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GENETIC ANALYSIS OF THE YEAST CYTOSKELETON

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INTRODUCTION

The fibrous elements of eukaryotic cells, including microtubules, microfilaments, and intermediate filaments, compose the cytoskeleton. These elements are thought to be involved in an array of cellular processes such as mitosis, cell motility, intracellular transport of organelles, the organization of the

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cytoplasm, and the maintenance of cellular morphology. To understand the molecular mechanisms that underlie cytoskeletal function, we must define the roles of the individual cytoskeletal elements, identify their protein components, and determine the factors that regulate the assembly of these components into the appropriate structures at the correct time and position in the cell.

The cytoskeleton has been genetically analyzed, to different degrees, in various mammalian cells (20) and organisms including *Drosophila melanogaster* (28a, 41, 64, 65), *Caenorhabditis elegans* (7, 26, 45), *Chlamydomonas reinhardtii* (4, 47), *Aspergillus nidulans* (53), *Physarum polycephalum* (67, 72), and two yeasts, *Saccharomyces cerevisiae* (86) and *Schizosaccharomyces pombe* (94). The yeasts are particularly tractable organisms for such studies. Their sophisticated genetic systems and the small number of genes specifying each cytoskeletal protein make mutants relatively easy to find or construct. In this review we concentrate primarily on the budding yeast, *Saccharomyces*. This focus allows us to illustrate genetic techniques and approaches using examples from our own laboratory. Many of the techniques for identifying and analyzing mutations affecting the yeast cytoskeleton can be applied to other systems, and indeed some of the ideas derive from successful applications in *Drosophila* and *Aspergillus*.

The surest and the most straightforward way to connect the function of proteins in vivo to their activities in vitro is through mutations (12). There are several ways to identify mutations that directly and specifically affect cytoskeletal function. One way (previously called *plan A;* 86) is to proceed from assumptions of the expected phenotype (e.g. tubulin mutants should be lethals blocked at mitosis) and simply screen for mutations with those properties. The proteins can be identified by cloning each gene (in yeast by complementing the defect in vivo) and then raising antibodies by using the gene to make fusion proteins or synthetic peptides. The alternate procedure *(plan B)* begins with proteins identified biochemically as part of the cytoskeleton. The gene can be obtained by standard cloning methods, and mutations can be produced in the gene in vitro. In yeast, these mutant genes can then be substituted for the normal gene in the living cell.

Both strategies achieve the same end: the association of gene, protein, and function. We believe that the combination of both plans is more effective than either alone. This is demonstrated by the failure to find β-tubulin mutants among mitosis-defective cell-cycle mutants even though the β-tubulin mutant constructed using the cloned gene had precisely this phenotype.

With the combination of genetic and biochemical analysis it should now be possible to identify all the essential components of the cytoskeleton. In this review, we first summarize the central features of the cell cycle and cytoskeleton of *Saccharomyces*. Second, we review the ways in which plan B

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(starting with proteins, proceeding to the gene, and then to the mutants) has allowed the definition of mutant phenotypes that characterize failure of cytoskeleton formation. Third, we summarize the various genetic technologies that have arisen from attempts to obtain mutants that specifically affect cytoskeletal functions (plan A). In the final section we describe briefly the phenotypes of the cytoskeletal mutants thus far identified in yeast.

THE CYTOSKELETON AND CELL CYCLE OF *SACCHAROMYCES CEREVISIAE*

Mitotic Cell Cycle

Saccharomyces divides by budding (reviewed in 61). The bud emerges early in the cell cycle and grows steadily as the cell cycle progresses, remaining attached to the mother cell by a short neck region. Near the end of the DNA synthetic period, the nucleus migrates toward the neck. After DNA synthesis is completed the nucleus elongates symmetrically into the mother and daughter cell bodies and then divides. At this point, the bud is nearly the size of the mother cell. Cytokinesis and cell separation complete the cell cycle (see Figure 1 and Figure 2).

As in other eukaryotic cells, yeast microtubules are found in both the mitotic spindle and the cytoplasm (2, 16, 44; reviewed in 13). In yeast all microtubules appear to have one end associated with the spindle pole body, which is embedded in the nuclear envelope. An unbudded cell contains a single spindle pole body, and microtubules extend from it both into the nucleus and outward to the cytoplasm. Near the time of bud emergence, the spindle pole body duplicates, and the two spindle pole bodies migrate to opposite sides of the nuclear envelope. A short spindle forms between the separated spindle pole bodies, and one of the bundles of cytoplasmic microtubules extends into the growing bud. Shortly after nuclear migration, the spindle poles move apart until they lie at the extremities of the nucleus. Spindle elongation appears to separate the two sets of chromosomes, which remain closely associated with the spindle pole bodies. The spindle then becomes interrupted at its midpoint as the daughter nuclei pinch apart. The spindle microtubules have been presumed to be involved in chromosome segregation and nuclear division. The role of the cytoplasmic microtubules has been less clear, but it has been suggested that they play a role in daughter cell formation by mediating the delivery of secretory vesicles or organelles to the bud (13, 15, 16) or ensuring the proper orientation of nuclear division (2).

