulatory *HEX2* gene necessary for glucose repression in yeast. *Mol. Gen. Genet.* **206,** 505–509.

Novick, A., and Szilard, L. (1950). Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **36,** 708–719.

Nurse, P., Thuriaux, P., and Naysmith, K. (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **146,** 167–178.

Paigen, K., and Williams, B. (1970). Catabolite repression and other control mechanisms in carbohydrate utilization. *Adv. Microbiol. Physiol.* **4,** 251–334.

Panek, A. D. (1985). Trehalose metabolism and its role in *Saccharomyces cerevisiae*. *J. Biotechnol.* **3,** 121–130.

Plietz, P., Damaschun, G., Kopperschläger, G., and Müller, J. J. (1978). Small-angle X-ray scattering studies on the quarternary structure of phosphofructokinase from bakers' yeast. *FEBS Lett.* **91,** 230–232.

Polakis, E. S., and Bartley, W. (1965). Changes in the enzyme activities of *Saccharomyces cerevisiae* during aerobic growth on different carbon sources. *Biochem. J.* **97,** 284–297.

Poole, R. K., and Lloyd, D. (1973). Oscillation of enzyme activities during the cell-cycle of a glucose-repressed fission yeast *Schizosaccharomyces pombe* 972 h^-. *Biochem. J.* **136,** 195–207.

Price, N. C., Duncan, D., and Ogg, D. J. (1985). Purification and preliminary characterization of phosphoglycerate mutase from *Schizosaccharomyces pombe*. *Int. J. Biochem.* **17,** 843–846.

Reed, L. J., and Cox, D. J. (1966). Macromolecular organization of enzyme systems. *Annu. Rev. Biochem.* **35,** 57–84.

Rieger, M., Käppeli, O., and Fiechter, A. (1983). The role of limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **129,** 653–661.

Roman, G. N., Jansen, N. B., and Tsao, G. T. (1984). Ethanol inhibition of D-xylulose fermentation by *Schizosaccharomyces pombe*. *Biotechnol. Lett.* **6**, 7–12.

Rothman-Denes, L. B., and Cabib, E. (1971). Glucose 6-phosphate dependent and independent forms of yeast glycogen synthetase. Their properties and interconversions. *Biochemistry* **10**, 1236–1242.

Roy, D. J., and Dawes, I. W. (1987). Cloning and characterization of the gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **133**, 925–933.

Russell, P. R., and Hall, B. D. (1983). The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **258**, 143–149.

Russell, D. W., Smith, M., Williamson, V. M., and Young, E. T. (1983). Nucleotide sequence of the yeast alcohol dehydrogenase II gene. *J. Biol. Chem.* **258**, 2674–2682.

Schlanderer, G., and Dellweg, H. (1974). Cyclic AMP and catabolite repression in yeasts. *Eur. J. Biochem.* **49**, 305–316.

Scopers, R. K. (1973). 3-Phosphoglycerate kinase. *Enzymes* **8**, 335–351.

Shill, J. P., Peters, B. A., and Neet, K. E. (1974). Monomer–dimer equilibria of yeast hexokinase during reacting enzyme sedimentation. *Biochemistry* **13**, 3864–3871.

Snow, P. G., and Gallander, J. F. (1979). Deacidification of white table wines through partial fermentation with *Schizosaccharomyces pombe*. *Am. J. Enol. Vitic.* **30**, 45–48.

Sols, A., Gancedo, C., and Dela Fuente, G. (1971). Energy-yielding metabolism in yeasts. *In* "The Yeasts" (A. H. Rose and J. S. Harrison, eds.), Vol. 2, pp. 271–307. Academic Press, New York.

Sonnleitner, B., and Käppeli, O. (1986). Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: Formulation and verification of a hypothesis. *Biotechnol. Bioeng.* **28**, 927–937.

Sprague, G. F., and Cronan, J. E. (1977). Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. *Eur. J. Biochem.* **159**, 171–174.

Steitz, T. A., Flatterick, R. J., Anderson, W. F., and Anderson, C. M. (1976). High resolution X-ray structure of yeast hexokinase, an allosteric protein exhibiting a nonsymmetric arrangement of subunits. *J. Mol. Biol.* **104**, 197–222.

Tempest, D. W. (1970). The place of continuous culture in microbiological research. *Adv. Microbiol. Physiol.* **4**, 223–250.

Tempest, D. W., Meers, J. L., and Brown, C. M. (1970). Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. *Biochem. J.* **117**, 405–407.

Thevelein, J. M. (1984). Regulation of trehalose mobilization in fungi. *Microbiol. Rev.* **48**, 42–59.

Tsai, C. S., Aveledo, A. J., McDonald, I. J., and Johnson, B. F. (1987). Diauxic growth of the fission yeast, *Schizosaccharomyces pombe*, in mixtures of D-glucose and ethanol or acetate. *Can. J. Microbiol.* **33**, 593–597.

Ueng, P. P., Hunter, C. A., Gong, C. S., and Tsao, G. T. (1981). D-Xylulose fermentation in yeasts. *Biotechnol. Lett.* **3**, 315–320.

Uhlinger, D. J., Yang, C. Y., and Reed, L. J. (1986). Phosphorylation–dephosphorylation of pyruvate dehydrogenase from bakers' yeast. *Biochemistry* **25**, 5673–5677.

Van Andel, J., and Brown, C. M. (1977). Ammonia assimilation in the fission yeast *Schizosaccharomyces pombe* 972. *Arch. Microbiol.* **111**, 265–270.

Vasiliadis, G. E., Sloan, J., Marshall, J. A., and May, J. W. (1987). Glycerol and dihydroxyacetone metabolizing enzymes in fission yeast of genus *Schizosaccharomyces*. *Arch. Microbiol.* **147**, 263–267.

Vraná, D. (1983a). Morphological properties of *Schizosaccharomyces pombe* in a continuous culture. *Folia Microbiol.* **28**, 414–419.

Vraná, D. (1983b). The fission yeast *Schizosaccharomyces pombe* in continuous culture. *Biotechnol. Bioeng.* **25,** 1989–1994.

Vraná, D., and Kalasová, M. (1982). Resistance of *Schizosaccharomyces pombe* to mechanical strain caused by mixing in continuous culture. *Folia Microbiol.* **27,** 363–364.

Wang, P. Y., Shopsis, C., and Schnieder, H. (1980). Fermentation of pentose by yeasts. *Biochem. Biophys. Res. Commun.* **94,** 248–254.

Welch, P., and Scopes, R. K. (1985). Studies on cell-free metabolism: Ethanol production by a yeast glycolytic system reconstituted from purified enzyme. *J. Biotechnol.* **2,** 257–273.

Wenger, J. I., and Bernofsky, C. (1971). Mitochondrial alcohol dehydrogenase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **227,** 479–490.

Wickerham, L. J. (1951). "Taxonomy of Yeasts." U.S. Dept. Agric. Tech. Bull. 1029.

Wills, C. (1976). Production of yeast alcohol dehydrogenase isozymes by selection. *Nature (London)* **261,** 26–29.

Wingender-Drissen, R., and Becker, J. U. (1983). Regulation of yeast phosphorylase by phosphorylase kinase and cAMP-dependent kinase. *FEBS Lett.* **163,** 33–36.

Witt, I., Kronau, R., und Holzer, H. (1966). Repression von alkoholdehydrogenase, malatdehydrogenase, isocitratlyase und malatsynthase in hefe durch glucose. *Biochim. Biophys. Acta* **118,** 522–537.

11

Plasma Membrane H^+-ATPase: Ion and Metabolite Transport in the Yeast *Schizosaccharomyces pombe*

ANDRÉ GOFFEAU,* ALAN CODDINGTON,† AND ALAIN SCHLESSER*

*Laboratoire d'Enzymologie
Faculté des Sciences Agronomiques
Université Catholique de Louvain
1348 Louvain-la-Neuve, Belgium

†School of Biological Sciences
University of East Anglia
Norwich NR4 7TJ, England

MOLECULAR BIOLOGY OF THE
FISSION YEAST

I. PLASMA MEMBRANE H^+-ATPase FROM *SCHIZOSACCHAROMYCES POMBE*

A. Early Physiological and Biochemical Properties

In 1973, Foury and Goffeau reported that the addition of cyclic AMP to *Schizosaccharomyces pombe* cells under nutritional step-down conditions stimulates the incorporation of uridine into RNA. It was later shown (Foury and Goffeau, 1975) that this stimulation is due to an increased uptake of uridine. The transport of other nutrients, adenine, amino acids, and even sucrose, is also stimulated under these conditions. Quite surprisingly, it was observed that cyclic AMP concomitantly enhances the glucose-dependent cellular ejection of protons. This process is totally inhibited by Dio-9, an ATPase inhibitor, in the absence of mitochondrial metabolism blocked by antimycin A. It was therefore proposed that the *S. pombe* plasma membrane contained a proton-translocating ATPase, stimulated by cyclic AMP under semistarvation conditions and generating an electrochemical gradient required for active transport of amino acids, uridine, adenosine, and sucrose.

This proposal received direct support by the identification of an ATPase activity in a membrane fraction of density 1.27 g cm^{-3} which separates by differential and isopycnic gradient centrifugations from the mitochondrial membrane of density 1.17 g cm^{-3} (Delhez *et al.*, 1977). This ATPase activity belongs to the plasma membrane as shown by cytochemical and chemical determination of mannans in the same fraction. The ATPase activity has an optimal pH of 6.0, has a K_m for MgATP of 3.3 m*M*, and is inhibited not only by Dio-9 but also by dicyclohexylcarbodiimide, triethyltin, miconazole, sodium fluoride, *p*-hydroxymercuribenzoate, and MgADP. This plasma membrane-bound ATPase activity is clearly different from the mitochondrial ATPase since it is not inhibited by the mitochondrial inhibitors oligomycin and venturicidin, nor by an antibody against the mitochondrial F_1-ATPase. It is also not stimulated by carbonate or maleate anions.

The physiological functions which are driven by the plasma membrane ATPase were further studied by using Dio-9 in intact cells. It was found that Dio-9 elicits an electrogenic exit of K^+ along the concentration gradient of this cation (Foury *et al.*, 1977). The electrical membrane potential so generated is balanced with a 1 to 1 stoichiometry by the uptake of protons when a proton-conducting agent is added to the medium in the absence of glucose. In the presence of glucose and Dio-9, protons or other cations such as Ca^{2+} or Na^+ are taken up by the cell in the absence of protonophores and serve as counterions for the K^+ efflux. These observa-

tions indicate that *S. pombe* plasma membranes contain carriers for cellular electrogenic potassium efflux and electrogenic H^+, Na^+, and Ca^{2+} influx which are highly dependent on the membrane potential generated by the Dio-9-sensitive plasma membrane ATPase.

The system responsible for uptake of Ca^{2+} was studied in more detail by Boutry *et al.* (1977). *Schizosaccharomyces pombe* cells carry out an energy-dependent calcium uptake of high affinity, the apparent K_m being 45 μM Ca^{2+} at pH 4.5. This uptake system which is inhibited by lanthanum, ruthenium red, and hexamine cobalt chloride is also able to transport other divalent cations with the following order of affinities: $Sr^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$. The uptake rate of calcium is about 10 times higher at pH 8.25 than at pH 4.0. An energy-dependent extrusion system for calcium is also observed under acid conditions. In the presence of glucose, Dio-9 enhances severalfold the uptake of calcium even at 2°C. This indicates that under these conditions the electrogenic K^+ efflux resulting from the action of Dio-9 is compensated by the electrogenic Ca^{2+} influx. That Dio-9 is not a K^+ ionophore and operates by its action on the plasma membrane ATPase is supported by the observation that three other ATPase inhibitors, *N,N'*-dicyclohexylcarbodiimide, miconazole, and octylaminoester, also induce a rapid efflux of K^+ compensated by the stoichiometric influx of H^+ when added at pH 4.5 to intact cells incubated in the presence of glucose (Dufour *et al.*, 1980). These studies carried out with ATPase inhibitors strongly support the concept that the membrane potential created by the ATPase drives the secretion of acid equivalents, the uptake of amino acids, bases, nucleosides, and sucrose, the efflux of K^+, and the uptake of Na^+ and Ca^{2+}.

B. Purification of ATPase and Properties

Purified plasma membranes of *S. pombe* were obtained by precipitation of mitochondria at pH 5.2 followed by differential centrifugations and isopycnic centrifugation in a discontinuous sucrose gradient. The specific activity of the Mg^{2+}-requiring plasma membrane ATPase activity was enriched from 0.3 μmol min^{-1} mg^{-1} of protein in the homogenate to 26 in the purified membranes. Owing to the proficient and protective properties of lysolecithin, a natural zwitterionic phospholipid detergent, it has been possible to solubilize the plasma membrane ATPase. A 6- to 7-fold purification of the solubilized ATPase activity was obtained by centrifugation of the lysolecithin extract in a sucrose gradient (Dufour and Goffeau, 1978). Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, of the purified enzyme showed only one band of M_r 100,000 stained with Coomassie blue. Another ATPase component of apparent

molecular weight lower than 10,000 was stained by periodic acid–Schiff reagent but not colored by Coomassie blue and shown to contain phospholipids.

The rate of sedimentation of the purified ATPase yields a sedimentation coefficient $s^{0.784}_{20,w}$ of 24 *s*. Calculation of the corresponding molecular weight shows that the ATPase is purified in an oligomeric form of molecular weight 800,000–1,000,000 corresponding to 8 to 10 monomers of molecular weight 100,000. Hofstee (1952) plots for the purified ATPase activity are linear when tested with or without lysophosphatidylcholine, showing that strict Michaelis–Menten kinetics apply within a large range of ATP and Mg^{2+} concentrations. Addition of egg lysophosphatidylcholine increases V_{max} from 1.4 to 26.8 mmol min^{-1} mg^{-1} whereas K_m is less modified. The lipid-reconstituted purified ATPase activity is strictly specific for ATP and is inhibited competitively by ADP with a K_i of 2.5 m*M*. The optimum temperature of the purified ATPase activity is 30° and 32.5°C in absence or presence of lysophosphatidilcholine, respectively. The lipid-reconstituted purified ATPase activity is cation-dependent with the following specificity: $Zn^{2+} > Co^{2+} > Mg^{2+} > Mn^{2+} > Ni^{2+}$ for cation concentrations below 2 m*M* (M^{2+}/ATP = 0.6). Above 2 mM (M^{2+}/ATP > 1), however, Mg^{2+} is the most efficient cofactor followed by $Co^{2+} > Mn^{2+} > Zn^{2+} > Ni^{2+}$. These properties are qualitatively similar to those of the membrane-bound ATPase activity indicating that the integrity of the lipid-reconstituted purified ATPase is not appreciably modified by the extraction, purification, and reconstitution procedures (Dufour and Goffeau, 1980).

The purified plasma membrane ATPase of *S. pombe* is sensitive to seven different inhibitors. Most of the inhibition kinetics yield straight lines when analyzed by the graphical methods of Lineweaver and Burk ($1/v$ versus 1/[S], 1934) Dixon ($1/v$ versus [I], 1953), and Webb ($1/i$ versus 1/[I], 1963), indicating classic Michaelis–Menten mechanisms and noninteracting binding sites for the substrate MgATP. New ATPase inhibitors, octylaminoester and miconazole, are strict competitive inhibitors. Purified Dio-9, the most powerful inhibitor among those tested, is presumably a competitive inhibitor. Orthovanadate and *N*,*N*′-dicyclohexylcarbodiimide exhibit a strictly noncompetitive inhibition pattern. *p*-Hydroxymercuribenzoate irreversibly inhibits the ATPase activity, and NaF exhibits a complex inhibition pattern, depending on the [Mg^{2+}]/[MgATP] ratio (Dufour *et al.*, 1980). A general similarity is observed in the responses of the membrane-bound and purified ATPases to all inhibitors tested. It is concluded that the integrity of the ATPase activity is not appreciably modified by its solubilization by lysolecithin and the subsequent purification and lipid reconstitution procedures.

C. Protein–Lipid Interactions

The plasma membrane ATPase solubilized by lysolecithin and purified by centrifugation through a sucrose gradient is essentially inactive during long-term incubation. The phospholipid distribution in the sucrose gradient indicated that inactivation of the ATPase may result from the partial delipidation occurring during purification. Taking into account the 100,000 Da of the ATPase polypeptide, it is concluded that 74 mol of phospholipids is bound per mole of purified ATPase monomer (Dufour and Goffeau, 1980).

The ATPase so purified is reactivated simply by mixing the enzyme with preformed lipid micelles of vesicles. Lysolecithins reactivate the enzyme at concentrations around the critical micellar concentration. Gel-exclusion chromatography indicates that the enzyme binds reversibly to the lysolecithin micelles. On the other hand, lecithins of varying chain length and unsaturation reactivate the enzyme to different extents and with different efficiencies. In addition, from binding studies, it is observed that each saturated lecithin combines equally well with the ATPase. Using other diacylphospholipids, no specificity for the polar head group is observed. Moreover, cardiolipin microvesicles are shown to bind to the protein but not to restore the enzyme activity. From lipid-reactivation titration curves, Arrhenius plots, and physical data of the phospholipids, it is concluded that the major parameter which governs the optimal reactivation of ATPase is the ability of the phospholipids to form amphipathic structures (micelles or vesicles) of sufficient fluidity and hydrophobicity.

In an attempt to clarify mechanisms for reactivation of the enzyme and to obtain information on the aggregational state of the lipid-reactivated plasma membrane ATPase, a binding study of radioactive plasma membrane ATPase to dimyristoylphosphatidylcholine microvesicles was carried out. The binding of the purified ATPase to lipid vesicles has been assessed by a flotation gradient technique using ^{35}S-labeled protein as a radioactive marker for the purified ATPase. The reactivation curve of the enzyme parallels the binding curve, indicating that formation of a lipoprotein complex is required for the reactivation of the enzyme. Moreover, the reconstitution of enzyme activity is maximal at a ratio of 1 mol of protein peptide (M_r 100,000) per microvesicle, suggesting that the minimal size of an active ATPase is the monomeric form (Dufour and Tsong, 1981). Fusion of the phospholipid microvesicles into larger unilamellar vesicles of 100 nm is triggered by the purified plasma membrane ATPase when incubated below the phase transition temperature of the phospholipid. The rate of fusion exhibits a maximum at 8°C and is second order to vesicle concentration, indicating that the rate-limiting step is the collision

between the microvesicles. Moreover, the collision and hence the fusion are diffusion controlled, with a second-order rate constant of 4.48×10^9 M^{-1} sec (in microvesicle concentration).