Through much of the mitotic cell cycle, actin is unevenly distributed (2, 44, 56). The mother cell contains cables of actin directed toward the bud neck, and the bud contains brightly staining dots just below the cell surface. This asymmetry persists until shortly before cytokinesis, when randomly directed

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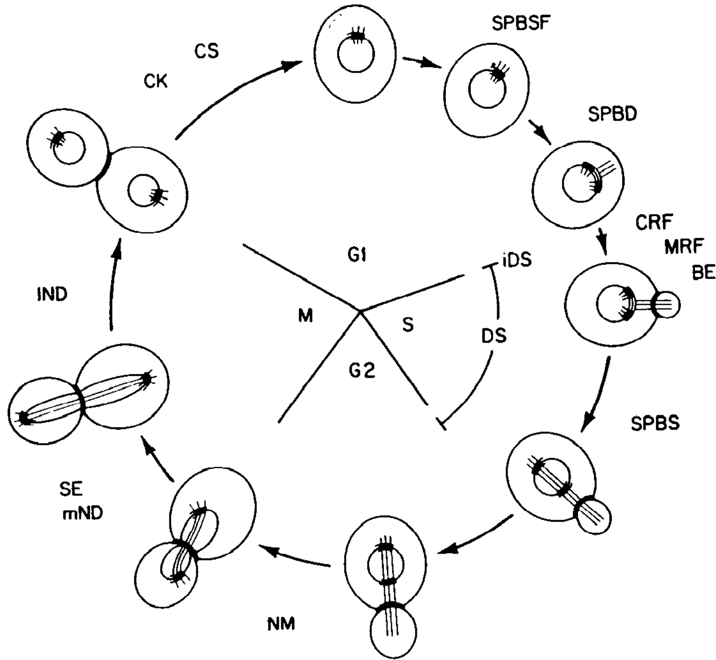

Figure 1 The *S. cerevisiae* cell cycle. Abbreviations: SPBSF, spindle-pole-body satellite formation; SPBD, spindle-pole-body duplication; CRF, formation of the chitin ring (shown in the diagram as a heavy line at the mother-bud junction); MRF, formation of the microfilament ring (not shown in the diagram, but found adjacent to the cell membrane in the region of the mother-bud junction); BE, bud emergence; iDS, initiation of chormosomal DNA synthesis; DS, chromosomal DNA synthesis; SPBS, spindle-pole-body separation (and formation of a complete spindle); NM, nuclear migration; mND, medial stage of nuclear division; SE, spindle elongation; lND, late stage of nuclear division; CK, cytokinesis; CS, cell separation. Figure and legend taken from Ref. 61.

cables and scattered dots are seen in both the mother and bud cells. Adams & Pringle (2) established a correlation between the growing regions of the yeast cell surface and the regions showing a concentration of actin patches. Based on this correlation they proposed a role for actin in the localization of surface growth. At the time of bud emergence, in addition to the dots and cables, a ring of actin dots forms on the mother cell's side of the neck. This ring of actin remains around the neck throughout the early portion of the cell cycle, but disappears later (see Figure 2).

Other features also mark the neck separating the mother cell and the bud. From the earliest stages of bud formation the neck is completely lined on the cytoplasmic side by a ring of 10-nm-diameter filaments (18). This filamentous ring is evident until immediately before cytokinesis. In addition, a collar of chitin is formed in the cell wall surrounding the neck (19).

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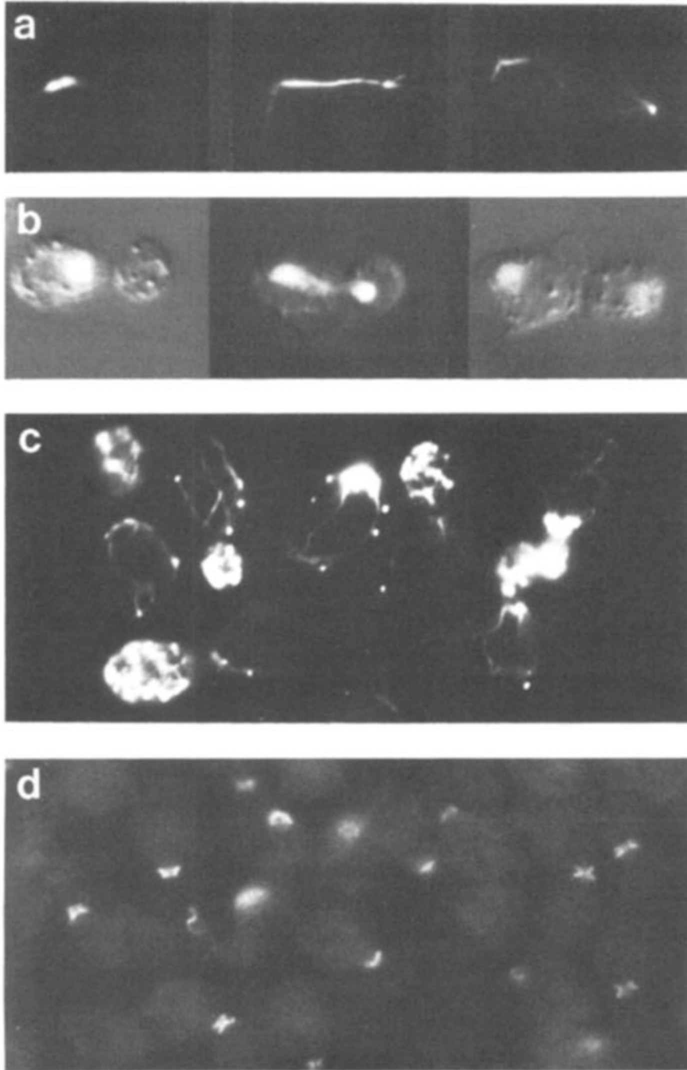

Figure 2 Immunofluorescence of yeast cytoskeletal structures: (*a*) microtubules of cells at successive stages of mitotis stained with tubulin-specific antibody; (*b*) DNA staining and Nomarski microscopy of the same cells shown in (*a*); (*c*) actin filaments of cells stained with actin-specific antibody; (*d*) 10-nm filament location revealed by CDC12-specific antibody.

Following cell division, the chitin collar remains with the mother cell as a ring called the *bud scar*. Noting that this collar is apparently coincident with the ring of actin patches, Kilmartin & Adams (44) proposed a role for actin in localizing the synthesis of chitin to the neck (see Figure 2).

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Conjugation

Saccharomyces can grow mitotically as haploids, diploids, or polyploids. Diploid cells are formed from two haploids of opposite mating type by conjugation (16; reviewed in 13). Under the influence of mating pheromones, haploid cells depart from the mitotic cell cycle in G_1 phase with a single spindle pole body. Conjugation begins with cytoplasmic fusion to produce a zygote containing two haploid nuclei. Karyogamy, the fusion of the two nuclei, generally begins soon after cytoplasmic fusion. The two nuclei, whose spindle pole bodies are oriented toward the site of cell fusion, migrate toward each other and fuse to form a zygote with a single diploid nucleus. These processes appear to be mediated by cytoplasmic microtubules that connect the nuclei at their spindle pole bodies. Once the nuclei have fused, the zygote gives rise to diploid daughter cells by mitotic budding.

Structural Genes Specifying Filamentous Cytoskeletal Components

Microtubules are composed of tubulin subunits; each subunit is a heterodimer of α-tubulin and β-tubulin (48). *Saccharomyces* contains a single essential gene encoding β-tubulin, *TUB2* (54), and two genes encoding α-tubulins, *TUB1* and *TUB3* (70). Actin is encoded by a single essential gene, *ACT1* (29, 30, 55, 76; see also Table 1). The protein composition of the 10-nm filaments has not been determined. However, recent evidence suggests *CDC3, CDC10, CDC11,* and *CDC12* may encode structural proteins of these filaments (see below).

GENERATING MUTANTS DEFECTIVE IN THE FUNCTIONS OF KNOWN PROTEINS (PLAN B)

Isolation of Genes For Known Proteins by Homology

The amino acid sequences of the structural proteins of the cytoskeleton seem remarkably conserved among eukaryotic species. For example, about 90% of the residues are identical in actin from *Saccharomyces* and mammals (30, 55), and more than 70% of the residues are identical in tubulins from *Saccharomyces* and chicken (54, 70). This extraordinary degree of conservation could result from a functional requirement for specific protein–protein interactions. Such a requirement would place constraints on the variation possible in the sequence of structural proteins that may not exist for enzymes whose function depends on the conservation of an active site.