Arrhenius plots of the enzyme activity in the microvesicles and in the fused vesicles show a break, respectively, at 18.4 and 23.2°C, corresponding to the phase transition temperatures of these structures. In both cases, the activation energy is around 170 kJ mol^{-1} at temperatures below and 100 kJ mol^{-1} at temperatures above the midpoints of the phase transition of the phospholipid vesicles. These results indicate that the physical state of lipid bilayers significantly affects the activation process of the enzyme–substrate interaction.

The purified ATPase incubated with L-α-dimyristoylphosphatidylcholine vesicles of 260 Å at 10°C and at a low ratio of enzyme to vesicle concentration forms fused macrovesicles of about 950 ± 200 Å. These ATPase trigger-fused vesicles are unilamellar and have an intact ionic permeation barrier at 30°C and a gel to liquid–crystalline transition temperature of 24.4°C with a transition heat of 5.64 kcal mol^{-1}. In contrast to data found in the literature, which show a lack of pretransition for unilamellar microvesicles, a pretransition around 15°C is observed for all the vesicle forms examined. Moreover, the transition widths of unilamellar vesicles are much broader than those of the multilamellar vesicles, suggesting that in the latter system interlayer interactions may contribute to the cooperativity of the transition (Dufour *et al.*, 1981). Unfortunately these macrovesicles have not yet been used for studying the proton-pumping activity of the ATPase.

D. Reconstitution of H^+-Pumping Activity

1. H^+ Movements

In a first approach (Dufour and Tsong, 1982; Dufour *et al.*, 1982), the purified plasma membrane ATPase was incorporated into azolectin vesicles using a freeze–thaw procedure. The reconstituted vesicles have a mean diameter of 59 nm compared to 53 nm for the unreconstituted lipid vesicles. ATP hydrolysis by the incorporated enzyme is inhibited by the electrochemical proton gradient as demonstrated by increased ATPase activity on addition of the proton carrier carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. This stimulation has been optimized to a value of 90%. The ATPase activity is maximally stimulated when both ΔpH and $\Delta\psi$ are collapsed.

Fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine is induced on addition of MgATP to the reconstituted vesicles and is quantita-

tively related to ATP hydrolysis. The initial rate of quenching is stimulated 10.3-fold when the membrane potential is collapsed by K^+ plus valinomycin. In the absence of valinomycin, the rate of quenching is stimulated by charge-compensating anions. These stimulations of the proton pump by cation or anion movements indicate that the primary translocation of H^+ is electrogenic. Since the leakiness of the reconstituted vesicles to H^+ is 240 times slower than the maximal rate of H^+ pumping, the reconstituted vesicles are tightly coupled. The fluorescence quenching of the dye is completely abolished by protonophores or proton–cation exchangers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, nigericin, monensin, and A23187. The fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine is therefore due only to the generation of a pH gradient ($pH_{in} < pH_{out}$) across the lipid membrane. By addition of HCl to the medium, the ΔpH formed by ATP hydrolysis is calculated to be 3.6 units.

In another approach (Villalobo *et al.*, 1981), the purified ATPase was incorporated in liposomes using a cholate–dialysis method. Under these conditions, the ATPase activity of the incorporated enzyme is stimulated by the H^+-conducting agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and to a lesser extent by the K^+ ionophore valinomycin in the presence of potassium. The K^+/H^+ exchanger nigericin (plus K^+) does not stimulate ATPase activity, whereas the combined addition of nigericin plus valinomycin is strongly stimulatory. The incorporated ATPase activity is controlled by the generated electrochemical H^+ gradient since only conditions which collapse both the membrane potential and the pH gradient stimulate fully the ATPase activity of the incorporated enzyme. Direct measurement of proton movement with a pH glass electrode shows a fast and transient proton entry into the proteoliposomes on addition of MgATP in the presence of the charge-compensating cation K^+ (plus valinomycin). Moreover, during the steady-state ATP hydrolysis, H^+ entry is again observed when the membrane potential is collapsed on addition of valinomycin in the presence of K^+. These data demonstrate that the plasma membrane ATPase is involved in electrogenic H^+ translocation coupled to ATP hydrolysis.

2. *K^+ Movements*

Effects of K^+ on ATPase activity have been observed in the proteolipid vesicles obtained by cholate dialysis (Villalobo, 1982, 1984). When a potassium concentration gradient (high K^+_{in}) is applied to the proteoliposome membrane, a significant drop in the CCCP-stimulated ATPase activity is observed. Inversion of the K^+ concentration gradient (high K^+_{out}) does not decrease the stimulation by CCCP. High Na^+_{in} also decreases the stimula-

tion induced by CCCP in the absence but not in the presence of external K^+. However, high Li^+_{in} has no effect.

Direct potassium efflux from the proteoliposomes is detected on addition of MgATP, using a selective K^+ electrode. The ATP-dependent potassium efflux is abolished in CCCP- and/or nigericin-pretreated proteoliposomes. H^+ and K^+ movements are also detected in ATPase-containing liposomes in the absence of ATP when a membrane potential of appropriate polarity is applied. Under these conditions, the membrane potential-induced K^+ translocation is strongly inhibited by high concentrations of vanadate. K^+_{in} but not K^+_{out} stimulates the rate of ATP hydrolysis in the absence but not in the presence of CCCP. Na^+ on either side of the membrane does not have any stimulatory effect.

The K^+ translocation driven by ATP hydrolysis has two different kinetic components. Although the ATP-dependent K^+ transport strictly requires the presence of a membrane potential, the rate of K^+ translocation is not affected by a broad modulation of the degree of coupling (q) between ATP hydrolysis and the electrogenic H^+ translocation. These experiments were interpreted to suggest that the plasma membrane Mg^{2+}-dependent ATPase in yeast cells not only carries out electrogenic H^+ ejection but also drives the uptake of K^+ via a voltage-sensitive gate which is closed in the absence and open in the presence of the membrane potential. It should, however, be clear that under none of the above conditions has an active transport of K^+ (e.g., against a concentration gradient) been measured and that the fluxes of K^+ reported are of more than one order of magnitude smaller than the H^+ fluxes. Therefore, one author of this chapter (A. Goffeau) considers that it has not been demonstrated that the K^+ movements measured *in vitro* have any physiological relevance to the active uptake of K^+ by intact cells.

E. Mechanism for ATP Hydrolysis

1. Phosphorylated Catalytic Intermediate

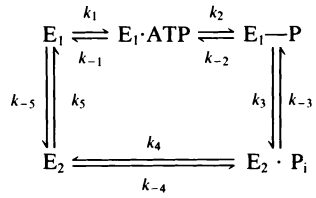
A new acid slab gel electrophoresis method of high resolving power has allowed detection of a phosphorylated form in the purified ATPase and its identification as a catalytic intermediate (Amory *et al.*, 1980). At a maximum steady-state rate of MgATP hydrolysis by the membrane-bound ATPase, 20–40% of the ATPase subunits of 100,000 Da are in a phosphorylated form, while only 0.8% of the subunits of the purified ATPase are phosphorylated under the same conditions. The phosphorylated intermediate reaches the steady-state level in less than 100 msec and rapidly turns over. The phosphorylated substance is cleaved by hydroxyl-

amine and is relatively stable in acids, but it is readily hydrolyzed in alkaline or in acid alcoholic media. These results suggest that the intermediate is an acylphosphate. The phosphorylated amino acid of the purified ATPase was reduced with [^{3}H]$NaBH_4$. After acid hydrolysis and separation of the amino acids by ion-exchange chromatography, the presence of radioactive homoserine shows that the phosphorylated residue is an aspartate. The phosphorylation reaction has an apparent K_m of 3.0 m*M* MgATP for the purified ATPase (Amory and Goffeau, 1982). The ATPase activity is directly proportional to the phosphoprotein concentration. The hydrolytic dephosphorylation of the intermediate is nearly irreversible under the tested conditions as the enzyme is not phosphorylated by [^{32}P]P_i.

Seven different inhibitors of the ATPase activity inhibit the phosphorylation of the 100,000-Da polypeptide. The parallel inhibition of the steady-state phosphorylation level and the ATP hydrolysis activity indicates that *N*,*N*′-dicyclohexylcarbodiimide, miconazole nitrate, and diethylstilbestrol act on a step leading to the formation of phosphoprotein and do not act on the dephosphorylation of the intermediate. On the other hand, vanadate and Dio-9 interact with the dephosphorylation. Suloctidil and *p*-hydroxymercuribenzoate exhibit both type of inhibition, depending on the inhibitor concentration.

2. *Partial Reactions*

The mechanisms for ATP hydrolysis summarized below has been identified from kinetic measurements of enzymatic exchange of oxygen between inorganic phosphate or ATP (Amory *et al.*, 1982, 1984), from $^{32}P_i$–ATP exchange (de Meis *et al.*, 1987), and from nucleotide binding data (Ronjat *et al.*, 1987).



a. Oxygen Exchange Data. *Evidence for steps 3 and 4.* During hydrolysis of [γ-^{18}O]ATP in unlabeled water, more than one oxygen atom derived from water is incorporated into the γ-phosphate group (intermediate P_i–H_2O exchange), suggesting the existence of the $E_2{\cdot}P_i$ species. The P_i in $E_2{\cdot}P_i$ tumbles in the active site so that each of its oxygens has an equal probability of exchange with water by reversal of step 3. The partition

coefficient [$P_c = k_{-3}/(k_3 + k_4)$] is 0.45. Varying the [ATP] has very little effect on P_c, indicating little or no cooperativity between different catalytic sites.

In the presence of Mg^{2+} and P_i and in the absence of ATP and ADP, the ATPase catalyzes a medium P_i–H_2O exchange occurring via steps 4 and 3. The P_c is the same as the one found for intermediate exchange, indicating that the same pathway operates for phosphorylation from both ATP and P_i. The total rate of oxygen exchange, V_{ex}, representing the rate of incorporation of water oxygens occurring during hydrolysis of E_1—P to $E_2 \cdot P_i$ ($V_{ex} = k_{-2}[E_1—P]$), is dependent on the [P_i] with an apparent K_m of 177 m*M*, reflecting the very low affinity of the enzyme for P_i. The maximal exchange rate is 6.7 mg atoms of oxygen min^{-1} mg^{-1} protein. The individual kinetic constants are evaluated as follows: $k_3 = 3.4 \times 10^3$ min^{-1}, $k_{-3} = 5.50 \times 10^5$ min^{-1}, and $k_4 = 4.11 \times 10^3$ min^{-1}. Under conditions of uncoupled transport, the hydrolysis of E_1—P is exergonic since $[E_2 \cdot P_i]/[E_1—P] = 164$.

Evidence for step 5. During hydrolysis of ATP, the rate of medium P_i–H_2O exchange activity as well as the extent of phosphorylation of the enzyme from P_i are markedly stimulated (7.9 and 5.3 times, respectively) whereas the P_c is not modified. These data are most simply interpreted by the existence of two isomeric forms of the enzyme, E_1 being specific for binding ATP and E_2 for binding P_i. This interpretation is in agreement with the stimulation by unlabeled ATP of the low phosphorylation of the enzyme from inorganic phosphate.

b. P_i–ATP Exchange Data. That the hydrolysis of ATP is highly irreversible is confirmed by the extremely low rate of $^{32}P_i$–ATP exchange which in uncoupled proteoliposomes is 55,000 times lower than the rate of ATP hydrolysis: 0.2 nmol ATP versus 11.1 mmol ATP min^{-1} mg^{-1} protein. Under conditions where the H^+ electrochemical gradient is allowed to develop, the rate for $^{32}P_i$–ATP exchange is stimulated 76 times, up to 15.3 nmol ATP min^{-1} mg^{-1} protein, still 720 times slower than the rate of ATP hydrolysis (A. Amory and A. Goffeau, personal communication). Under certain conditions, alkaline pH and in the presence of the organic solvent dimethyl sulfoxide, it has been possible to obtain $^{32}P_i$–ATP exchange in the absence of a proton gradient. Under optimal conditions exchange rates of 1.3 nmol ATP min^{-1} mg^{-1} protein have been obtained. This rate is only 12 times lower than that observed in the presence of a proton gradient and is only 100 times lower than the rate of ATP hydrolysis measured under the same conditions (pH 7.5 and 20% dimethyl sulfoxide). The effect of organic solvent on the P_i–ATP exchange is related to a decrease of the apparent K_m for P_i. These observations suggest the occur-

rence of changes of water activity and of H^+-binding energy during the catalytic cycle for ATP hydrolysis (de Meis *et al.*, 1987).

c. Nucleotide Binding Data. In the presence of Mg^{2+}, formycin triphosphate (FTP) is hydrolyzed by the H^+-ATPase and supports H^+ transport. When combined with terbium ion, FTP (TbFTP) and ATP (TbATP) are no longer hydrolyzed. Competition between MgATP and TbFTP for ATP hydrolysis indicates that terbium-associated nucleotides bind to the catalytic site of the H^+-ATPase (Ronjat *et al.*, 1987).

The fluorescent properties of the TbFTP complex were used to study the active site of the H^+-ATPase. Fluorescence of TbFTP is greatly enhanced on binding into the nucleotide site of H^+-ATPase with a dissociation constant of 1 m*M* whereas TbATP, TbADP, and TbITP are competitive inhibitors of TbFTP binding with K_i values of 4.5, 5.0, and 6.0 μM, respectively. Binding of TbFTP is observed only in the presence of an excess of Tb^{3+} with an activation constant K_a of 25 μM for Tb^{3+}. Analysis of the data reveals that the sites for TbFTP and Tb^{3+} binding are independent entities. In standard conditions, these sites would be occupied by MgATP and Mg^{2+}, respectively. These findings suggest an important regulatory role of divalent cation on the activity of H^+-ATPase. Replacement of H_2O by D_2O in the medium suggests that existence of two types of nucleotide binding sites differing by the hydration state of the terbium ion in the bound TbFTP complex.

The range of K_m values reported for the hydrolysis of MgATP by the H^+-ATPase of yeast and fungi is very large. For different conditions the *S. pombe* purified enzyme exhibits K_m values ranging from 160 μM (Dufour *et al.*, 1982) to 3.5 μM (Dufour and Goffeau, 1981). Dissociation constants of the order of 10 μM were observed for the complex of the enzyme not only with nonhydrolyzed fluorescent nucleotide analogs, such as terbium formycin triphosphate (Ronjat *et al.*, 1987), but also with radioactive magnesium trinitrophenyl-ATP (M. Ronjat and Y. Dupont, personal communication). Accordingly, in the presence of high concentrations of lipids and at low pH values, the K_m values may be extrapolated to as low as 10 μM MgATP (J. P. Blanpain and A. Goffeau, personal communication).

The K_m value for MgATP hydrolysis can thus be interpreted by the following simplified scheme:

$$\mathrm{ATP} + \mathrm{E_1} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ATP{\cdot}E_1} \xrightarrow{k_{cat}} \mathrm{ADP} + \mathrm{P_i} + \mathrm{E_1}$$

with K_m being $(k_{-1} + k_{cat})/k_1$ where k_{cat} is a lumped rate constant. Since $K_d = k_{-1}/k_1$ is low, the K_m value is strongly dependent on k_{cat}. It is only at

very high lipid concentrations and at low pH values that k_{cat} becomes so small that the K_m and K_d values for MgATP are similar (~10 μM). The lumped rate constant k_{cat} includes the steps $E_1 \cdot ATP$ to E_1—P (step 2), E_1—P to $E_2 \cdot P_i$ (step 3), $E_2 \cdot P_i$ to E_2 (step 4), and E_2 to E_1 (step 5). The effect of ATP on P_i–H_2O medium exchange indicates that this last step is the rate-limiting step at pH 6.0. This is also supported by the observation that lowering the pH from 6.0 to 5.0 not only decreases K_m but also brings about modifications of the intrinsic fluorescence of the protein, similarly to the conformational transition of E_2 to E_1 reported for the Ca^{2+}-ATPase (M. Ronjat, Y. Dupont, and J. P. Dufour, personal communication).

F. Structural Studies

1. Chemical Modifications

The purified ATPase is inactivated on incubation with the arginine modifier 2,3-butanedione. The modified enzyme is reactivated when incubated in the absence of borate after removal of 2,3-butanedione. The extent of inactivation is half-maximal at 10 m*M* 2,3-butanedione for an incubation of 30 min at 30°C at pH 7.0. Under the same conditions, the time dependence of inactivation is biphasic in a semilogarithmic plot with half-lives of 10.9 and 65.9 min. Incubation with 2,3-butanedione while lowering markedly the maximal rate of ATPase activity does not modify the K_m for MgATP. These data suggest that two classes of arginyl residuces play an essential role in the plasma membrane ATPase activity (Di Pietro and Goffeau, 1985).

MgATP and MgADP, the specific substrate and product, partially protect against enzyme inactivation by 2,3-butanedione. Free ATP or MgGTP, which are not enzyme substrates, do not protect. Free magnesium, another effector of enzyme activity, exhibits partial protection at concentrations up to 0.5 m*M*, while increased inactivation is observed at higher Mg^{2+} concentrations. These protective effects indicate either the existence of at least one reactive arginyl in the substrate binding site or a general change of enzyme conformation induced by MgATP, MgADP, and free Mg^{2+}.