Such sequence conservation suggests that cytoskeletal proteins from all eukaryotes have also retained common biochemical properties. Several lines of evidence support this notion. *Saccharomyces* tubulin coassembles with animal-cell tubulin to form microtubules in vitro (43). A chimeric β-tubulin

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Table 1 Genes and their functions

Gene	Function
ACT1	encodes actin
CDC3	essential for 10-nm filament formation
CDC4	essential for karyogamy
CDC10	essential for 10-nm filament formation
CDC11	essential for 10-nm filament formation
CDC12	essential for 10-nm filament formation
CDC13	essential for medial stage of nuclear division
CDC14	essential for late stage of nuclear division
CDC15	essential for late stage of nuclear division
CDC16	essential for medial stage of nuclear division
CDC17	essential for medial stage of nuclear division
CDC20	essential for medial stage of nuclear division
CDC23	essential for medial stage of nuclear division
CDC28	essential for karyogamy
CDC31	essential for spindle pole body duplication
CDC34	essential for karyogamy
CDC37	essential for karyogamy
CIN1	essential for fidelity of mitotic chromosome transmission
ESP1	essential for control of spindle pole body number
KAR1	essential for karyogamy
KAR2	essential for karyogamy
KAR3	essential for karyogamy
NDC1	essential for attachment of chromosomes to poles
SAC1	identified as a suppressor of *act1-3*
SAC2	identified as a suppressor of *act1-3*
SAC3	identified as a suppressor of *act1-3*
SPA1	probably encodes a spindle pole body component
TUB1	encodes the major α-tubulin
TUB2	encodes β-tubulin
TUB3	encodes the minor α-tubulin

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that contains chicken and *Saccharomyces* sequences is incorporated efficiently into all of the microtubule structures of mouse fibroblasts in vivo (9). Actin from yeast and rabbit muscle copolymerizes in vitro (31). Yeast actin filaments exhibit ATP-dependent movement on rabbit skeletal-muscle myosin filaments (S. J. Kron, D. Drubin, J. A. Spudich & D. Botstein, unpublished observations), a property common to actin filaments from several diverse species (46). The similarity of these proteins suggests that cytoskeletal structures assembled by yeast and other eukaryotes are much alike and probably perform analogous basic functions in vivo. Conservation of amino acid sequence (sometimes even nucleotide sequence) provides information about cytoskeletal proteins and genes from one species, which can then be applied, through recombinant DNA, immunological, and biochemical methods to

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isolate the proteins and genes of any other species. For *Saccharomyces*, the isolation of each new cytoskeletal protein in virtually any species presents an opportunity to find the analogous protein (if there is one) in yeast.

Indeed, the actin and β-tubulin genes of *Saccharomyces* were both cloned by DNA hybridization to probes consisting of the corresponding genes from higher eukaryotes (29, 54, 55). The α-tubulin genes of *Saccharomyces* were cloned by DNA homology with the α-tubulin genes of *Schizosaccharomyces* (70). The usefulness of heterologous clones as DNA probes in the general case depends, of course, on the degree of conservation of the amino acid sequences throughout the proteins and the similarity of codon use between different species. The problems caused by such differences may be mitigated by identifying the most highly conserved regions of a protein and using as probes oligonucleotide mixtures that encode those sequences and represent all of the possible combinations of codon usage.

A method that may be more generally applicable takes advantage of antibody cross-reactivity between homologous proteins from different species. Antibodies to actin and tubulin cross-react with these proteins from several different eukaryotes. Many proteins that associate with microtubules, microfilaments, and intermediate filaments in diverse cell types have been used to produce antibodies. Initial results suggest that some of these antibodies can be used to identify similar proteins in yeast. An antibody raised against *Dictyostelium* myosin identifies a 195-kd protein in yeast that binds to a yeast filamentous actin column and is eluted with ATP (D. Drubin, D. Botstein, unpublished observations). Monoclonal antibodies against nematode myosin also react with a high-molecular-weight myosin like yeast protein (91). Human autoantibodies isolated from scleroderma patients react with the mammalian spindle pole and were used to identify a 59-kd yeast polypeptide that may be a spindle pole body protein (79; see "Nontubulin Mutants That Affect Spindle Function").

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Isolation of Genes via Yeast Cytoskeletal Proteins

Many techniques are available for molecular cloning of genes encoding particular proteins. Therefore, genetic analysis of a known protein or expected class of proteins is often most readily approached by using biochemical techniques to detect and purify the protein directly. The protein can then be used to generate antibodies that allow the screening of recombinant DNA expression libraries. If the protein cannot be obtained in pure form, monoclonal antibodies can be raised against a protein mixture and then screened for their ability to identify the specified protein.

Oligonucleotide probes are the alternative to antibody probes. This approach requires that the amino acid sequence of the protein be partially determined. Synthetic oligonucleotide probes (usually mixtures to avoid

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ambiguities in codon usage in the predicted nucleotide sequence) are then used to screen libraries by hybridization.

Various biochemical procedures have been used to isolate proteins that associate with microtubules and actin filaments in many cell types. A description of these experiments and discussion of their relative merits are outside the scope of this review. However, we briefly discuss some procedures successfully applied to the identification of cytoskeletal proteins in yeast.

Pillus & Solomon (59) have applied a technique to yeast that has been used to define microtubule-associated proteins in vertebrate cells. They used detergent extraction of [^{35}S]methionine-labeled cells to produce a cytoskeletal preparation containing the microtubules assembled in vivo. Parallel extraction of cells pretreated with the microtubule-destabilizing drug nocodazole produced a cytoskeleton devoid of microtubules and tubulin. Following cycles of microtubule depolymerization and polymerization, the electrophoretic patterns of these two fractions were compared. Several proteins are present in the control fraction but absent in the drug-treated fraction, which suggests that they are yeast microtubule-associated proteins.

D. Drubin & D. Botstein (unpublished observations) have isolated yeast actin-associated proteins by virtue of their binding to a yeast filamentous-actin column. A 195-kd protein is eluted from the column with ATP. This protein cross-reacts with an antibody raised against *Dictyostelium* myosin. Subsequent salt washes elute two additional proteins of 67 kd and 85 kd. Immunofluorescence, using antibody raised to these latter two proteins, has demonstrated that each colocalizes with dots of actin in yeast cells.

Watts et al (91) have also isolated a 200-kd myosinlike protein from yeast that binds to a novobiocin–Sepharose column. This protein cross-reacts with nematode muscle myosin antibody, exhibits Ca^{2+}-dependent ATPase activity, and is present in actomyosin complexes isolated from yeast.

H. Liu & A. Bretscher (personal communication) have purified a 33-kd protein that they have tentatively identified as a yeast tropomyosin. It displays the same isoelectric point, sedimentation coefficient, and heat stability as bovine brain tropomyosin, and binds to filamentous actin in a Mg^{2+}-dependent manner. In addition, antibody raised against this yeast protein cross-reacts with bovine brain tropomyosin.

Constructing Null Mutations

Disruption of the functional gene in the chromosome is indispensable for determining whether a gene's function is essential for growth. Two strategies have been used for gene disruption in yeast (69, 76), and their results show that many yeast genes are essential for growth, including *ACT1* (76), *TUB1* (71), and *TUB2* (54). Each of these techniques destroys the integrity of one allele of the gene in a diploid and tests whether haploid segregants carrying

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the interrupted and/or deleted gene can grow. When the null-disruption haploid strain is viable, it is the best object for further study of phenotype; however, when the disruption haploid is not viable, conditional-lethal mutations are usually required to study the consequences of loss of the gene function.

Two properties of a gene are generally inferred from the finding that its null phenotype is lethality: (*a*) the gene encodes a protein whose function is required for cell growth and (*b*) the gene is the only one that encodes this protein. The actin gene and the β-tubulin gene in *Saccharomyces* have these attributes. However, not all essential genes are quite so simple. *Saccharomyces* contains two genes that encode α-tubulin, *TUB1* and *TUB3*, and both genes are expressed (70). Under standard growth conditions, *TUB1* is essential and *TUB3* is not. This discrepancy is apparently not due to any functional difference between the two gene products, but reflects the fact that *TUB1* is expressed at a higher level than *TUB3*. The *TUB3* gene on a high-copy-number plasmid allows the growth of a strain that contains a disrupted *TUB1* gene (71). Interestingly, the two α-tubulin genes of *Schizosaccharomyces* behave in an analogous manner (1).

Analysis of null phenotypes is also complicated by the observation that some gene disruptions produce conditional-lethal phenotypes. *SAC1* (*s*uppressor of *ac*tin) and *CIN1* (*c*hromosome *in*stability) are essential only at cold temperatures (14°C) (P. Novick, M. A. Hoyt, D. Botstein, unpublished observations), and *SPA1* (*s*pindle *p*ole *a*ntigen) is required only at high temperature (38°C) (79). The proteins encoded by these genes are expressed at all temperatures, but are apparently not essential except at extreme temperatures. One possible explanation of this observation is that these proteins stabilize cytoskeletal structures either by association with them or by modification of their components. This stabilization may be advantageous at all temperatures but is essential only at high or low temperatures.