2. Primary Structure

The structural gene *pma1*$^+$ for the *S. pombe* H^+-ATPase has been isolated by hybridization with the corresponding gene from *Saccharomyces cerevisiae* (Ghislain *et al.*, 1988). The deduced amino acid sequence is presented in Fig. 1 and compared to those of the H^+-ATPases

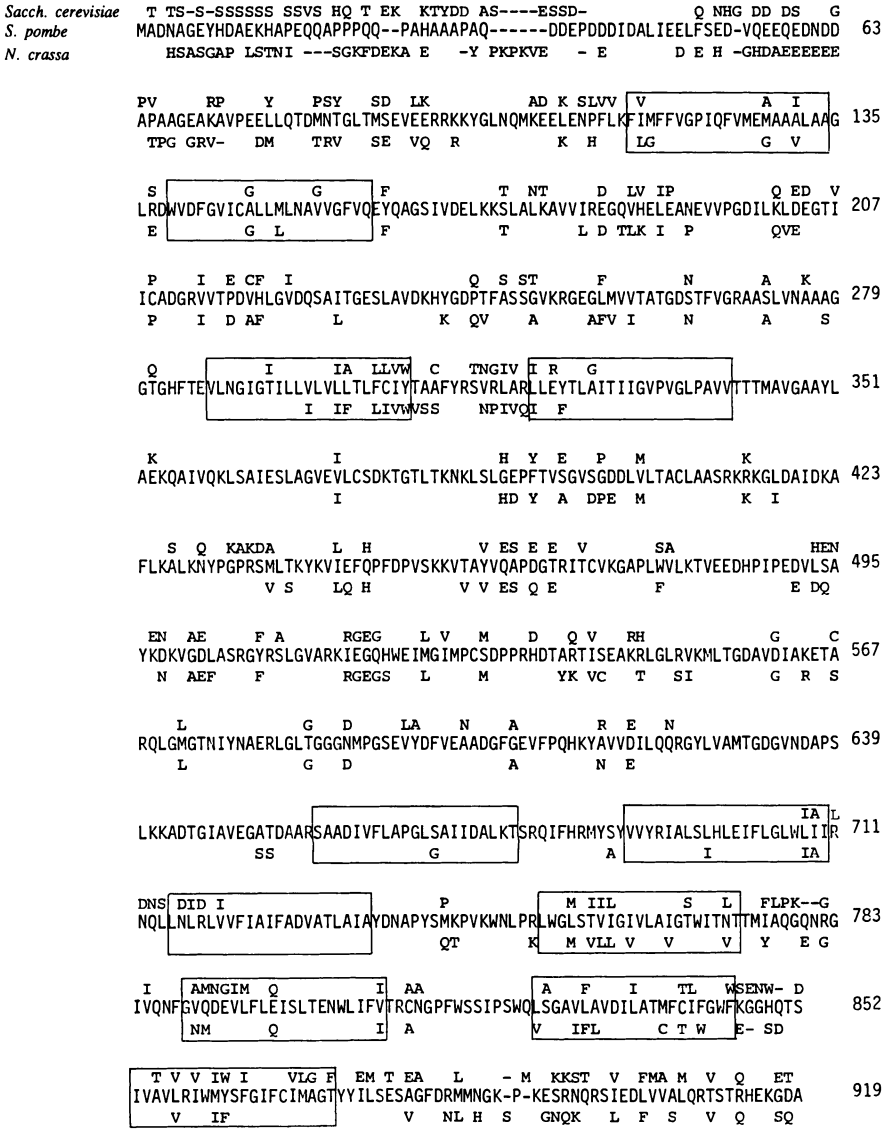


Fig. 1. Deduced acid sequences for fungal H⁺-ATPases. The sequence from *S. pombe* (middle) is the reference. The sequences from *Sacch. cerevisiae* (top) and *N. crassa* (bottom) have been aligned with that of *S. pombe* in order to obtain maximal homology. Postulated deletions are indicated by small dashes (–). When the amino acid residues from *Sacch. cerevisiae* or *N. crassa* are identical to those from *S. pombe*, only the *S. pombe* sequence is given. Boxed regions represent hydrophobic regions that are possible membrane-spanning segments of the protein.

from *Neurospora crassa* (Hager *et al.*, 1986; Addison, 1986) and *Sacch. cerevisiae* (Serrano *et al.*, 1986).

a. Hydrophobicity Profile. The *S. pombe* H^+-ATPase deduced amino acid sequence contains 11 long hydrophobic segments of about 21 amino acids, according to the method of Kyte and Doolittle (1982). If each of these segments (boxed in Fig. 1) were spanning the membrane the topology illustrated in Fig. 2A would be obtained, where the amino and carboxy termini would be on opposite sides of the membrane. In order to assess the probability of each of these segments being transmembranous, the mean hydrophobicity value averaged over a span of 11 amino acids was plotted against the maximal mean hydrophobicity moment as described by Eisenberg *et al.* (1984). Segments 1, 2, 3, 7, 8, 10, and 11 are predicted as being multimeric and segment 4 as monomeric transmembrane segments. We conclude that the protein probably contains at least 8 transmembrane segments; 4 of these segments are located between amino acid residues 114 and 340 and 4 between amino acid residues 690 and 873. Segments 2 and 3 mark the boundaries of a hydrophilic region. Another large hydrophilic region comprises residues 340 to 658. It is, however, not excluded that this large hydrophilic region includes segments 4 (see below) as well as segments 5 and 6. Indeed, segments 5 and 6 are predicted to be of amphipathic and globular nature, respectively, according to Eisenberg's procedure. Segment 9, predicted to be multimeric, might or not be considered as a transmembrane segment depending on the hydrophobicity scale chosen, since its hydrophobicity value is near the threshold value.

Among the 11 hydrophobic stretches predicted by the Eisenberg plot, segments 2, 4, 5, and 6 are well conserved among the three fungal ATPases; each segment shows only four or fewer single amino acid differences among 21 residues. Conservation of segment 6 is particularly striking because the Eisenberg plot does not define this hydrophobic segment as being of transmembrane nature but instead predicts a globular structure. Segment 6 (residues 690–710) is highly conserved among all fungal H^+-ATPases but has no counterpart in the Na^+,K^+-, Ca^{2+}-, or H^+,K^+-ATPases. It is therefore tempting to speculate that it might be a candidate for a putative proton-binding and/or proton-transport region. It contains the histidine-699 residue which could be protonated at physiological pH and might act in concert with glutamate-701 in a charge relay system, as proposed for the lactose permease of *Escherichia coli* (Carrasco *et al.*, 1986). This proposal is not necessarily in contradiction with the recent suggestion for the participation of glutamate-129 from segment 1 in the proton-translocating function of the *N. crassa* H^+-ATPase (Sussman *et al.*, 1987). Indeed, several charged residues belonging to different mem-

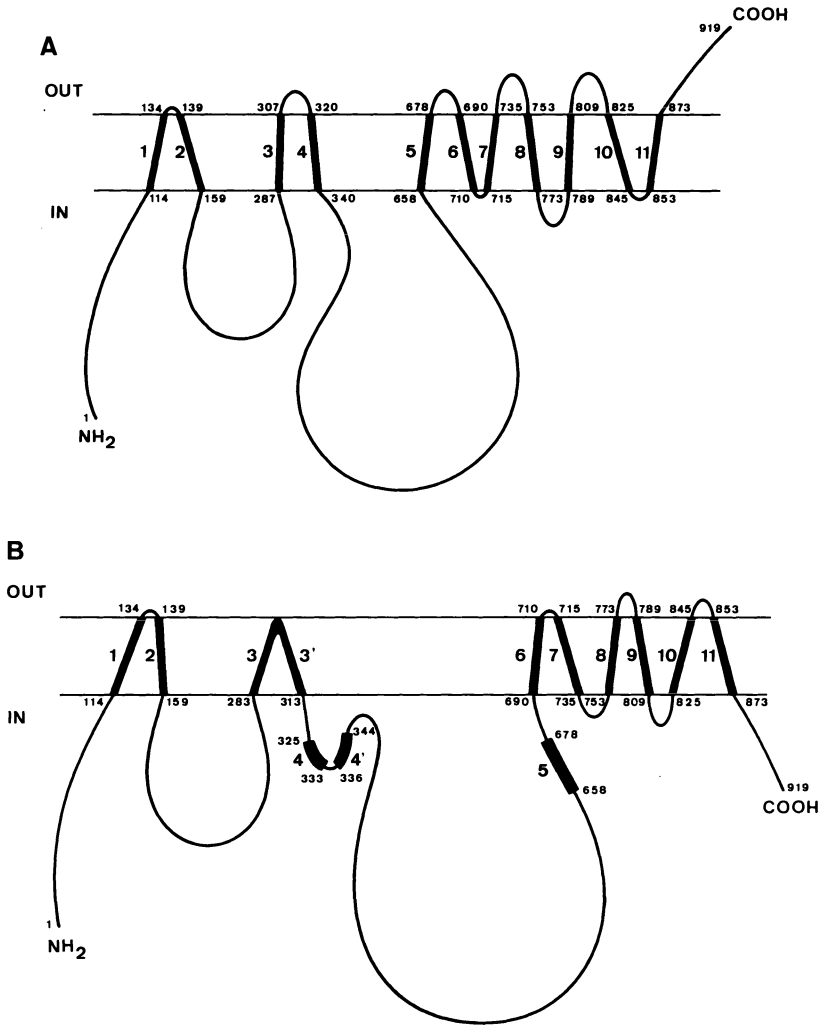


Fig. 2. Structural predictions for the *S. pombe* H⁺-ATPase. (A) Putative topology allowing 11 transmembrane segments (numbered from 1 to 11). (B) Alternative putative topology allowing 10 transmembrane segments and 2 hydrophobic regions (4 plus 4′ and 5) included in the large hydrophilic loop. (C) Conserved regions among all transport ATPases (numbered a through f). (D) Distribution of negative (aspartyl and glutamyl) and positive (histidyl, arginyl, and lysyl) residues. Those located in the hydrophobic segments are circled. (E) Amino acids residues of particular interest. (F) Predicted α-helices and β sheets in the hydrophilic regions. See the text for details.

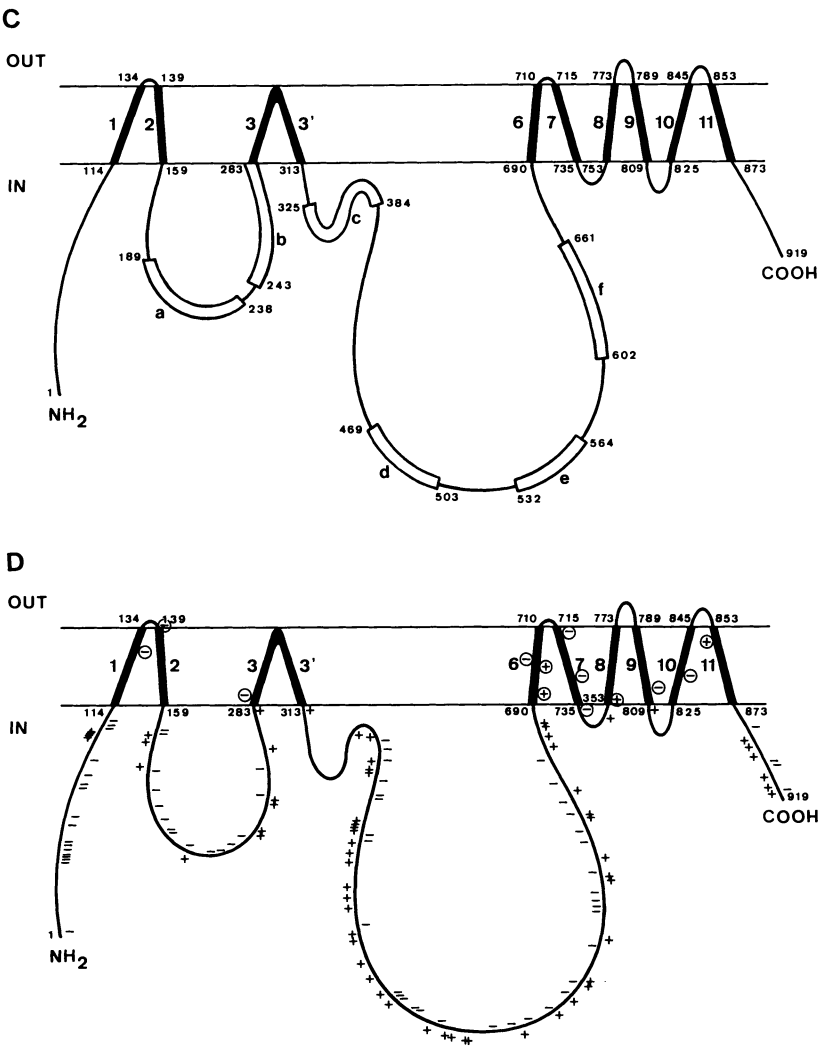


Fig. 2C and D. See legend on p. 411.

brane spans as well as to nonmembrane regions (called stalks by Brandl *et al.*, 1986) might contribute to the ion-exchange pathway across the membrane.

Segment 4 (residues 320–340) overlaps with the region called b in Fig. 2C, which shows the six regions (a through f) that are conserved in all transport ATPases. Segment 4 contains two proline and two glycine residues, known to be strong α breakers (Chou and Fassman, 1974). These

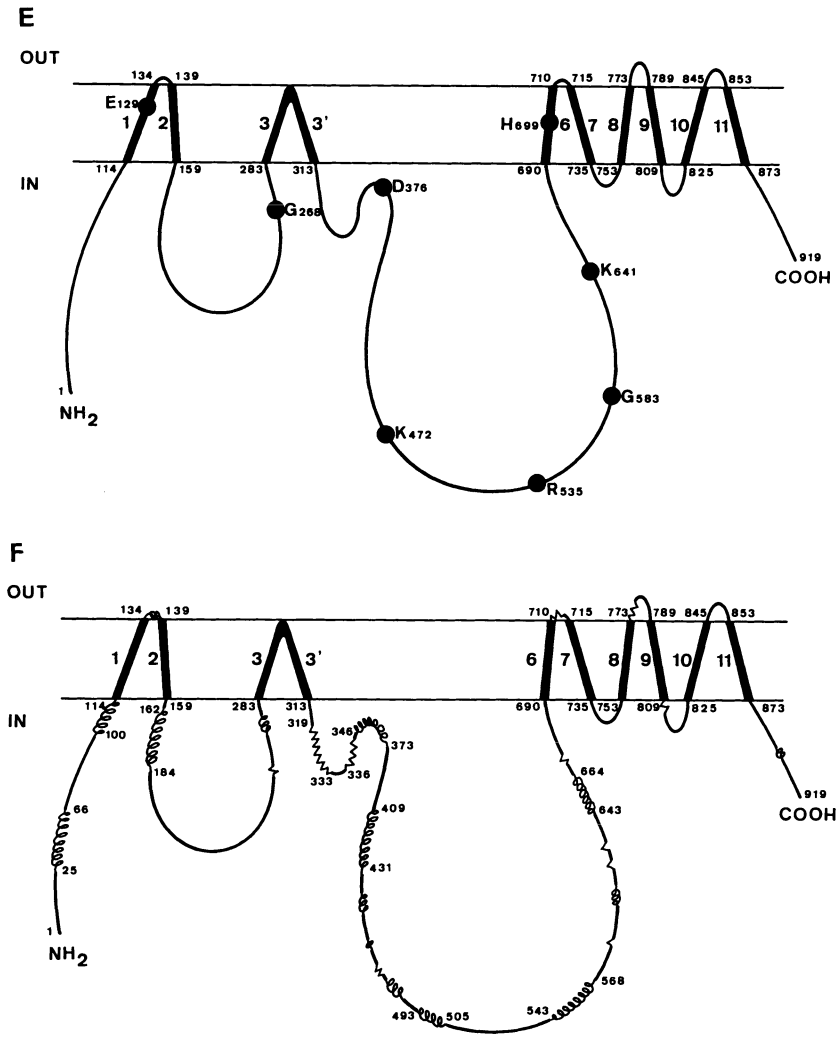


Fig. 2E and F. See legend on p. 411.

prolines and glycines are conserved in the three yeast H+-ATPases and can be interpreted as forming a sharp β turn between two predicted β sheets (Fig. 2F). Therefore, as shown in Fig. 2B, it is possible that this structure might be not inserted in membrane. It is also tempting to propose that the large hydrophobic segment 3, when slightly enlarged (e.g., from residues 283 to 313), is able to cross the membrane twice. The resulting topology described Fig. 2B has several consequences. First, it exposes to the outside the putative glycosylation site located between

segments 10 and 11 as well as the two amino acid deletion observed between *S. pombe* and *Sacch. cerevisiae* (780–781) and the one amino acid deletion observed between *S. pombe* and *N. crassa* (847). Second, it generates an optimal distribution of positively charged residues near the cytoplasmic side of the membrane (see Fig. 2D) as proposed by van Heijne (1986) for bacterial membrane proteins. Third, a second putative charge relay system E-X-X-H^{283} becomes included in membrane segment 3.

b. Comparison with Other ATPases. Fundamental mechanistic similarities between all transport ATPases and ATP synthases have been pinpointed by Scarborough (1985). Gross structural similarities between fungal, mammalian, and plant transport ATPases have been previously noticed, and many enzymatic properties are similar in all studied fungal ATPases (Goffeau and Slayman, 1981; Serrano, 1984). The three fungal H^+-ATPase sequences available today have identical sizes: 918 amino acids for *Sacch. cerevisiae*, 919 amino acids for *S. pombe*, and 920 amino acids for *N. crassa* and show an overall amino acid sequence similarity of 66%. Among 238 amino acid residues that are unique to the *S. pombe* H^+-ATPase, 185 are conservative replacements (Fig. 1). This means that the 53 nonconserved residues are not likely to be involved in major catalytic functions. The major differences are concentrated in the first 100 amino-terminal amino acids, which show, however, high percentage of conserved acid residues.

The existence of homologous regions in the amino acid sequences of the different transport ATPases (Ca^{2+}, Na^+,K^+) was first pointed out by Hesse *et al.* (1984). Six regions labeled a to f are conserved throughout all transport ATPases (Fig. 2C) including the aspartyl phosphorylation site (in c) and the two lysine residues labeled, respectively, by fluorescein isothiocyanate (in d) (Mitchison *et al.*, 1982; Farley *et al.*, 1984) or by 5′-*p*-fluorosulfonylbenzoyladenosine (in f) (Ohta *et al.*, 1986) which seem to form part of the ATP binding site (Fig. 2E). The conserved region e is the only one containing arginine residues and therefore might be the putative butanedione and phenylglyoxal targets which are protected by MgATP and which have been observed in several cation transport ATPases including fungal H^+-ATPases (Di Pietro and Goffeau, 1985; Kasher *et al.*, 1986). It can thus be suggested that all conserved regions of the large hydrophilic loop are directly involved in MgATP binding and hydrolysis. No strictly defined ADP binding β,α,β-fold structure -GXGXXG- (Wierenga *et al.*, 1986) is observed in *S. pombe*; however, analogy with *Sacch. cerevisiae* and *N. crassa* allows us to define such a putative inverted structure in -G^{583}XXGXG- (Fig. 2E). So far no experimental evi-

dence is available to allow one to suggest specific functions for the conserved regions a and b situated in the small hydrophilic loop, called the transduction domain by Brandl *et al.* (1986) for the Ca^{2+}-ATPase.

Another point of particular interest is the very likely existence of an H^+-ATPase in plant plasma membranes which resembles the fungal H^+-ATPase (Scalla *et al.*, 1983; Vara and Serrano, 1983). It contains an M_r 110,000 polypeptide in maize roots (Scalla *et al.*, 1983) or an M_r 90,000 polypeptide in maize shoots (Clement *et al.*, 1986), indicating possible tissue-specific ATPase isoforms. The plant ATPase forms a catalytic phosphorylated intermediate (Scalla *et al.*, 1983), and a purified plant ATPase is inhibited by an *S. pombe* ATPase antibody, indicating close structural similarities between the fungal and plant enzymes. This has been recently confirmed by the hybridization of a plant ATPase gene with the yeast structural gene for ATPase and by the observation of extended similarities in the respective DNA-deduced amino acid sequences (M. Boutry and A. Goffeau, personal communication).

c. Secondary Structure. The potential secondary structures of the putative extramembranous domains were analyzed using the Garnier algorithm (Garnier *et al.*, 1978). The prediction was further improved by averaging the values calculated from *S. pombe*, *Sacch. cerevisiae*, and *N. crassa* sequences. The first 100 amino-terminal amino acids are predicted to contain a majority of α helices, whereas the two large hydrophylic regions and the carboxy-terminal end are predicted to contain alternating α helix and β strand. On this basis the H^+-ATPase would belong to the α/β protein family. Figure 2F indicates the exact location of the predicted α and β structures. Curiously, using this method, the α helix and β sheet predicted for the so-called transduction domain (residues 159–283) and phosphorylation domain (residues 346–465) of the *S. pombe* H^+-ATPase do not coincide with those predicted for the mammalian Ca^{2+}-ATPase (residues 133–238 and 330–505, respectively; see MacLennan *et al.*, 1985). However, some of the α/β alternation typical of the nucleotide-binding domain in the Ca^{2+}-ATPase (residues 506–680) is also seen in the H^+-ATPase (residues 466–612).

G. ATPase Mutants

1. Genetics of pma1-1 and Its Phenotypes

An *S. pombe* mutant JV66 has been selected for resistance to Dio-9 and assigned to a new single locus on nuclear DNA (Ulaszewski *et al.*, 1986)

which by analogy with *Sacch. cerevisiae* (Ulaszewski *et al.*, 1983) is called *pma1* (for plasma membrane ATPase). The phenotypes of *pma1* mutants from *S. pombe* and *Sacch. cerevisiae* are quite similar: both are resistant *in vivo* to Dio-9, ethidium bromide, decamethylene guanidine, and *N,N'*-(*p*-xylylidene)bisaminoguanidine, and the ATPase activities tested *in vitro* were resistant to vanadate. In addition, the *pma1* mutant from *S. pombe* exhibits hypersensitivity to protamine sulfate at 37°C. This phenotype strongly suggests a modification of the general permeability barrier in the *pma1* mutant, possibly resulting from a primary defect in the H^+-pumping ATPase of the plasma membrane.

The *S. pombe pma1* locus is found on chromosome I at 5.3 map units from *cyh1-C7* and about 20.7 map units from the centromere. Although the *pma1* gene is not allelic to the *cyh1-C7* locus, some phenotypic interference is observed, since the double mutant *pma1 cyh1-C7* lost resistance to decamethylene diguanidine. However, the *pma1* mutation can still be detected *in vitro* by its vanadate-resistant ATPase activity (see later).

Schizosaccharomyces pombe pma1 is thus a new type of multiple drug resistant mutant. Indeed, the multiple drug resistance phenotype shown in *cyh3* and *cyh4* mutants previously described by Coddington and coworkers (Ibrahim and Coddington, 1976; Johnson and Coddington, 1982, 1983) is different from the multiple drug resistance phenotype of *pma1*. The *cyh3* and *cyh4* mutants are resistant to cycloheximide and other protein synthesis inhibitors to which *pma1* mutants are sensitive. In contrast *pma1* confers resistance to diguanidine and other positively charged compounds to which *cyh3* and *cyh4* mutants are sensitive. Moreover, the ATPase activity is normal (qualitatively and quantitatively) in *cyh3* and *cyh4* while that of *pma1* is reduced in quantity and is resistant to vanadate. Finally, the mutations are genetically distinct since they map on different chromosomes.

The plasma membrane ATPase has been purified from the *S. pombe pma1-1* mutant (Ulaszewski *et al.*, 1987). It contains an M_r 100,000 major polypeptide which is phosphorylated by [γ-^{32}P]ATP. Proton pumping is not impaired since the isolated mutant ATPase is able, in reconstituted proteoliposomes, to quench the fluorescence of the ΔpH probe 9-amino-6-chloro-2-methoxyacridine. The isolated mutant ATPase is sensitive to Dio-9 as well as to seven other plasma membrane H^+-ATPase inhibitors. The mutant H^+-ATPase activity tested *in vitro* is, however, insensitive to vanadate. Its K_m for MgATP is modified, and its ATPase specific activity is decreased. A single modification into aspartate of the glycine-268 residue located in the conserved region b is the only change observed in the mutant sequence (Ghislain *et al.*, 1988). Mitotic cosegregation in an *S.*

pombe transformant of the mutant phenotype with the *Pvu*I site created by the *pma1-1*$^-$ mutation demonstrates that the modified amino acid is responsible for the vanadate resistance of the H^+-ATPase.

The wild-type *S. pombe* allele on a multicopy plasmid is able to complement the *S. pombe pma1-1*$^-$ mutant. The low frequency of this complementation suggests that overexpression of the H^+-ATPase might be lethal for yeast transformants and would explain the observed plasmid loss or integration into the chromosome. The *pma1* mutation decreases the rate of extracellular acidification induced by glucose when cells are incubated at pH 4.5 under nongrowing conditions. During growth, the intracellular pH of mutants is more acid than the wild-type one. The derepression by ammonia starvation of methionine transport is decreased in the mutant. The growth rate of *pma1* mutants is reduced in minimal medium compared to rich medium, especially when combined with an auxotrophic mutation.

These data fully support the earlier suggestions on the function of the H^+-ATPase obtained through the use of Dio-9. It is concluded that the H^+-ATPase activity from yeast plasma membranes controls the intracellular pH as well as the uptake of amino acids, purines, and pyrimidines. The *pma1* mutation modifies several transport properties of the cells including those responsible for the uptake of Dio-9 and other inhibitors.

2. *Vanadate-Resistant ATPase Activity in pma1-1*

In the oxidation state +5, orthovanadium is a potent inhibitor of all cation transport ATPases. It has been proposed that this inhibition results from the binding of vanadate to the ATPase activity site where it would mimic a trigonal-bipyramidal structure analogous to the transition state for aspartyl phosphate hydrolysis (Cantley *et al.*, 1978). This proposal has yet to be confirmed at the molecular level. However, it is generally accepted that vanadate binds preferentially to the E_2 conformation of the enzyme (Pick, 1982), which in contrast to the E_1 conformation is not able to bind and to hydrolyze ATP but instead binds P_i (Amory *et al.*, 1982). Participation of glycine-268 in the vanadate sensitivity of the *S. pombe* H^+-ATPase is quite specific since the *pma1-1*$^-$ mutant ATPase activity is not modified in its sensitivity to seven other ATPase inhibitors (Ulaszewski *et al.*, 1987). It might be premature, however, to conclude that the *S. pombe* glycine-268, which is highly conserved in all mammalian and fungal transport ATPases, necessarily defines a vanadate binding site. The mutation might indirectly produce, during steady-state ATP hydrolysis, a higher concentration of vanadate-insensitive E_1 forms and fewer E_2 vanadate-sensitive forms. Such a shift of equilibrium in favor of E_1 might

increase the vanadate resistance and lower the ATPase activity without affecting the proton/ATP stoichiometry as observed in the *S. pombe pma1-1*$^-$ mutant (Ulaszewski *et al.*, 1987). Consistent with this hypothesis is the fact that in the Na^+,K^+ATPase, the conserved glycine-261 residue is located next to a chymotrypsin cut which is only obtained when the enzyme is in the E_1 form. This cut interrupts the conformational transition between aspartyl phosphate hydrolysis and cation exchange (Jørgensen *et al.*, 1982). A similar site was also described for the Ca^{2+}-ATPase (Scott and Shamoo, 1982). In the *N. crassa* H^+-ATPase, the presence of magnesium vanadate markedly modifies the pattern of trypsin digestion (Addison and Scarborough, 1982), indicating again that vanadate reacts with a particular enzyme conformation.

H. Associated Membrane-Bound Protein Kinase Activity

During incubation of isolated plasma membranes with [γ-^{32}P]ATP, several peptides and in particular a polypeptide of M_r 50,000 are slowly phosphorylated by a membrane-bound, cAMP-independent protein kinase (3 min for maximal phosphorylation). The kinase activity is sensitive to three distinct plasma membrane ATPase inhibitors, vanadate, *N*,*N'*-dicyclohexyl-carbodiimide, and diethylstilbestrol. Only vanadate inhibits both the ATPase and kinase activities in parallel. The kinase activity, however, proceeds in the presence of the four other ATPase inhibitors miconazole, suloctidil, Dio-9, and *p*-hydroxymercuribenzoate (Amory *et al.*, 1980; Amory and Goffeau, 1982).

A similar kinase activity exists in *Sacch. cerevisiae* (Foury *et al.*, 1981) and in plant cell plasma membranes (Scalla *et al.*, 1983). The plasma membrane protein kinase from *Sacch. cerevisiae* has recently been purified and shown to phosphorylate the M_r 100,000 ATPase polypeptide at a seryl residue (Kolarov *et al.*, 1988). This kinase activity is inhibited by 200 μM free Zn^{2+} and by 10 μg heparin ml^{-1}. A similar kinase activity copurifies with the *S. pombe* ATPase (A. Goffeau, personal communication). Its exact role in the regulation of ATPase activity remains to be assessed.

II. ACTIVE TRANSPORT OF AMINO ACIDS, NUCLEIC ACID BASES AND RIBOSIDES, AND MALATE

A. Introduction

It is generally accepted that metabolites and ions are actively transported into all yeast cells against a concentration gradient and that this

transport is driven by a proton gradient (higher $[H^+]$ outside) across the plasma membrane (Eddy, 1982). According to Mitchell's chemiosmotic hypothesis (Mitchell, 1973) this proton gradient is generated by the hydrolysis of ATP, the two processes being coupled by the plasma membrane-bound ATPase. As well as utilizing the driving force of a proton gradient, active transport also requires the presence in the membrane of a specific protein carrier, usually referred to as a permease.

All active transport systems that have been studied are saturable and exhibit Michaelis–Menten type kinetics. This means that they can be characterized in terms of a K_m (in this case a half-saturation constant) and V. Inhibition studies can define competitive and noncompetitive inhibitors which can in turn give information as to whether a transport system is specific for a particular metabolite or general for a class of metabolites. A further complication which often arises is the possibility of transport systems being regulated. For example, amino acids and nucleic acid bases can act as nitrogen sources for yeasts. In the presence of ammonium ions not only is the synthesis of enzymes involved in their metabolism repressed but also that of their transport into the cell. Finally a very useful feature of yeasts is the ability to isolate mutants in which transport is reduced or sometimes completely abolished. Characterization of such mutants can often give valuable information as to the transport mechanism itself. Keeping the above points in mind, specific examples of metabolite transport in *S. pombe* are considered.

B. Energy Requirements for Transport and Involvement of Plasma Membrane ATPase

In all the transport systems studied there is a general requirement for an energy source, usually glucose, whose function is to generate ATP. ATP hydrolysis is coupled to proton translocation via the membrane ATPase, the resulting proton gradient driving the transport of metabolites into the cell. Collapse of the proton gradient by uncouplers, such as azide, CCCP, and dinitrophenol (DNP), drastically reduces transport. Evidence for the direct involvement of the plasma membrane ATPase in transport processes in *S. pombe* has already been reviewed in Sections I,A and I,G and are not discussed further.

C. Kinetic Constants for Transport of Metabolites and Ions

A list of the published values for kinetic constants for transport of amino acids, nucleic acid bases, ribosides, malate, and Ca^{2+} is given in Table I.

TABLE I

Kinetic Constants for Transport of Metabolites and Ions into *Schizosaccharomyces pombe*

Metabolite or ion	Strain	Conditions	K_m (μM)	V	Reference
Amino acids					
Asp	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	5000	230 nmol/min/g protein	Foury and Goffeau (1975)
Gly	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	1000	290 nmol/min/g protein	Foury and Goffeau (1975)
Leu	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	200	800 nmol/min/g protein	Foury and Goffeau (1975)
Lys	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	250	690 nmol/min/g protein	Foury and Goffeau (1975)
Try	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	2000	590 nmol/min/g protein	Foury and Goffeau (1975)
Val	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	550	480 nmol/min/g protein	Foury and Goffeau (1975)
α-Aminoisobutyric acid	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	3000	100 nmol/min/g protein	Foury and Goffeau (1975)
Arg	972 *h*$^-$	EMM,[a] pH 5.5	13	70 amol/min/cell	Fantes and Creanor (1984)
Arg	972 *h*$^-$	Acetate, 50 m*M*, pH 5.5	10	11 nmol/min/mg dry wt.	Coddington and Schweingruber (1986)
Gly	NCYC 132	LM,[b] pH 5.6	27	6.6 amol/min/cell	Vaughan (1971)
Leu	NCYC 132	LM, pH 5.6	400	5.9 amol/min/cell	Vaughan (1971)
Tyr	972 *h*$^-$	Acetate, 50 m*M*, pH 5.5	150	2.3 nmol/min/mg dry wt.	Coddington and Schweingruber (1986)
Bases					
Adenine	NCYC 132	EMM, pH 5.5	2.5	70 amol/min/cell	Cummins and Mitchison (1967)
Guanine	972 *h*$^-$	EMM, pH 5.5	0.8	ND[c]	Pourquie (1970)
Guanine	972 *h*$^-$	EMM, pH 5.5	1.1	15 amol/min/cell	Housset *et al.* (1975)
Xanthine	972 *h*$^-$	Citrate, 50 m*M*, pH 5.5	6.0	4 amol/min/cell	Seipel and Reichert (1975)
Ribosides					
Adenosine	*COB5*	Tris–citrate, 50 m*M*, pH 5.5	2000	640 nmol/min/g protein	Foury and Goffeau (1975)
Guanosine	*COB5*	Tris–citrate, 50 m*M*, pH 5.5	>5000	ND	Foury and Goffeau (1975)
Uridine	*COB5*	Tris–citrate, 50 m*M*, pH 5.5	14	90 nmol/min/g protein	Foury and Goffeau (1975)
Malate	972 *h*$^-$	KCl, 100 m*M*, pH 3.5	3700	40 nmol/min/mg dry wt.	Osothsilp and Subden (1986)
Calcium ion	*COB5*	Tris–citrate, 50 m*M*, pH 4.5	44	0.66 amol/min/cell	Boutry *et al.* (1977)

[a]EMM, Edinburgh minimal medium (Mitchison, 1970).
[b]LM, Leupold's minimal medium (Gutz *et al.*, 1974).
[c]ND, Not determined.

1. Strains

The majority of workers have used the standard wild-type strain 972 h^-. Strain NCYC 132 which was used by early workers subsequently turned out to contain a mixture of mating types (J. M. Mitchison, personal communication). COB5 is a mitochondrial respiratory-proficient mutant derived from 972 h^-; in this mutant respiration can be completely suppressed by added glucose.

2. Experimental

To measure transport, cells are usually grown to exponential phase in either a complete or a synthetic medium (Gutz *et al.*, 1974) with shaking at 30°C. They are then washed and resuspended in the uptake medium at a cell density of around 5×10^7 per ml. After allowing the cells to equilibrate, transport is determined by following the incorporation of a radiolabeled metabolite with time into the cells. Kinetic constants are calculated either from Lineweaver and Burk (1934) or Hofstee (1952) plots. An alternative approach is to grow the cells to exponential phase in a synthetic medium and determine the transport of a labeled metabolite without washing and transfer to fresh medium (Fantes and Creanor, 1984; Vaughan, 1971). The main disadvantage of the latter technique is that the concentration of salts and metabolites in the medium, except those which are added with the radiolabeled metabolite; is not known with any certainty.

D. Detailed Consideration of Each Transported Species

1. Amino Acids

In all cases studied transport is saturable and exhibits Michaelis–Menten kinetics indicating the involvement of a protein permease in the plasma membrane. There is also evidence, from the kinetic constants, from inhibitor studies with other amino acids and their analogs, and from studies with mutants, for both a general amino acid permease and a separate one for the basic amino acids. The transport of tyrosine is likely to be via a general amino acid permease. It is competitively inhibited by phenylalanine, histidine, and glutamic acid with inhibitor constants of the same order of magnitude as the K_m for tyrosine (Coddington and Schweingruber, 1986). Mutations at three loci, *cyh3*, *cyh4* (Johnston and Coddington, 1982), and *pho1* (Coddington and Schweingruber, 1986) significantly decrease transport. Indeed, in the case of strains carrying the *cyh3* and *pho1* mutations there is no longer any saturable transport. *cyh3* and *cy4* muta-

tions qualitatively reduce the transport of glutamic acid, glutamine, threonine, glycine, lysine, and arginine; the *pho1* mutation qualitatively reduces the transport of glutamic acid, glutamine, and glycine but not of arginine.

Evidence for a basic amino permease comes from the work of Fantes and Creanor (1984). They showed that arginine could be taken up by two systems, I and II. System I was of high affinity (K_m 13 μM), was inhibited by lysine, ornithine, and canavanine but not by tryptophan, and was unaffected by the presence of ammonium ion. In contrast, system II was repressed by ammonium ion and not significantly affected by the above amino acids. A mutant strain *can1*, isolated as resistant to canavanine (an arginine analog), had much reduced system I activity. The best interpretation of these findings is that system I represents a basic amino acid permease and that the *can1*$^+$ gene codes for a component of that system. System II shows parallels with the general amino acid uptake system in *Sacch. cerevisiae* (Grenson *et al.*, 1966, 1970). Additional evidence for the existence of a basic amino acid permease is provided by Coddington and Schweingruber (1986) who also showed the existence of a high-affinity (K_m 10 μM) arginine transport system which was competitively inhibited by lysine and canavanine. *pho1* strains, which completely lack the saturable transport system for tyrosine, show normal arginine transport kinetics, further evidence for there being at least two distinct uptake systems for amino acids in *S. pombe*.