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Constructing Conditional-Lethal Mutations

In theory, it should be possible to obtain conditional-lethal alleles of any essential gene that has been cloned. The gene of interest is usually subjected to in vitro mutagenesis and introduced into yeast such that the mutant allele replaces the wild-type allele (12). These transformants are then screened for conditional-lethal phenotypes. This general approach has now been used to identify conditional-lethal alleles of several of the yeast cytoskeletal genes, including *ACT1* (77), *TUB1* (P. J. Schatz & D. Botstein, unpublished observations), *TUB2* (T. C. Huffaker & D. Botstein unpublished observations), and *KAR1* (68). The specific phenotypes of these alleles are discussed below.

Conditional-lethal alleles are extremely valuable for genetic studies. Cells

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can be grown at a permissive temperature at which a protein functions normally and then shifted to the nonpermissive temperature to observe the effects of a nonfunctional or partially functional protein on various cellular processes. A similar type of experiment can be performed without mutant alleles by using an inducible promoter. Typically, the promoter of the gene is replaced by the yeast *GAL1–GAL10* promoter (40). A shift from galactose to glucose medium inhibits expression of the gene and produces, eventually, a mutant phenotype. This approach was used to determine the phenotype of cells lacking histone H2B (32) and of the *ras* homologues of yeast encoded by the *RAS1*, *RAS2*, and *YPT1* genes (42, 74).

There is, however, a fundamental difference between a block in synthesis and mutational inactivation of a protein. Following a block in synthesis, a mutant phenotype can be observed only when protein already present in the cells is eliminated either by degradation or by dilution with cell growth. This removal may require several generations if the protein is relatively stable. In addition, different conditional-lethal alleles affecting a single protein may produce different phenotypes. These partially functional alleles can provide detailed information that simply blocking new synthesis does not (e.g. see "Tubulin Mutants").

The intact wild-type *ACT1* (D. Shortle & D. Botstein, unpublished observations), *TUB2* (83), and *KAR1* (68) genes can not be cloned on high-copy-number plasmids, indicating that their overexpression is lethal to yeast cells. In this sense, the assembly of cytoskeletal structures in the cell may be analogous to the well-studied morphogenesis of the bacteriophages T4 and P22 (28, 38, 78, 82), whose structural proteins must be produced in appropriate stoichiometry for optimal phage production. When the proper stoichiometry of a protein is essential to the cell, overproduction of this protein can be used to generate a mutant phenotype.

The genes encoding yeast proteins that the cell requires in proper stoichiometry can be cloned on high-copy-number plasmids if their expression is controlled by the *GAL1–GAL10* promoter and the cells are grown in glucose medium. Shifting these cells to galactose medium causes overexpression of the genes, and the cells stop growing. Interestingly, when this experiment is performed with the *TUB2* (D. Burke & L. Hartwell, personal communication) and *KAR1* (68) genes, the arrest phenotypes caused by overexpression are similar to the arrest phenotypes caused by shifting conditional-lethal alleles to the nonpermissive temperature. These results suggest that in certain cases, overexpression, as well as underexpression, can be used to determine the role of proteins in the cell cycle.

Extending this idea, Meeks-Wagner et al (50) screened yeast genomic libraries made in high-copy-number vectors for plasmids that caused a high frequency of chromosome loss to identify new genes that function in mitotic

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chromosome transmission. Two genes identified by this protocol presumably encode proteins that interfere with chromosome transmission when overexpressed.

IDENTIFICATION OF GENES BY MUTANT PHENOTYPE (PLAN A)

Identification of Mutations That Affect Cytoskeletal Function

Screening or selecting for a mutant phenotype has led to the identification of many yeast genes. This approach requires that the phenotype of the desired mutant be anticipated. In addition, mutations that alter a protein encoded by duplicate genes may be missed because they produce recessive phenotypes. Several phenotypes have been used successfully to identify gene products associated with the yeast cytoskeleton.

CELL DIVISION CYCLE ARREST The classical conditional-lethal *cdc* (*c*ell *d*ivision *c*ycle) mutants arrest at a specific point the cell cycle under nonpermissive conditions (see 61 for a review). Some of these should result from alterations in cytoskeletal elements. In particular, tubulin mutants were expected to have this property, and the phenotype of cold-sensitive mutations in the β-tubulin gene *(TUB2)* made according to plan B is indeed cell-cycle arrest at mitosis (see "Tubulin Mutants" for detail). Additional mitosis-defective *cdc* mutations might well alter other components of the mitotic spindle. Examples include the *CDC31* gene: *cdc31* mutants fail to duplicate the spindle pole body, and thus cannot form a spindle (14). Analysis of other mutants shows that the *CDC13, CDC16, CDC17, CDC20,* and *CDC23* genes are necessary for spindle elongation, and the *CDC14* and *CDC15* genes are needed for nuclear division after the formation of the elongated spindle (15).