2. *Purine Bases*

The purine bases adenine, guanine, and xanthine all show saturable, high-affinity transport systems with similar K_m values. Cummins and Mitchison (1967) showed that adenine transport was competitively inhibited by diaminopurine and hypoxanthine, the inhibitor constants being similar to the K_m for adenine (2.5 μM). Guanine, xanthine, guanosine, and adenosine also inhibited competitively but with inhibitor constants of a higher order of magnitude. In a study of the kinetics of guanine transport, Pourquié (1970) showed that adenine, hypoxanthine, and 2,6-diaminopurine were all competitive inhibitors, with inhibitor constants very similar to the K_m for guanine transport (0.8 μM). No inhibition by xanthine, guanosine, inosine, and adenosine was found. Taken together the evidence would suggest a single transport system for purine bases.

3. *Uracil*

Uracil transport is saturable and of high affinity (Vaughan, 1971). The uracil permease gene (*fur4*) has been identified by Chevallier and La-

croute (1982), who used a plasmid carrying the *Sacch. cerevisiae* orotidyl monophosphate (OMP)decarboxylase and uracil permease genes and an *S. pombe ars* sequence to transform a *ura4 fur4* double mutant of *S. pombe* which lacked OMPdecarboxylase and was resistant to 5-fluorouracil because of its inability to take up the uracil analog. The transformants were prototrophic, they could actively transport uracil, and transport activity was abolished by DNP, indicating that energy coupling was occurring in the heterologous membrane.

4. Purine and Pyrimidine Ribosides

Adenosine and possibly guanosine showed saturation kinetics but at low affinity. However, uridine uptake was saturable and of high affinity (Foury and Goffeau, 1975).

5. Malate

Osothsilp and Subden (1986) showed that malate transport was constitutive and saturable and had an optimum at pH 3.5 and 30°C. It was inhibited by dicarboxylic acids in general, succinate being a competitive inhibitor. This suggests a common transport system for dicarboxylic acids. Mutants unable to transport malate were isolated but not characterized genetically.

E. Mutations Affecting Transport

Mutations of at least five well-characterized genetic loci (*cyh3*, *cyh4*, *fur4*, *pho1*, and *pma1*) affect metabolite transport. In two of these, *fur4* and *pma1*, the respective gene products would seem to affect the transport process directly, and the proteins involved, uracil permease in the case of *fur4* and the plasma membrane ATPase in the case of *pma1* may be specified. Of the remaining three, *cyh3* and *cyh4* were first identified as conferring resistance to cycloheximide but subsequently were shown to confer cross-resistance to a wide variety of ribosomal and mitochondrial inhibitors (Ibrahim and Coddington, 1976). As well as having the specific effect on tyrosine transport discussed earlier, they also showed a reduced transport of cycloheximide, trichodermin, and chloramphenicol (Johnston and Coddington, 1982). Although it was initially reported that crude membrane preparations from *cyh3* and *cyh4* strains had a lowered mitochondrial and plasma membrane ATPase specific activity compared to wild type which might be responsible for the reduced transport (Johnston

and Coddington, 1983), this was not confirmed in a later, more careful study (Ulaszewski *et al.*, 1986). Indeed, crude membrane preparations from *cyh3* and *cyh4* and the purified ATPase from *cyh4* were shown to have properties identical to those of their wild-type analogs.

In a later paper Johnston and Coddington (1984) compared the fatty acid and sterol composition of total lipids from the *cyh3* and *cyh4* strains with that of the wild type. The mutant lipids had reduced amounts of the unsaturated oleic acid and increased amounts of the saturated longer chain ($>C_{18}$) fatty acid side chains. The sterol content of the membranes also differed: *cyh3* and *cyh4* showed a reduced percentage of ergosterol relative to 24(28)-dehydroergosterol. If these alterations in fatty acid side chain and sterol composition of total lipids were reflected in the plasma membrane lipids, this could provide a membrane environment sufficiently altered in the mutants to lead to a general reduction in transport. However, it might not be expected to lead to the complete lack of saturable tyrosine transport showed by *cyh3*, which would argue for the lack of a general amino acid permease in that strain.

The remaining locus *pho1* is equally intriguing. Protein purification and sequencing together with gene cloning and DNA sequencing studies (Elliott *et al.*, 1986) show that this gene codes for the phosphate-repressible acid phosphatase. The *pho1* mutant used by Coddington and Schweingruber (1986), probably a deletion, had very much reduced acid phosphatase activity and completely lacked the saturable tyrosine transport system (presumably a general amino acid permease). Acid phosphatase is a highly glycosylated extracellular enzyme and not a normal membrane component. Hence it is difficult to see how it could be a component of the general amino acid permease. Although *cyh3* and *pho1* strains are phenotypically similar with regard to tyrosine transport, they are not linked genetically and *cyh3* has normal levels of phosphate-repressible acid phosphatase activity.

F. Conclusions

Although relatively few active transport systems for metabolites have been studied in *S. pombe* the available evidence suggests that the mechanisms are probably identical to those in other yeast species. The H^+-ATPase translocates protons to form a gradient (higher concentration outside), and a specific permease catalyzes the movement inward of metabolites by a process probably involving proton symport.

REFERENCES

Addison, R. (1986). Primary structure of the *Neurospora* plasma membrane H^+-ATPase deduced from the gene sequence. *J. Biol. Chem.* **261,** 14896–14901.

Addison, R., and Scarborough, G. A. (1982). Conformational changes of the *Neurospora* plasma membrane H^+ATPase during its catalytic cycle. *J. Biol. Chem.* **257,** 10421–10426.

Amory, A., and Goffeau, A. (1982). Characterization of the β-aspartyl phosphate intermediate formed by the H^+-translocating ATPase from the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **257,** 4723–4730.

Amory, A., Foury, F., and Goffeau, A. (1980). The purified plasma membrane ATPase of the yeast *Schizosaccharomyces pombe* forms a phosphorylated intermediate. *J. Biol. Chem.* **255,** 9353–9357.

Amory, A., Goffeau, A., McIntosh, D., and Boyer, P. (1982). Exchange of oxygen between phosphate and water catalyzed by the plasma membrane ATPase from the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **257,** 12509–12516.

Amory, A., Goffeau, A., McIntosh, D., and Boyer, P. (1984). Contribution of ^{18}O technology to the mechanism of the H^+-ATPase from yeast plasma membrane. *Curr. Top. Cell. Regul.* **24,** 471–483.

Boutry, M., Foury, F., and Goffeau, A. (1977). Energy-dependent uptake of calcium by the yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **464,** 602–612.

Brandl, C. J., Green, N. M., Korczak, B., and MacLennan, D. H. (1986). Two Ca^{2+}-ATPase genes: Homologies and mechanistic implications of deduced amino acids sequences. *Cell* **44,** 597–607.

Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978). A characterization of vanadate interactions with the (Na,K)-ATPase. Mechanistic and regulatory implications. *J. Biol. Chem.* **253,** 7361–7368.

Carrasco, N., Antes, L. M., Poonian, M. S., and Kaback, H. J. (1986). Lac permease of *Escherichia coli:* Histidine-322 and glutamic acid-325 may be components of a charge-relay system. *Biochemistry* **25,** 4486–4488.

Chevallier, M. R., and Lacroute, F. (1982). Expression of the cloned uracil permease gene of *Saccharomyces cerevisiae* in a heterologous membrane. *EMBO J.* **3,** 375–377.

Chou, P. Y., and Fassman, G. D. (1974). Prediction of protein conformation. *Biochemistry* **13,** 222–295.

Clement, J. D., Ghislain, M., Dufour, J. P., and Scalla, R. (1986). Immunodetection of 90,000 M_r polypeptide related to yeast plasma membrane ATPase in plasma membrane from maize shoots. *Plant Sci.* **5,** 43–50.

Coddington, A., and Schweingruber, M. E. (1986). Acid phosphatase deficient mutants of *Schizosaccharomyces pombe* are defective in tyrosine uptake. *Curr. Genet.* **11,** 113–118.

Cummins, J. E., and Mitchison, J. M. (1967). Adenine uptake and pool formation in the fission yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **136,** 108–120.

Delhez, J., Dufour, J. P., Thinès, D., and Goffeau, A. (1977). Comparison of the properties of plasma membrane-bound and mitochondria-bound ATPases in the yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **79,** 319–328.

de Meis, L., Blanpain, J. P., and Goffeau, A. (1987). $P_i \rightleftharpoons$ ATP exchange in the absence of proton gradient by the H^+-ATPase from yeast plasma membranes. *FEBS Lett.* **212,** 323–327.

Di Pietro, A., and Goffeau, A. (1985). Essential arginyl residues in the H^+-translocating

ATPase of plasma membrane from the yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **148,** 35–39.

Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.* **55,** 170–171.

Dufour, J. P., and Goffeau, A. (1978). Solubilization by lysolecithin and purification of the plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **253,** 7026–7032.

Dufour, J. P., and Goffeau, A. (1980). Molecular and kinetic properties of the purified plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **105,** 145–154.

Dufour, J. P., and Goffeau, A. (1980). Phospholipid reactivation of the purified plasma membrane ATPase of yeast. *J. Biol. Chem.* **255,** 10591–10598.

Dufour, J. P., and Tsong, T. Y. (1981). Plasma membrane ATPase of yeast. Activation and interaction with dimyristoylphosphatidycholine vesicles. *J. Biol. Chem.* **256,** 1801–1808.

Dufour, J. P., and Tsong, T. Y. (1982). ATP-dependent proton pumping catalyzed by the purified yeast plasma membrane ATPase reconstituted into phospholipid vesicles. *Biophys. J.* **37,** 96–97.

Dufour, J. P., Boutry, M., and Goffeau, A. (1980). Plasma membrane ATPase of yeast. Comparative inhibition studies of the purified and membrane-bound enzymes. *J. Biol. Chem.* **255,** 5735–5741.

Dufour, J. P., Nunnally, R., Buhle, L., and Tsong, T. Y. (1981). Comparative study of an adenosine triphosphatase trigger-fused lipid vesicle and other vesicle forms of dimyristoylphosphatidycholine. *Biochemistry* **20,** 5576–5584.

Dufour, J. P., Goffeau, A., and Tsong, T. Y. (1982). Active proton uptake in lipid vesicles reconstituted with the purified yeast plasma membrane of ATPase. Fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine. *J. Biol. Chem.* **257,** 9365–9371.

Eddy, A. A. (1982). Mechanisms of solute transport into selected eukaryotic microorganisms. *Adv. Microb. Physiol.* **23,** 1–78.

Eisenberg, D. A., Schwartz, E., Komaromy, M., and Wall, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179,** 125–142.

Elliott, S., Chang, C., Schweingruber, M. E., Schaller, J., Rickli, E. R., and Carbon, J. (1986). Isolation and characterisation of the structural gene for secreted acid phosphatase from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **261,** 2936–2941.

Fantes, P. A., and Creanor, J. (1984). Canavanine resistance and the mechanism of arginine uptake in the fission yeast *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **130,** 3265–3273.

Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., and Shively, J. E. (1984). The amino acid sequence of a fluorescein-labeled peptide from the active site of (Na,K)-ATPase. *J. Biol. Chem.* **259,** 9532–9535.

Foury, F., and Goffeau, A. (1973). Stimulation of yeast RNA synthesis by cycloheximide and 3′,5′-cyclic AMP. *Nature (London) New Biol.* **245,** 44–47.

Foury, F., and Goffeau, A. (1975). Stimulation of active uptake of nucleosides and amino acids by cyclic adenosine 3′:5′-monophosphate in the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **250,** 2354–2362.

Foury, F., Boutry, M., and Goffeau, A. (1977). Efflux of potassium induced by Dio-9, a plasma membrane ATPase inhibitor in the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **252,** 4577–4583.

Foury, F., Amory, A., and Goffeau, A. (1981). Large-scale purification and phosphorylation

of a detergent-treated adenosine triphosphatase complex from plasma membrane of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **119,** 395–400.

Garnier, J., Osguthorpe, D. S., and Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120,** 97–120.

Ghislain, M., Schlesser, A., and Goffeau, A. (1988). A conserved glycine residue modifies the vanadate-sensitivity of the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **262,** 17549–17555.

Goffeau, A., and Slayman, C. W. (1981). The proton-translocating ATPase of the fungal plasma membrane. *Biochim. Biophys. Acta* **639,** 197–223.

Grenson, M., Mousset, M., Wiame, J.-M., and Bechet, J. (1966). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine transporting system. *Biochim. Biophys. Acta* **127,** 325–338.

Grenson, M., Hou, C., and Crabeel, M. (1970). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. *J. Bacteriol.* **103,** 770–777.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. *Handb. Genet.* **1,** 394–446.

Hager, K. M., Mandala, S. M., Davenport, J. W., Speincher, D. W., Benz, E. J., Jr., and Slayman, C. W. (1986). Amino acid sequence of the plasma membrane ATPase of *Neurospora crassa:* Deduction from genomic and cDNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7693–7697.

Hesse, J. E., Wieczorek, L., Altendorf, K., Reicin, A. S., Dorus, E., and Epstein, W. (1984). Sequence homology between two membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4746–4750.

Hofstee, B. H. J. (1952). On the evaluation of the constants V_m and K_m in enzyme reactions. *Science* **116,** 329–331.

Housset, P., Nagy, M., and Schwenke, J. (1975). Protoplasts of *Schizosaccharomyces pombe:* An improved method for their preparation and the study of their guanine uptake. *J. Gen. Microbiol.* **90,** 260–264.

Ibrahim, M. A. K., and Coddington, A. (1976). Genetic studies on cycloheximide resistant strains of *Schizosaccharomyces pombe*. *Heredity* **37,** 179–191.

Johnston, P. A., and Coddington, A. (1982). Multiple drug resistance in the fission yeast *Schizosaccharomyces pombe:* Evidence for the existence of pleiotropic mutations affecting energy dependent transport systems. *Mol. Gen. Genet.* **185,** 311–314.

Johnston, P. A., and Coddington, A. (1983). Multiple drug resistance in the fission yeast *Schizosaccharomyces pombe:* Correlation between drug and amino acid uptake and membrane ATPase activities. *Curr. Genet.* **7,** 299–307.

Johnston, P. A., and Coddington, A. (1984). Multiple drug resistance in the fission yeast *Schizosaccharomyces pombe:* Pleiotropic mutations affecting the oleic acid and sterol composition of cell membranes. *Curr. Genet.* **8,** 37–43.

Jørgensen, P. L., Skriver, E., Herbert, H., and Maunsbach, A. B. (1982). Structure of the Na,K pump: Crystallization of pure membrane-bound Na,K-ATPase and identification of functional domains of the alpha-subunit. *Biochim. Biophys. Acta* **731,** 290–303.

Kasher, J., Allen, K. E., Kasamo, K., and Slayman, C. W. (1986). Characterization of an essential arginine residue in the plasma membrane H^+-ATPase of *Neurospora crassa*. *J. Biol. Chem.* **261,** 10808–10813.

Kolarov, J., Kulpa, J., Baijot, M., and Goffeau, A. (1988). Characterization of a protein serine kinase from yeast plasma membrane. *J. Biol. Chem.* **263,** 10613–10619.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Biol. Chem.* **157,** 105.

Lineweaver, H., and Burk, D. (1934). The determination of enzyme dissociation contents. *J. Am. Chem. Soc.* **56,** 658–666.

MacLennan, D. H., Brandl, C. J., Korczak, B., and Green, N. M. (1985). Amino-acid sequence of a Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature* (*London*) **316,** 696–700.

Mitchell, P. (1973). Performance and conservation of osmotic work by proton-coupled solute transporter systems. *Bioenergetics* **4,** 63–91.

Mitchison, C., Wilderspin, A. F., Trinnaman, B. J., and Green, N. M. (1982). Identification of a leballed peptide after stoichiometric reaction of fluorescein isothiocyanate with the Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum. *FEBS Lett.* **146,** 87–92.

Mitchison, J. M. (1970). Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.* **4,** 131–165.

Ohta, T., Nagano, K., and Yoshida, M. (1986). The active site structure of Na^+/K^+-transporting ATPase: Location of the 5′(*p*-fluorosulfonyl)benzoyladenosine binding site and soluble peptides released by trypsin. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 2071–2075.

Osothsilp, C., and Subden, R. E. (1986). Malate transport in *Schizosaccharomyces pombe*. *J. Bacteriol.* **168,** 1439–1443.

Pick, V. (1982). The interaction of vanadate ions with the Ca-ATPase from sarcoplasmic reticulum. *J. Biol. Chem.* **257,** 6111–6119.

Pourquie, J. (1970). Antagonism by adenine in the nutrition of *Schizosaccharomyces pombe* mutants. Inhibition at the level of guanine uptake. *Biochim. Biophys. Acta* **209,** 269–277.

Ronjat, M., Lacapere, J. J., Dufour, J. P., and Dupont, Y. (1987). Study of the nucleotide binding site of the yeast *Schizosaccharomyces pombe* plasma membrane H^+-ATPase using formycin triphosphate–terbium complex. *J. Biol. Chem.* **262,** 3146–3153.

Scalla, R., Amory, A., Rigaud, J., and Goffeau, A. (1983). Phosphorylated intermediate of a transport ATPase and activity of protein kinase in membranes from corn roots. *Eur. J. Biochem.* **132,** 525–530.

Scarborough, G. E. (1985). Binding energy, conformational change, and the mechanism of transmembrane solute movements. *Microbiol. Rev.* **49,** 214–231.

Scott, T. L., and Shamoo, A. E. (1982). Disruption of energy transduction in sarcoplasmic reticulum by trypsin cleavage of (Ca^{2+} + Mg^{2+})-ATPase. *J. Membr. Biol.* **84,** 137–144.

Seipel, S., and Reichert, U. (1975). Untersuchungen zur Aufnahme von Xanthin durch *Schizosaccharomyces pombe* aus der stationären Wachstumsphase. *Protoplasma* **84,** 127–135.