Saccharomyces strains with mutations in *CDC3, CDC10, CDC11,* or *CDC12* form multiple buds and undergo several rounds of nuclear division, but their cytoplasmic masses fail to separate at the nonpermissive temperature (34). They are also the only *cdc* mutants that lack the highly ordered rings of 10-nm filaments that normally lie under the inner surface of the plasma membrane within the bud neck. This finding suggests that these cytoskeletal elements play a role in cytokinesis (17).

Cell division cycle mutants thus can help to identify proteins that play a role in the assembly and function of the cytoskeleton. However, the primary defects of most of these mutations have not been identified, and other than the tubulin mutations, none is known to alter a component of the spindle apparatus. This specific approach (*cdc* phenotype) is obviously limited to the identification of genes whose products are essential for progression through the cell cycle. Not all cytoskeletal elements have this property. For instance, temperature-sensitive actin mutants do not show a cell division cycle pheno-

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type; instead cells are arrested at many points in the cell cycle (56). In addition, some mutants blocked in a specific mitotic function do not arrest with uniform cell morphology. For example, *ndc1* mutants fail to separate chromosomes, but can complete the morphological cell cycle (84).

DRUG SENSITIVITY Drugs that specifically inhibit the assembly of cytoskeletal structures have been used to identify mutations that alter these proteins in different cell types (20, 75, 90). Fungi are sensitive to the benzimidazole class of microtubule inhibitors that includes methyl benzimidazole-2-yl carbamate (MBC), benomyl, and nocodazole (22, 63, 75). Benzimidazole compounds block several cellular processes in fungi that are thought to be mediated by microtubules, such as nuclear migration (57, 62), chromosome segregation (93), and karyogamy (23). Resistance to high concentrations of benomyl is conferred in *Saccharomyces* (54, 85), *Schizosaccharomyces* (37), and *Aspergillus* (75) by missense alleles of β-tubulin genes; sometimes these mutations confer conditional lethality as well as drug resistance. Resistance due to a missense change in an α-tubulin polypeptide has not been observed. Interestingly, *Saccharomyces* strains that contain extra copies of *TUB1* or *TUB3* and thereby overproduce α-tubulin are slightly more resistant than wild-type cells to benomyl (71).

In *Saccharomyces* and *Schizosaccharomyces* alleles of both α-tubulin and β-tubulin genes have been identified in screens for benzimidazole supersensitive strains (89; T. Stearns & D. Botstein, unpublished observations). In addition, *Saccharomyces* strains that contain a disruption of *TUB3*, the minor α-tubulin gene, are also more sensitive than wild-type cells to benomyl (71). However, supersensitivity to microtubule inhibitors is not a phenotype restricted to tubulin mutants. Mutant alleles of three other genes in *Saccharomyces* have been identified in screens for benomyl supersensitivity as well (T. Stearns & D. Botstein, unpublished observations). These genes have been identified independently by mutations that reduce the fidelity of mitotic chromosome transmission (M. A. Hoyt & D. Botstein, unpublished observations).

Unfortunately, no inhibitor of the actin filament system has yet been reported that affects growth of yeast.

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FIDELITY OF MITOTIC CHROMOSOME TRANSMISSION One of the major roles for microtubules is the partitioning of replicated chromosomes at mitosis. The rate of loss of individual chromosomes is typically about once in every 10^5 cell divisions (27, 35, 49). Impairment of tubulin activity, either by drug (93) or by mutation (T. C. Huffaker & D. Botstein, unpublished observations), dramatically increases this rate. Mutations affecting other mitosis-specific functions can be expected to show a similar phenotype.

Genes encoding mitotic spindle components can presumably be identified

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by recessive mutations that decrease the fidelity of chromosome transmission. Hartwell & Smith (35) observed that most of the *cdc* mutants that are defective in the nuclear division pathway of the cell cycle show increased rates of loss of a marked chromosome V. However, many of these mutations increase the frequency of mitotic recombination as well. For this class of mutants, the elevated chromosome loss frequencies are probably not due to nondisjunction, as might be expected for a cytoskeletal defect, but to defects in DNA metabolism. A defect in DNA metabolism may leave lesions in the DNA that promote increases in both mitotic recombination and mitotic loss.