Serrano, R. (1984). Plasma membrane ATPase of fungi and plants as a novel type of proton pump. *Curr. Top. Cell. Regul.* **23,** 87–126.

Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+- and Ca^{2+}-ATPase. *Nature* (*London*) **319,** 689–693.

Sussman, M. R., Strickler, J. E., Hager, K. M., and Slayman, C. W. (1987). Location of a dicyclohexylcarbodiimide-reactive glutamate residue in the *Neurospora crassa* plasma membrane H^+-ATPase. *J. Biol. Chem.* **262,** 4569–4573.

Ulaszewski, S., Grenson, M., and Goffeau, A. (1983). Modified plasma-membrane ATPase in mutants of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **130,** 235–239.

Ulaszewski, S., Coddington, A., and Goffeau, A. (1986). A new mutation for multiple drug resistance and modified plasma membrane ATPase activity in *Schizosaccharomyces pombe*. *Curr. Genet.* **10,** 359–364.

Ulaszewski, S., Van Herck, J. C., Dufour, J. P., Kulpa, J., Nieuwenhuis, B., and Goffeau, A. (1987). A single mutation confers vanadate resistance to the plasma membrane H^+-ATPase from the yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **262,** 223–228.

van Heijne, G. (1986). The distribution of positively charged residues in bacterial inner membrane proteins correlates with the transmembrane topology. *EMBO J.* **5,** 3021–3027.

Vara, F., and Serrano, R. (1983). Phosphorylated intermediate of the ATPase of plant plasma membranes. *J. Biol. Chem.* **258,** 5334–5336.

Vaughan, G. L. (1971). Oxygen toxicity in a fission yeast. *J. Cell Physiol.* **77,** 363–372.

Villalobo, A. (1982). Potassium transport coupled to ATP hydrolysis in reconstituted proteoliposomes of yeast plasma membrane ATPase. *J. Biol. Chem.* **257,** 1824–1828.

Villalobo, A. (1984). Energy-dependent H^+ and K^+ translocation by the reconstituted yeast plasma membrane ATPase. *Can. J. Biochem. Cell Biol.* **62,** 865–877.

Villalobo, A., Boutry, M., and Goffeau, A. (1981). Electrogenic proton translocation coupled to ATP hydrolysis by the plasma membrane Mg^{2+}-dependent ATPase of yeast in reconstituted proteoliposomes. *J. Biol. Chem.* **256,** 12081–12087.

Webb, J. L. (1963). "Enzyme and Metabolic Inhibitors," Vol. 1. Academic Press, New York.

Wierenga, R. K., Terpotra, P., and Hol, W. G. J. (1986). Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$-fold in proteins using amino acid sequence fingerprint. *J. Mol. Biol.* **187,** 101–107.

12

Taxonomy and Phylogenesis

M. SIPICZKI

Department of Genetics
University of Debrecen
H-4010 Debrecen, Hungary

I. TAXONOMY

A. Taxonomy of Genus *Schizosaccharomyces* Lindner

Most yeast taxonomists follow the principles of naming of organisms laid down in the *International Code of Botanical Nomenclature*, which is reedited after each International Botanical Congress (for a review, see Barnett *et al.*, 1983). The last edition of the International Code was published in 1978. Article 36 of the Code rules that a Latin description must be given for any taxon published after January 1, 1935. Another requirement for the acceptibility of a name is the indication of the type. In the case of plants, the type of the name must be a dried herbarium specimen (Article 9.5). For microorganisms, where the classification is based

MOLECULAR BIOLOGY OF THE
FISSION YEAST

mainly on physiological and biochemical characteristics, this provision is replaced by the requirement to deposit a strain in one of the internationally accepted collections as the type of the species (Barnett *et al.*, 1983). For the genus *Schizosaccharomyces*, the type cultures are maintained in the Centraalbureau voor Schimmelcultures (CBS; Baarn and Delft, The Netherlands), but they are also available in a number of other collections.

According to the latest Latin description (Sipiczki *et al.*, 1982) the fission yeasts comprise a single genus: *Schizosaccharomyces* Lindner (Table I). Its type species is *Schizosaccharomyces pombe* Lindner. The genus contains one four-spored species with two varieties and two eight-spored species. One of the latter two taxa is also divided into two varieties (Sipiczki *et al.*, 1982; Yarrow, 1984).

The introduction of a new genus, *Octosporomyces*, suggested by Kudriawzew (1960), that would include the eight-spored *Schizosaccharomyces japonicus* and *Schizosaccharomyces octosporus* seems unjustified, especially in view of the ability of the latter to undergo somatic hybridization with the four-spored taxa (Sipiczki, 1979; Sipiczki *et al.*, 1982) and the strong similarities of the cytochrome spectrum (Claisse and Simon-Becam, 1978; Sipiczki *et al.*, 1982) and long-chain fatty acid profile (Kock and van der Walt, 1986) to those of the four-spored group. Comparison of β-1,3-glucanase and β-1,6-glucanase activities among the fission yeasts also showed a closer relationship of *S. octosporus* to *S. pombe* than to *S. versatilis:* the latter showed activities higher by factors of 300 and 25, respectively (Fleet and Phaff, 1975). A numerical classification using 60 strains and 100 characters did not reveal a significantly higher similarity between *S. japonicus* and *S. octosporus* than between either of them and *S. pombe* (Bridge and May, 1984). The number of serine tRNA genes and genes for $tRNA_i^{Met}$ are identical in *S. octosporus* and *S. pombe* and differ from that in *S. japonicus* var. *versatilis* (Stadelmann, 1985).

Nevertheless, the idea of division of the fission yeasts into two genera cannot be completely rejected, because certain findings still seem to support it. For instance, the four-spored taxa contain coenzyme Q_{10}, while the eight-spored species are characterized by the coenzyme Q_9 system (Yamada *et al.*, 1973). A numerical taxonomy based mainly on tests of extracellular hydrolytic activities revealed two phenons among 22 strains, one for the four-spored and one for the eight-spored strains (Kocková-Kratochvilová *et al.*, 1985).

The division of *S. pombe* and *S. japonicus* into varieties is not accepted by all taxonomists either. The numerical taxonomy of Bridge and May (1984) did not reveal sufficient evidence for the subdivision of any of these species. A major problem of most numerical classification, however, is that the characters involved are considered with equal value, that is,

TABLE I

Taxonomy of *Schizosaccharomyces* Lindner

Genus	Species	Variety	Type strain	Synonyms
Schizosaccharomyces Lindner 1893	*S. pombe* Lindner 1893	*S. pombe* Lindner var. *pombe* Sipiczki *et al.* 1982	CBS 356	*S. vordermani* Wehmer 1906 *S. mellacei* Jörgensen 1909 *S. formosensis* Nakazawa 1914 *S. formosensis* Nakazawa var. *akoensis* Nakazawa 1914 *S. formosensis* Nakazawa var. *tapaniensis* Nakazawa 1914 *S. santawensis* Nakazawa 1914 *S. pinan* Nakazawa 1919 *S. taito* Nakazawa 1919 *S. liquefaciens* Osterwalder 1924 *S. acidevoratus* Tchalenko 1941 *S. pombe* Lindner var. *acidevoratus* (Tschalenko) Dittrich 1964
		S. pombe Lindner var. *malidevorans* (Rankine et Fornachon) Sipiczki *et al.* 1982	CBS 5557	*S. malidevorans* Rankine et Fornachon 1964
	S. octosporus Beijerinck 1894		CBS 371	*Octosporomyces octosporus* (Beijerinck) Kudriawzew 1960 *S. sloofiae* Kumbhojkar 1972
	S. japonicus Yukawa et Maki 1931	*S. japonicus* Yukawa et Maki var. *japonicus*	CBS 351	*Octosporomyces japonicus* (Yukawa et Maki) Kudriawzew 1960
		S. japonicus Yukawa et Maki var. *versatilis* (Wickerham et Duprat) Sloof 1970	CBS 103	*S. versatilis* nom. nud. (Wickerham et Duprat, 1945)

without distinction as to their biological importance or phylogenetic conservatism. This approach may hide certain phylogenetically important traits and overrate those which are variable or highly dependent on changes of other characters or environmental conditions. Since varieties, when defined in terms of genetics, are slightly differing but distinct subunits of a species that are partially isolated from each other in the exchange of genetic information, the comparison of their genomes may reveal how advanced they are in the phylogenetic divergence. The DNA–DNA reassociation analysis of various strains revealed 98% homology between *S. pombe* var. *pombe* and *S. pombe* var. *malidevorans* and 84% homology among the varieties of *S. japonicus* (A. Vaughan-Martini, personal communication). Interfertility was detected both between the four-spored varieties and between the eight-spored ones (Sipiczki *et al.*, 1982). In the former crosses, spore viability was rather low (no complete tetrad among 50 asci), indicating that their genomes are different to some extent.

In addition, there are a number of morphological and physiological differences, such as the rough outer membrane on the spores of *S. pombe* var. *malidevorans*, its inability to ferment maltose, and its increased ability to decompose L-malic acid and produce hydrogen sulfide compared to *S. pombe* var. *pombe* (Rankine and Fornachon, 1964), which all offer a possibility to distinguish these two taxa. The varieties of *S. japonicus* differ in their cytochrome spectra: *S. japonicus* var. *versatilis* lacks all cytochromes $a + a_3$, b, c_1, and c, while in *S. japonicus* var. *japonicus* only $a + a_3$ and c are absent (Sipiczki *et al.*, 1982). The ascospores of the former become reniform to allantoid; those of the latter variety do not change shape (Yarrow, 1984). The former segregates heterothallic types, the latter is strictly homothallic (M. Sipiczki, unpublished). Thus, it seems justified to retain these two taxa in variety status as well.

B. Origin and History of Laboratory Strains

Most laboratory strains used in genetics and molecular biology originate from a Swiss isolate, *Schizosaccharomyces liquefaciens* Osterwalder (Osterwalder, 1924), which was introduced to genetic analysis by Leupold (1950). Since in the meantime it was placed in the species *S. pombe* Lindner (Stelling-Dekker, 1931), most authors use this name. A recent emendation of the taxonomy of the genus connected it with *Schizosaccharomyces malidevorans* (Sipiczki *et al.*, 1982), and, thus, its new legitimate name is *S. pombe* var. *pombe*. The original isolate is maintained in the CBS collection with the number CBS 1042. Strains of other origin are used only occasionally.

C. Taxonomic Relationship of Fission Yeasts to Other Yeasts

The yeasts are a taxonomically and phylogenetically diverse group of fungi and include ascomycetes, basidiomycetes, and taxa belonging to Deuteromycotina (imperfect yeasts). Because of the lack of absolute criteria for use in speciation, one may find the taxonomy of yeasts rather confusing and controversial. Delimitation of the genera and even the higher taxa is also mostly arbitrary and depends on the choice of characters. Actually, the development of criteria have always paralleled the development of other areas of biology, especially the advances in molecular biology. The changes in the taxonomy of yeasts have been reviewed from time to time in monumental taxonomic works like the three editions of "The Yeasts—A Taxonomic Study" (third edition: Kreger-van Rij, 1984a), edited by taxonomists associated with the CBS. For more details, the reader is referred to these publications.

The taxonomic system proposed by Kreger-van Rij (1984b) is the most comprehensive one and includes all yeast genera recognized by the CBS, but it is not necessarily agreed to by all yeast biologists. It classifies all yeasts forming saclike meiosporangia (ascosporogenous yeasts) in the class Hemiascomycetes (Table II). The Hemiascomycetes are differentiated from the other ascomycetes by their lack of ascocarps and ascogenous hyphae (von Arx, 1980). The fission yeasts comprise a subfamily which contains the genus Schizosaccharomyces only. Other systems consider them as a separate family (e.g., Kocková-Kratochvilová, 1982). A very new system proposed by a group of taxonomists (Gams *et al.*, 1987) also working in the CBS, however, classifies them in the family Dipodascaceae, together with the genus *Dipodascus* (anamorph: *Geotrichum*) (Table III).

The diversity of the ranks of this taxon in the individual systems illustrates how perplexed taxonomists are by the unique morphological and physiological features of the fission yeasts. All efforts to link them with other genera in a common family seem to be compromises in the endeavour to provide a complete system harboring all yeasts and yeastlike organisms. For instance, the connection of *Schizosaccharomyces* with *Dipodascus* (Table III) is based on the seemingly similar mode of propogation of the two taxa: formation of arthroconidia (in *Dipodascus*) or fission cells (in *Schizosaccharomyces*) and single asci which develop immediately from conjugating cells. The larger number of other traits in which these two genera differ from each other, however, makes this classification unjustified. Furthermore, the rapid development of the molecular biology of one of the fission yeasts, *S. pombe* var. *pombe* (as illustrated by this book), has provided a great deal of new data which suggest that the fission

TABLE II

Classification of Ascosporogenous Yeasts According to Kreger-van Rij (1984b)

Subdivision	Class	Order	Family	Subfamily
Ascomycotina	Hemiascomycetes	Endomycetales	Spermophtoraceae	
			Saccharomycetaceae	Schizosaccharomycetoideae
				Nadsonioideae
				Lipomycetoideae
				Saccharomycetoideae

TABLE III

Classification of Ascosporogenous Yeasts and Yeastlike Organisms According to Gams *et al.* (1987)

Division	Class	Order	Selected families
Ascomycota	Hemiascomycetes	Endomycetales	Endomycetaceae Dipodascaceae Saccharomycataceae Saccharomycodaceae
		Torulopsidales	
		Taphrinales	Taphrinaceae Protomycetaceae

yeasts should be separated from the other ascosporogenous yeasts and raised to a higher rank in the system of fungi. This would better fit its phylogenetic position (see below) which is a major goal of all natural taxonomic systems.

II. PHYLOGENESIS

The unexpected extent of divergence between *S. pombe* and *Saccharomyces cerevisiae* revealed during the 1970s and 1980s has raised doubts whether the taxonomic allocation of the fission yeasts corresponds to their phylogenetic position among fungi. This inspired me to review and analyze certain recent findings and some older data and thus contribute to a better understanding of the phylogenetic history of *Schizosaccharomyces*.

A. Sequence Comparison of rRNAs, tRNAs, and Ribosomal Proteins: Three Possible Phylogenies

Owing to its high conservatism and relatively small size (approximately 120 nucleotides), the 5 S RNA is an attractive model for analysis of phylogenetic relationships. Sequence comparisons of *S. pombe* 5 S rRNA with its counterparts in other organisms revealed a considerable evolutionary distance between this species and the ascosporogenous budding yeasts. The nucleotide sequence of *S. pombe* 5 S rRNA shows more differences from that of *Sacch. cerevisiae* than from that of human or rat (Erdman *et al.*, 1983; Komiya *et al.*, 1981). In the phylogenetic trees constructed by

Hori's group using a clustering method (Hori and Osawa, 1979; Hori *et al.*, 1982), the above-mentioned distance is reflected in the independent origin of fission yeasts and other ascomycetes. The lineage leading to *Schizosaccharomyces* originates somewhat later than that leading to Ascomycota (including *Sacch. cerevisiae*). Assuming that the human and *Saccharomyces* divergence occurred 1200 ± 75 million years ago (Kimura and Ohta, 1973; Hori and Osawa, 1979), the divergence between *Schizosaccharomyces* and higher eukaryotes could have taken place about 1000 million years ago, as calculated from their dendograms.

Huysmans *et al.* (1983), using a somewhat different method and including more species in the analysis (82 sequences), came basically to the same conclusion. In contrast to metazoa and green plants, which form homogenous clusters in their tree, the fungi are represented by more branches, suggesting a polyphyletic origin, in which *Schizosaccharomyces* does not belong to any main fungal group. In contrast, in the tree of Küntzel *et al.* (1983) compiled also by clustering the 5 S rRNA sequences, the fungi ramify from a single branch. This is due, however, to the different method of tree construction (Küntzel *et al.*, 1981). They first classified the sequences on the basis of established phylogenetic affinities as eubacteria, fungi, plants, prototoa, and metazoa and then constructed subtrees for each group separately. Although in this case all examined fungi had a common origin, *S. pombe* still appeared to diverge from the fungal branch very early, together with the lower fungus *Phycomyces blakesleeanus*.

Phylogenetic trees inferred from cluster analyses are critically dependent on proper alignment of sequences and can be valid when the rates of sequence divergence are sufficiently similar in all lineages (Colles, 1970). When the pace of evolution is different in the various lineages, the rapidly changing sequence tends to branch too early in the inferred phylogeny. On the other hand, when so much time has passed after the divergence of two taxa that new mutations obscure old ones, the evolutionary comparison is confounded, because only one mutation per site can be recognized.

One approach to these problems is to exclude positions of high variability and to assign so-called signature nucleotides to specific taxa (Wolters and Erdmann, 1986). This may be useful, however, only if combined with cladistic evaluation. Application of this method to the analysis of 5 S rRNA and 16 S rRNA sequences confirmed the monophyletic origin of a number of higher taxa; in fungi the basidiomycetes proved to be biphyletic, while the ascomycetes were found monophyletic (Wolters and Erdmann, 1986). Two ascomycetous genera, *Protomyces* and *Schizosaccharomyces*, however, exhibited plesiomorphic (primitive, ancient) characteristics, because they did not have an insertion at position 52.1

which was characteristic of all examined members of the division *Ascomycota*. The lack of this signature nucleotide raises the question as to whether their classification in *Ascomycota* is sufficiently justified.

As for *Schizosaccharomyces* it is tempting to return to the suggestion of Hori's trees, that this genus arose later than the ancestors of Ascomycota, by branching off from the lineage of animals. However, in the system of Wolters and Erdmann (1986), the Metazoa and Mesozoa share an UU odd base pair at position 81:95 which is unique in eukaryotes. *Schizosaccharomyces* has AU at this position, suggesting that if the fission yeasts are indeed of later origin they must have diverged from the animals very early, prior to the fixation of this base pair. Somewhat supportive of this speculation is the tree of Huysmans *et al.* (1983) which suggests that *Schizosaccharomyces* is related to protists rather than to ascomycetes. According to Wolters and Erdmann (1986), however, the Euglenozoa (including also trypanosomes) and the Cyanaphora share a C to U mutation at position 47 which is missing in *Schizosaccharomyces*. The other protist groups either have not been involved in this analysis or their position in the phylogenetic scheme could not be determined.

The results reviewed demonstrate that the phylogenies based on molecular analysis of 5 S rRNA sequences contradict one another, and in their present form they do not allow the reconstruction of the evolutionary history of *Schizosaccharomyces*. Although all authors are unanimous in the conclusion that this genus is not closely related to any present-day ascomycetes analyzed, they cannot resolve the question of whether the fission yeasts are an archaic ascomycete which diverged very early from the Ascomycota lineage or rather emerged later from the lineage leading to Metazoa. The two possibilities are depicted in Fig. 1A and 1B.

Present data from tRNA sequences, the other RNA family frequently used for mapping evolutionary relationships, cannot resolve the question either. In their early study on phenylalanine tRNA family, Cedergen *et al.* (1980) also found more differences between *S. pombe* and *Sacch. cerevisiae* than between *Schizosaccharomyces* and mammals or *Euglena*. On the other hand, however, of the two yeasts, *Sacch. cerevisiae* was more similar to them. Although in the tree *S. pombe* was placed with *Sacch. cerevisiae*, the authors noted that the fission yeasts could also be branched prior to the divergence of the *Saccharomyces* lineage. Thus, their results not only failed to clear the confusion but even raised a third possibility, namely, that *Schizosaccharomyces* had separated first, before Ascomycota emerged (Fig. 1C). Although they later rejected this possibility and rather preferred a version like Fig. 1A (supported also by the analysis of $tRNA^{Ser}$) (Cedergen *et al.*, 1981), the idea cannot be abandoned completely.

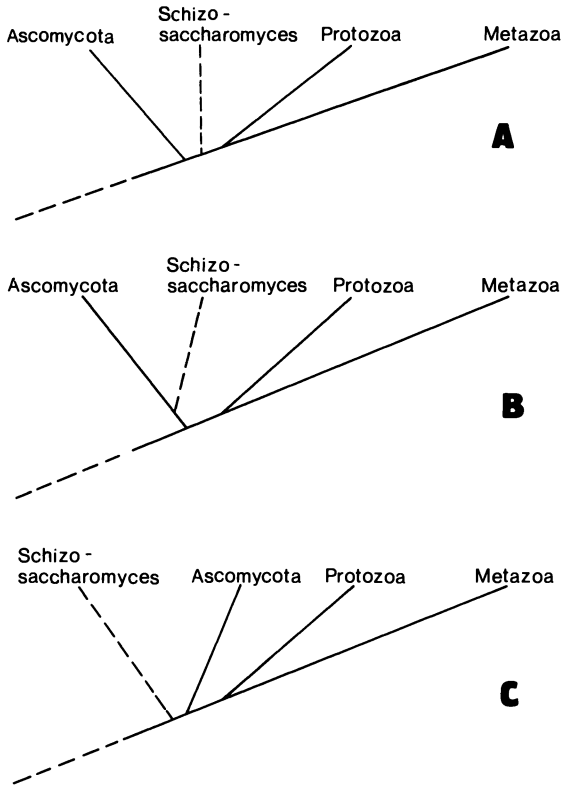


Fig. 1. (A–C) Evolutionary trees of the three possible phylogenies discussed in the text.

For example, if one accepts the method of Wickerham (1951), who distinguished between primitive and more advanced species by regarding the change from haploidy to diploidy as a fundamental evolutionary trend [a trend toward diploidy is a frequent component of theories on evolution (e.g., Bernstein *et al.*, 1985)], this genus with its strictly haplontic life cycle must have been an early product of evolution. Surprisingly, a $tRNA_i^{Met}$ gene was even found to show greater sequence similarity with prokaryotes (68–76%) than with eukaryotes (63–69%) (Kohli *et al.*, 1980). In addition, in the structure of the D-stem of $tRNA^{Phe}$, *S. pombe* also resembles *Escherichia coli* (Kohli *et al.*, 1980). The tyrosine tRNA, however, is more closely related to that of *Xenopus laevis* than to those of budding yeasts (Kohli *et al.*, 1980), and so supports the phylogeny illustrated in Fig. 1B. These discrepancies demonstrate that the tRNA sequence comparison alone is no reliable means for studying phylogenetic

relatedness. Supportive of this conclusion is the finding that the *S. pombe sup3-e* and *sup3-i* genes are accurately transcribed and active in *Sacch. cerevisiae*, suggesting a much closer relationship than that observed for many other corresponding tRNA species of these two organisms (Hottinger *et al.*, 1982, 1984).

In most eukaryotes the 5 S rRNA genes and the other three species of rRNA genes are independently organized in tandem arrays of repeating units. *Schizosaccharomyces pombe* also shows this type of rRNA organization. In contrast, in certain lower eukaryotes, such as *Dictyostelium* and budding yeasts, the 5 S rRNA gene is located near the repeating unit composed of the 18, 5.8, and 26 S rRNA genes (Tabata, 1981; Schaak *et al.*, 1982). These facts could also be interpreted to indicate that *S. pombe* is more closely related to higher eukaryotes than to ascomycetes. However, the rRNA gene organization in *Neurospora crassa* is also similar to that of the higher eukaryotes, so *S. pombe* is not unique in this respect.

The nucleotide sequence of the *S. pombe* 5.8 S rRNA gene shows a greater homology to those of *Sacch. cerevisiae* and *N. crassa* than to those of higher eukaryotes (Schaak *et al.*, 1982). Similar relationships were revealed by comparison of ribosomal proteins. The sequences of the proteins SP-S28 (*S. pombe*) and YS25 (*Sacch. cerevisiae*) show much greater similarity to each other than to their counterpart RL-S21 from rat liver (Otaka *et al.*, 1986). Practically the same conclusion can be drawn from the findings of Kwok *et al.* (1986) who studied the phylogenetic relatedness by measuring the thermal stability of DNA–DNA hybridizations between a cloned basidiomycete 28 S rRNA gene and whole DNA of selected species of fungi and other simple eukaryotes. The ΔT_m of hybridization was in the range 1.2–3.0 for the basidiomycetes, 4.7–5.7 for the ascomycetes, and 9.5 for *Dictyostelium*. With its 5.2 value, *S. pombe* unequivocally belongs to the ascomycetes.

B. Comparison of Amino Acid Sequences: Contradictory Conclusions

The conclusions drawn from comparative analyses of the amino acid sequences published to date are also rather controversial. Examples of *S. pombe* proteins where the sequences of their counterparts from other organisms are also available are listed in Table IV. As for phylogenesis, the informational value of these proteins varies because they do not show the same conservation in the course of evolution. Surprisingly, however, both the molecules presumed to be highly conservative and thus more suitable for phylogenetic analysis (such as the histones and tubulins) and

TABLE IV

Amino Acid Sequence Homologies of Certain *Schizosaccharomyces pombe* var. *pombe* Proteins with Their Counterparts in *Saccharomyces cerevisiae* and Higher Eukaryotes

Protein	Homology (%) with		References
	Sacch. cerevisiae	Higher eukaryotes	
H2A	83	79 (Human)	Matsumoto and Yanagida (1985)
H2B	82	68 (Human)	Matsumoto and Yanagida (1985)
H3	93	92 (Human)	Matsumoto and Yanagida (1985)
H4	91	91 (Human)	Matsumoto and Yanagida (1985)
α-Tubulin		76 (Porcine)	Toda *et al.* (1984)
β-Tubulin	73	75 (Chicken)	Hiraoka *et al.* (1984)
Protein kinase (encoded by *cdc2*$^+$)	62 (Encoded by *CDC28*)	63 (Human, *CDC2*Hs)	Hindley and Phear (1984), Lee and Nurse (1987)
Ribosomal proteins	66	69 (Rat liver)	Otaka *et al.* (1983)
Alcohol dehydrogenase	51	25 (Horse)	Russell and Hall (1983)

the proteins thought to be rather variable show almost the same degree of homology with their counterparts in *Sacch. cerevisiae* and with those in higher eukaryotes. In other words, the *S. pombe* proteins appear, in general, to be equidistantly related to those of *Sacch. cerevisiae* and vertebrates. Alcohol dehydrogenase is the only exception in Table IV. The yeast enzymes are much more closely related to each other than either is to horse alcohol dehydrogenase, suggesting that they have diverged from a common ancestral protein (Russell and Hall, 1983). However, when considering this finding it must be borne in mind that this molecule is probably less conservative than the other proteins listed.

α-Tubulin and β-tubulin are more closely related to vertebrate tubulins in sequence (Table IV), but not in gene organization. A striking similarity has been found between the two yeasts both in organization and expression of the tubulin genes. Both of them have two genes for α-tubulin and one for β-tubulin, but one of the α-tubulin genes is dispensable in both organisms (Adachi *et al.*, 1986). In higher eukaryotes, the number of these genes is much greater, and many of them are developmentally regulated. This contradiction between the sequence homologies and the similarities found in gene organization of the same molecules leads us to question whether the sequence or the organization of a gene (or a gene family) has a higher informative value. Since rearrangements of regions are quite frequent events in genomes of many species, the former seems to be a more reliable trait for studying phylogenesis.

Grill *et al.* (1986) recently reported the occurrence of a whole set of heavy metal-complexing peptides (phytochelatins) which were known from higher plants. It is remarkable that unlike *Sacch. cerevisiae* which follows the animal pattern of complexing heavy metals via metallothionein, *S. pombe* uses a pathway hitherto found only in plants. Their finding is especially interesting if one takes into consideration that the branching order of fungi and green plants is not known yet (see Whittaker, 1969; Hori *et al.*, 1985) and, on the other hand, urges the extension of the comparative analysis to plants.

C. Unique Fungal-Type Mitochondria

As for the *S. pombe* mitochondrial genome, it differs much in size from that of most ascomycetes (for reviews see Wolf, 1983; Lang *et al.*, 1983). Although it turned out that the different isolates cover a wide range of mitochondrial genome size [from 17.3 to 22.3 kilobase pairs (kbp)] (Zimmer *et al.*, 1984), the *S. pombe* mtDNA is still one of the smallest among the fungi, together with that of *Torulopsis glabrata* (CBS 138: 19.9 kbp)

(Clark-Walker and Sriprakash, 1981). In this it resembles mammalian mitochondria (16.5 kbp) (Anderson *et al.*, 1982), which raises the question of whether this similarity might have a value in mapping the phylogenesis of the fission yeasts. When considering this possibility, it must be borne in mind that the evolutionary relationship between the fungal and animal mitochondria is rather controversial. The trees published by Küntzel's group (e.g., Küntzel and Köchel, 1981) suggest a biphyletic origin, while others argue that they are of monophyletic origin (e.g., Gray *et al.*, 1984).

According to Lang *et al.* (1983), however, the mitochondrial genome of *S. pombe* is derived from a fungal-type ancestor, which is apparent from the relatively high AT content of structural genes, their separation by AT-rich sequences, and the occurrence and conservation of intron sequences. Unlike animal mitochondria, the *S. pombe* mitochondrial genes do not evolve faster than the nuclear DNA (see Lang *et al.*, 1985). Nevertheless, comparative analysis of mitochondrial genes (e.g., *cob* gene, ATPase subunit 6 gene, *cox1* gene) also placed *S. pombe* at a distance far from other fungi (Lang *et al.*, 1983, 1985).

In view of this, it is somewhat puzzling that the second intron of *cox1* is very similar in a number of features to its counterpart in *Aspergillus nidulans*. Two additional introns (A/2 and A/3), which were found in strain EF1, are located at positions identical to those of introns A/4 and A/5, respectively, in the *cox1* gene of *Sacch. cerevisiae*. A possibility to explain the occurrence of similar introns in different organisms is horizontal gene transfer, which may have occurred in the course of evolution, once between the *Aspergillus* lineage and *Schizosaccharomyces* (Lang, 1984), then again between *Saccharomyces* and *Schizosaccharomyces* (Trinkl and Wolf, 1986). Laboratory evidence for transfer of mitochondria between the two yeasts (isolated mitochondria into protoplasts) was reported by Yoshida and Takeuchi (1980). Thus, the data available on mitochondrial genomes reinforce the unique status of *Schizosaccharomyces* among fungi, but do not contribute to the disclosure of its evolutionary roots.

D. *Schizosaccharomyces* Does Belong to Ascomycota

Under the influence of certain striking differences recently revealed between *Sacch. cerevisiae* and *S. pombe* and supported by the phylogenetic trees discussed above, many pombeologists have begun to believe that *S. pombe* is much more closely related to Metazoa and thus represents a better model than the more frequented *Sacch. cerevisiae* for studying the eukaryotic cell. From their comparative analysis they have

inferred that *Schizosaccharomyces* separated quite late in the course of evolution, and with this conclusion they involuntarily raised the question of whether it is an ascomycete at all. It seems rather hard to reconcile its later emergence with the concept of a monophyletic origin of *Ascomycota* (see above). Either *S. pombe* is not an ascomycete or the considerable differences between the two yeasts are due to the heterogeny of the division. The data discussed in the previous sections suggest that *S. pombe* is equidistantly related to higher metazoans and *Sacch. cerevisiae* and are compatible with the phylogenies shown in Fig. 1A and 1B. Let us consider some additional data.

One main character distinguishing the ascomycetes from all other organisms is the ascus, a saclike sprorangium containing the ascospores formed by a free cell after meiosis. The sexual sporangium of the fission yeasts is unambiguously ascuslike, both in morphology and in development (Tanaka and Hirata, 1982; Sipiczki, 1983).

Like all higher fungi including the ascomycetous yeasts, the fission yeasts have a noncentric mitosis (without centrioles) with an intact nuclear envelope and spindle pole bodies intimately associated with the nuclear membranes. Phylogenetically, it evolved from centric mitosis, which is characteristic of most eukaryotes (Fuller, 1976). In the details of the cell cycle and mitosis, however, *S. pombe* differs considerably from *Sacch. cerevisiae* (see Nurse, 1985, for a review) and is more similar to higher eukaryotes. This is in spite of the fact that the basic patterns of growth and division seem simpler in the fission yeasts than in *Saccharomyces* and in most other eukaryotes (see Johnson *et al.*, 1982). The differences between the two yeasts may be due to the different modes of propagation (fission versus budding), and the lesser complexity in the fission yeast might be a phylogenetically older feature rather than a more advanced stage on the metazoan lineage. To resolve this question, more should be known about the organization of the cell division cycle in other fungi. The same holds true for centromere structure. In this case the *S. pombe* centromeres are more complex and, again, more similar to those of higher eukaryotes (Nakaseko *et al.*, 1986; Clarke *et al.*, 1986). However, owing to the lack of data, their relationship to other fungal centromeres is unknown.

The necessity of including more fungal species in the investigation of the phylogenesis of *Schizosaccharomyces* is also apparent from the findings which suggest that this genus may be more closely related to the filamentous ascomycetes *Neurospora* and *Aspergillus* than to *Sacch. cerevisiae*. Let us consider a few examples. In *Schizosaccharomyces*, like in *Neurospora*, one of two carbamoyl-phosphate synthases (CPSase A) that takes part in the arginine biosynthesis is localized in the mitochon-

dria. Since the other enzyme (CPSase P) used for pyrimidine synthesis occurs in the cytosol, the channeling of carbamoyl phosphate produced by them between the arginine and pyrimidine pathways is ensured (Vissers and Thuriaux, 1985). In *Sacch. cerevisiae*, where both enzymes are in the cytosol, the same goal is achieved by a regulatory system. The physiological and genetic organization of the purine catabolic pathway in *S. pombe* also appears to be similar to that found in filamentous ascomycetes and is unlike that found in *Sacch. cerevisiae* (Fluri and Kinghorn, 1985). Comparative analysis of the complex locus *trp1* in *S. pombe*, *Sacch. cerevisiae*, and *N. crassa* led Thuriaux *et al.* (1982) to suggest that these three species derived from a common ancestor that had a *trp1* organization similar to the one still found in *S. pombe*.

An advantage of *S. pombe* over *Sacch. cerevisiae* in molecular biology is its higher tolerance to the foreign genetic signals, since this renders it more amenable to cloning of genes of higher eukaryotes. These differences are probably due to the differences in their own signals and recognition mechanisms. Certain *Sacch. cerevisiae* genes such as *CDC9* and *CDC28* can complement defective alleles of their counterparts in *S. pombe*, but the *S. pombe* genes are not expressed in the budding yeast (Beach *et al.*, 1982; Johnston *et al.*, 1986). Further, splicing of the intron-containing *S. pombe* tRNA precursors in *Sacch. cerevisiae* extracts capable of transcription was found inefficient (Summer-Smith *et al.*, 1984, and references therein). *Saccharomyces cerevisiae* intron signal sequences such as the 5′ intron/exon junction and the heptanucleotide TACTAAC were found to be absolutely conserved (Guthrie *et al.*, 1986), which may account for the inefficient expression of foreign genes in this organism. Its stringency in splicing is unique, because other fungi are less conservative in this respect (Table V). These data indicate that the splicing process must be fundamentally similar in fungi, including *S. pombe*, and in Metazoa. It is the budding yeast *Sacch. cerevisiae* that is unique with its stringent splicing mechanism rather than the fission yeast, which is more similar both to other fungi and to metazoans. Further support for this was provided by comparisons of snRNAs (Hughes *et al.*, 1987, and references therein). In *Sacch. cerevisiae* they have diverged to the extent that none are present in the same amounts as the mammalian ones, while other fungi such as *Aspergillus*, *Neurospora*, and *S. pombe* seem to have retained a complement of snRNAs much more similar to the mammalian types both in number and in sequence homology (Hughes *et al.*, 1987; and references therein). In this context, it is remarkable that in *Sacch. cerevisiae* only a few genes contain introns, while the distribution of introns in *S. pombe* genes resembles that found in *Neurospora* or in plants and vertebrates (Fink, 1987).

TABLE V

Consensus Sequences in Eukaryotic Introns

Organism	5′ Splice site	"TACTAAC box"
Saccharomyces cerevisiae[a]	$\begin{matrix}A\\T\end{matrix}$ G G T A PyG T $\begin{matrix}A\\T\end{matrix}$	T A C T A A C
Schizosaccharomyces pombe[b,c]	G T A – G T T T T T[b]	C T Pu A Py[c]
Other fungi[a]	G T Pu A G T	$\begin{matrix}A\\T\end{matrix}$ Pu C T Pu A C
Metazoans[a]	$\begin{matrix}C\\A\end{matrix}$ A G T Pu A G T	C T Pu A Py

[a]Guthrie *et al.* (1986).
[b]In *cdc2:* Hindley and Phear (1984).
[c]Mertins and Gallwitz (1987).

Taken together, the data discussed do not provide enough evidence to reject the taxonomist's view that *Schizosaccharomyces* is an ascomycetous fungus. Despite the extreme divergence of certain RNA sequences from those of the other fungi and the striking similarities of numerous genes and proteins to their counterparts in higher eukaryotes, the fission yeasts still share a set of fundamental features which delimit the division Ascomycota (e.g., life cycle, ascus-type sporangium, mode of ascospore formation, noncentric mitosis). Their appearance via convergent evolution in a taxon which separated much later than the ancestors of Ascomycota had emerged would hardly be conceivable. One major difficulty in the mapping of *Schizosaccharomyces* phylogenesis is the paucity of molecular data in other fungi. Thus, the comparative analysis had to be restricted to a very limited number of species. Moreover, *Sacch. cerevisiae* seems to be rather unsuitable for phylogenetic comparison, because it appears peculiar among the fungi in a number of features. In many characteristics, the fission yeasts exhibit greater similarity to *Neurospora* and *Aspergillus* than to *Saccharomyces*.

On the basis of all these considerations, the phylogeny depicted in Fig. 1B seems to be the most plausible. The ancestors of fission yeasts presumably diverged from the lineage leading to the ascomycetous fungi at the time when most characteristic features of the division had already evolved. However, it must have occurred early enough for the conservative rRNA and other sequences to diverge to the extent revealed. On the other hand, owing to their early separation, the fission yeasts may have retained some common traits of the ancient fungi and metazoans which then disappeared during the formation of the modern budding yeasts but are still detectable in higher eukaryotes. Certain features for which the

filamentous species and *Schizosaccharomyces* were found more related to vertebrates than to *Sacch. cerevisiae* can be examples of this.

REFERENCES

Adachi, Y., Toda, T., Niwa, O., and Yanagida, M. (1986). Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **6,** 2168–2178.

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlick, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, J. H., Staden, R., and Young, I. G. (1982). Comparison of the human and bovine mitochondrial genomes. *In* "Mitochondrial Genes" (P. P. Slonimski, P. Borst, and G. Attardi, eds.), pp. 5–43. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Barnett, J. A., Payne, R. W., and Yarrow, D. (1983). "Yeasts. Characteristics and Identification." Cambridge Univ. Press, London and New York.

Beach, D., Durkacz, B., and Nurse, P. (1982). Functionally homologous cell cycle control genes in budding and fission yeasts. *Nature (London)* **300,** 706–709.

Bernstein, H., Byerly, H. C., Hopf, F. A., and Michod, R. E. (1985). The evolutionary role of recombinational repair and sex. *Int. Rev. Cytol.* **96,** 1–28.

Bridge, P. O., and May, J. W. (1984). A numerical classification of fission yeasts of the genus *Schizosaccharomyces* Lindner. *J. Gen. Microbiol.* **130,** 1921–1932.

Cedergren, R. J., LaRue, B., Sankoff, D., Lapalme, G., and Grosjean, H. (1980). Convergence and minimal mutation criteria for evaluating early events in tRNA evolution. *Proc. Natl. Acad. Sci. U.S.A.* **71,** 2791–2795.

Cedergren, R. J., LaRue, B., and Grosjean, H. (1981). The evolving tRNA molecule. *CRC Crit. Rev. Biochem.* **11,** 35–104.

Claisse, M. L., and Simon-Becam, A.-M. (1978). Cytochrome *c* from *Schizosaccharomyces pombe* I. Purification, spectral properties and amino acid composition. *Eur. J. Biochem.* **86,** 399–406.

Clarke, L., Amstutz, M., Fishel, B., and Carbon, J. (1986). Analysis of centromeric DNA in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8253–8257.

Clark-Walker, G. D., and Sriprakash, K. S. (1981). Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. *J. Mol. Biol.* **151,** 367–387.

Colles, D. H. (1970). The phenogram as an estimate of phylogeny. *Syst. Zool.* **19,** 352–362.

Erdman, V. A., Huysmans, E., Vanderberghe, A., and de Wachter, R. (1983). Collection of plasmid 5 S and 5.8 S ribosomal RNA sequences. *Nucleic Acids Res.* **11r,** 105–133.

Fink, G. R. (1987). Pseudogenes in yeast? *Cell* **49,** 5–6.

Fleet, G. H., and Phaff, H. J. (1975). Isolation and properties of an exo-β-glucanase from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. *versatilis*. *Biochim. Biophys. Acta* **401,** 318–332.

Fluri, R., and Kinghorn, J. R. (1985). The *all2* gene is required for the induction of the purine deamination pathway in *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **131,** 527–532.

Fuller, M. S. (1976). Mitosis in fungi. *Int. Rev. Cytol.* **45,** 113–153.

Gams, W., van der Aa, H. A., van der Plaats-Niterink, A. J., Samson, R. A., and Stalpers, J. A. (1987). "CBS Course on Mycology." Centraalbureau voor Schimmelcultures, Baarn and Delft.

Gray, M. W., Sankoff, D., and Cedergren, R. J. (1984). On the evolutionary descent of organisms and organelles: A global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Res.* **12,** 5837–5852.

Grill, E., Winnacker, E.-L., and Zenk, M. H. (1986). Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. *FEBS Lett.* **197,** 115–120.

Guthrie, L., Riedel, N., Parker, R., Swerdlow, H., and Patterson, B. (1986). Genetic analysis of snRNAs and RNA processing in yeast. *In* "Yeast Cell Biology" (J. Hicks, ed.), pp. 301–321. Liss, New York.

Hindley, J., and Phear, G. A. (1984). Sequence of the cell division gene *cdc2* from *Schizosaccharomyces pombe:* Patterns of splicing and homology to protein kinases. *Gene* **31,** 129–134.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes β-tubulin: A cold-sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39,** 349–358.

Hori, H., and Osawa, S. (1979). Evolutionary change in 5 S RNA secondary structure and a phylogenic tree of 54 5 S RNA species. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 381–385.

Hori, H., Itoh, T., and Osawa, S. (1982). The phylogenic structure of the metabacteria. *Zentralbl. Bakteriol. Hyg., I. Abt. Orig.* **C3,** 18–30.

Hori, H., Lim, B.-K., and Osawa, S. (1985). Evolution of green plants as deduced from 5 S rRNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 820–823.

Hottinger, H., Pearson, D., Yamao, F., Gamulin, U., Coolen, L., Cooper, T., and Söll, D. (1982). Nonsense suppression in *Schizosaccharomyces pombe:* The *S. pombe sup-e* $tRNA^{Ser}_{UGA}$ gene is active *S. cerevisiae. Mol. Gen. Genet.* **188,** 219–224.

Hottinger, H., Stadelmann, B., Pearson, D., Frendewey, D., Kohli, J., and Söll, D. (1984). The *Schizosaccharomyces pombe sup3-i* suppressor recognizes ochre, but not amber codons *in vitro* and *in vivo. EMBO J.* **3,** 423–428.

Hughes, J. M. X., Koninngs, D. A. M., and Cesareni, G. (1987). The yeast homologue of U3 snRNA. *EMBO J.* **6,** 2145–2155.

Huysmans, E., Dams, E., Vanderberghe, A., and de Wachter, R. (1983). The nucleotide sequence of the 5 S rRNAs of four mushrooms and their use in studying the phylogenetic position of basidiomycetes among the eukaryotes. *Nucleic Acids Res.* **11,** 2871–2880.

Johnson, B. F., Calleja, G. B., Yoo, B. Y., Zuker, M., and McDonald, I. J. (1982). Cell division: Key to cellular morphogenesis in the fission yeast, *Schizosaccharomyces. Int. Rev. Cytol.* **75,** 167–208.

Johnston, L. H., Barker, D. G., and Nurse, P. (1986). Cloning and characterization of the *Schizosaccharomyces pombe* DNA ligase gene *CDC17. Gene* **41,** 321–325.

Kimura, H., and Ohta, T. (1973). Eukaryotes–prokaryotes divergence estimated by 5 S ribosomal RNA sequences. *Nature (London) New Biol.* **243,** 199–200.

Kock, J. L. F., and van der Walt, J. P. (1986). Fatty acid composition of *Schizosaccharomyces* Lindner. *Syst. Appl. Microbiol.* **8,** 163–165.

Kocková-Kratochvilová, A. (1982). "Yeasts and Yeast-like Microorganisms." ALFA, Bratislava–SNTL, Praha. (In Slovak.)

Kocková-Kratochvilová, A., Sláviková, E., Zemek, J., Kadlečíková, B., and Kuniak, L. (1985). Hydrolytic activity in the genus *Schizosaccharomyces* Lindner. *Folia Microbiol. (Praha)* **30,** 443–451.

Kohli, J., Mao, J., and Söll, D. (1980). Transfer RNA and nonsense suppression in *Schizosaccharomyces pombe*. *In* "Genetics and Evolution of RNA Polymerase, tRNA and Ribosomes" (S. Osawa, H. Ozeki, H. Uchida, and T. Yura, eds.), pp. 233–250. Univ. of Tokyo Press, Tokyo.

Komiya, H., Miyazaki, M., and Takamura, S. (1981). The nucleotide sequence of 5 S ribosomal RNA from *Schizosaccharomyces pombe*. *J. Biochem.* **89,** 1663–1666.

Kreger-van Rij, N. J. W., ed. (1984a). "The Yeasts—A Taxonomic Study." Elsevier, Amsterdam.

Kreger-van Rij, N. J. W. (1984b). System of classification of the yeasts. *In* "The Yeasts—A Taxonomic Study" (N. J. W. Kreger-van Rij, ed.), pp. 2–15. Elsevier, Amsterdam.

Kudriawzew, W. I. (1960). "Die Systematik der Hefen." Akademie-Verlag, Berlin.

Küntzel, H., and Köchel, H. G. (1981). Evolution of rRNA and origin of mitochondria. *Nature* (*London*) **293,** 751–755.

Küntzel, H., Heidrich, M., and Piechulla, B. (1981). Phylogenetic tree derived from bacterial, cytosol and organelle 5 S rRNA sequences. *Nucleic Acids Res.* **9,** 1451–1461.

Küntzel, H., Piechulla, B., and Hahn, U. (1983). Consensus structure and evolution of 5 S rRNA. *Nucleic Acids Res.* **11,** 893–900.

Kwok, S., White, T. J., and Taylor, J. W. (1986). Evolutionary relationships between fungi, red algae, and other simple eukaryotes inferred from total DNA hybridizations to a cloned basidiomycete ribosomal DNA. *Exp. Mycol.* **10,** 196–204.

Lang, B. F. (1984). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe:* Highly homologous introns are inserted at the same position of the otherwise less conserved *cox1* genes in *Schizosaccharomyces pombe* and *Aspergillus nidulans*. *EMBO J.* **3,** 2129–2136.

Lang, B. F., Ahne, F., Distler, S., Trinkl, H., Kaudewitz, F. M., and Wolf, K. (1983). Sequence of the mitochondrial DNA, arrangement of genes and processing of their transcription in *Schizosaccharomyces pombe*. *In* "Mitochondria 1983" (R. J. Schweyen, K. Wolf, and F. Kaudewitz, eds.), pp. 313–329. De Gruyter, Berlin.

Lang, B. F., Ahne, F., and Bonen, L. (1985). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. The cytochrome *b* gene has an intron closely related to the first two introns in the *Saccharomyces cerevisiae cox1* gene. *J. Mol. Biol.* **184,** 353–366.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* (*London*) **327,** 31–35.

Leupold, U. (1950). Die Vererbung von Homothallie und Heterothallie bei *Schizosaccharomyces pombe*. *C. R. Lab. Carlsberg* **24,** 381–480.

Matsumoto, S., and Yanagida, M. (1985). Histone gene organization of fission yeast: A common upstream sequence. *EMBO J.* **4,** 3531–3538.

Mertins, P., and Gallwitz, D. (1987). Nuclear pre-mRNA splicing in the fission yeast *Schizosaccharomyces pombe* strictly requires an intron-contained, conserved sequence element. *EMBO J.* **6,** 1757–1763.

Nakaseko, Y., Adachi, Y., Funakashi, S., Niwa, O., and Yanagida, M. (1986). Chromosome walking shows a highly homologous repetitive sequence present in all the centromere regions of fission yeast. *EMBO J.* **5,** 1011–1021.

Nurse, P. (1985). Cell cycle control genes in yeast. *Trends Genet.* **1,** 51–55.

Osterwalder, A. (1924). *Schizosaccharomyces liquefaciens* n. sp., eine gegen freie schweflige Säure widerstandsfähige Gärhefe. *Mitt. Gebiete Lebensmittelunters. Hyg.* **15,** 5–28.

Otaka, E., Higo, K., and Itoh, T. (1983). Yeast ribosomal proteins: VII. Cytoplasmic ribosomal proteins from *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **191,** 519–524.

Otaka, E., Ooi, T., Itoh, T., and Kumazaki, T. (1986). Examination of protein sequence

homologies: II. Ribosomal protein YS25 from *Saccharomyces cerevisiae* and its counterparts from *Schizosaccharomyces pombe*, rat liver, and *Escherichia coli*. *J. Mol. Evol.* **23,** 337–343.

Rankine, B. C., and Fornachon, J. C. M. (1964). *Schizosaccharomyces malidevorans* sp. n., a yeast decomposing L-malic acid. *Antonie van Leeuwenhoek* **30,** 73–75.

Russell, P. R., and Hall, B. D. (1983). The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* **258,** 143–149.

Schaak, J., Mao, J., and Söll, D. (1982). The 5.8 S RNA gene sequence and the ribosomal repeat of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **10,** 2851–2864.

Sipiczki, M. (1979). Interspecific protoplast fusion in fission yeasts. *Curr. Microbiol.* **3,** 37–40.

Sipiczki, M. (1983). Diploid protoplasts in *Schizosaccharomyces pombe:* Formation, growth, and sporulation. *Can. J. Microbiol.* **29,** 593–595.

Sipiczki, M., Kucsera, J., Ulaszewski, S., and Zsolt, J. (1982). Hybridization studies by crossing and protoplast fusion within the genus *Schizosaccharomyces* Lindner. *J. Gen. Microbiol.* **128,** 1989–2000.

Stadelmann, B. (1985). Charakterisierung der Serin-tRNAs und der Serin-tRNA Gene in *Schizosaccharomyces pombe*. Inauguraldissertation, Universität Bern.

Stelling-Dekker, N. M. (1931). "Die Hefesammlung des Centraalbureau voor Schimmelcultures. Beiträge zu einer Monographie der Hefearten. Erster Teil. Die Sporogenen Hefen." Verhandel. Akad. Wetenschappen Amsterdam, Afdeel. Natuurkunde. 2d Sect.

Summer-Smith, M., Hottinger, H., Willis, I., Koch, T. L., Arentzen, R., and Söll, D. (1984). The *sup8* $tRNA^{Leu}$ gene of *Schizosaccharomyces pombe* has an unusual intervening sequence and reduced pairing in the anticodon stem. *Mol. Gen. Genet.* **197,** 447–452.

Tabata, S. (1981). Nucleotide sequences of the 5 S ribosomal RNA genes and their adjacent regions in *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **9,** 6429–6437.

Tanaka, K., and Hirata, A. (1982). Ascospore development in the fission yeasts *Schizosaccharomyces pombe* and *S. japonicus*. *J. Cell. Sci.* **56,** 263–279.

Thuriaux, P., Heyer, W.-D., and Strauss, A. (1982). Organization of the complex locus *trp1* in the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **6,** 13–18.

Toda, T., Adachi, Y., Hiraoka, Y., and Yanagida, M. (1984). Identification of the pleiotropic cell division cycle gene *NDA2* as one of two different α-tubulin genes in *Schizosaccharomyces pombe*. *Cell* **237,** 233–242.

Trinkl, H., and Wolf, K. (1986). The mosaic *cox1* gene in the mitochondrial genome of *Schizosaccharomyces pombe:* Minimal structural requirements and evolution of group I introns. *Gene* **45,** 289–297.

Vissers, S., and Thuriaux, P. (1985). Genetical evidence of carbamoyl phosphate compartmentation in *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus*. *Curr. Genet.* **9,** 561–565.

Von Arx, J. A. (1980). A mycologist's view of yeasts. *In* "Biology and Activities of Yeasts" (F. A. Skinner, S. M. Passmore, and D. R. Davenport, eds.), pp. 53–61. Academic Press, London.

Whittaker, R. H. (1969). New concepts of kingdoms of organisms. *Science* **163,** 150–160.

Wickerham, L. J. (1951). Taxonomy of yeasts. 1. Techniques of classification 2. A classification of the genus *Hansenula*. U.S. Department of Agriculture Technical Bulletin No. 1029.

Wolf, K. (1983). Mitochondrial genome divergence in the petite negative yeast *Schizosaccharomyces pombe*. *In* "Endocytobiology," Vol. II, pp. 229–240. De Gruyter, Berlin.

Wolters, J., and Erdmann, V. A. (1986). Cladistic analysis of 5 S rRNA and 16 S rRNA

secondary and primary structure. The evolution of eukaryotes and their relation to Archaebacteria. *J. Mol. Evol.* **24,** 152–166.

Yamada, Y., Arimoto, M., and Kondo, K. (1973). Taxonomic significance of the coenzyme Q system in the classification of the ascosporogenous yeast genus *Schizosaccharomyces* and the yeast-like genus *Endomyces*. *J. Gen. Appl. Microbiol.* **19,** 353–358.

Yarrow, D. (1984). Genus 25. *Schizosaccharomyces* Lindner. *In* "The Yeasts—A Taxonomic Study" (N. J. W. Kreger-van Rij, ed.), pp. 414–422. Elsevier, Amsterdam.

Yoshida, K., and Takeuchi, I. K. (1980). Cytological studies on mitochondria-induced cytoplasmic transformation in yeasts. *Plant Cell Physiol.* **21,** 497–509.

Zimmer, M., Lückemann, G., Lang, B. F., and Wolf, K. (1984). The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 3. Gene mapping in strain EF1 (CBS 356) and analysis of hybrids between the strain EF1 and *ade7-50* h^-. *Mol. Gen. Genet.* **196,** 473–481.

Index

R