M. A. Hoyt & D. Botstein (unpublished observations) isolated over 550 *Saccharomyces* mutants with an elevated rate of loss of a marked chromosome III; most do not have increased mitotic recombination. Many of these *cin* (chromosome *in*stability) mutants show additional phenotypes, which indicates that they have a mitosis-specific defect. For example, many of the mutants are conditional-lethal for growth, and at the nonpermissive temperature, cause a cell division cycle arrest similar to the arrest caused by tubulin mutants. Approximately one-fifth of the *cin* mutants are also supersensitive to benomyl. Since tubulin is believed to be the only cellular target for benomyl, these loci are likely to encode proteins that functionally interact with microtubules. Seven of the benomyl supersensitive *cin* mutants are new α-tubulin mutants; three are alleles of *TUB1*, and four are alleles of *TUB3*. F. A. Spencer, C. Connelly & P. Hieter (personal communication) have also collected chromosome loss mutants by screening for increased loss of a marked telocentric fragment of either chromosome III or chromosome VII. Three of their mutants show a tubulinlike cell division cycle arrest in addition to elevated chromosome loss.

Since chromosome loss can be achieved by routes other than nondisjunction (e.g. chromosomal damage or failure to replicate), a more specific assay for nondisjunction may be chromosome gain or diploidization. Mutant alleles of four genes result in an increased frequency of diploidization. These include *NDC1* (84), *CDC31* (73), *ESP1* (6), and *KAR1* (68). Other mutant phenotypes of these genes suggest an involvement in mitosis for all four (see "Nontubulin Mutants That Affect Spindle Function").

KARYOGAMY DEFICIENCY The mutant isolation schemes described above rely on the identification of phenotypes expressed during mitotic growth. Cytoskeletal components are also thought to be essential during conjugation. The microtubule-depolymerizing drug benomyl (23) and cold-sensitive β-tubulin mutations (T. C. Huffaker & D. Botstein, unpublished observations) block karyogamy in yeast.

Conde & Fink (21) identified *kar1-1* as a mutation that causes a defect in nuclear fusion so that zygotes produce haploid, rather than diploid, daughter

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cells. This mutation also affects the assembly of microtubules in mating cells (68). More recently, two other genes (*KAR2* and *KAR3*) have been identified by isolating mutants defective for nuclear fusion (60). In addition, several of the *cdc* mutants (*cdc4, cdc28, cdc34,* and *cdc37*) exhibit a defect in nuclear fusion (25), indicating that their gene products are required for both mitotic growth and karyogamy. It is not yet known whether any of these gene products, other than *KAR1,* specifically influence the microtubule network during conjugation. However, mutations in *CIN1* and *SPA1,* which appear by other criteria to affect microtubule function, display a Kar^- phenotype as well.

Identification of Genes Whose Products Interact

The morphogenesis and function of a complex cytoskeletal structure depend on many specific interactions between components of the system. A functional interaction between two gene products may be revealed by the phenotype of cells that contain mutant forms and/or altered levels or activities of both gene products. Below, we describe three approaches that share this theme. Besides revealing possible functional interactions between previously identified gene-products, they also can be used to identify new genes. Note that in this review we use "functional interaction" broadly to indicate gene products that participate in the same subpathway of the cell cycle (e.g., mitotic chromosome segregation). Whether a functional interaction between two proteins is physical in nature can sometimes be inferred from genetic data, but direct proof always requires a biochemical or physical demonstration.

PSEUDOREVERSION OR SUPPRESSOR ANALYSIS Extragenic suppression of a mutant phenotype has long been recognized as a useful method for identifying interacting gene products (11, 33). A particularly relevant example comes from the work of Morris et al with *Aspergillus* (52). They demonstrated that an extragenic suppressor of a temperature-sensitive β-tubulin mutant is an allele of an α-tubulin gene. Recently, additional suppressors have been isolated that are nonallelic to any of the tubulin genes and are, therefore, good candidates for genes whose products interact with tubulin (92).

In practice, selecting pseudorevertants that overcome a mutant phenotype is simple (especially if the phenotype is conditional lethality). The difficulty lies in determining the mode of suppression and the level, if any, at which the suppressor gene product interacts with the reference gene product. Suppression due to a functional interaction between gene-products might be expected to be identifiable by one or both of the following criteria:

1. Suppressors that result from a compensatory change in a physically interacting protein are limited in the types of mutant alleles they affect. Thomas et al (86) identified three genes that can mutate to yield suppressors

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of a temperature-sensitive actin allele *(act1-1)* in *Saccharomyces*. These *s*uppressor of *ac*tin genes (*sac1, sac2,* and *sac3*) show allele specificity for suppression; they do not suppress another temperature-sensitive actin allele, *act1-2*.

2. The suppressing allele of a gene may independently confer a phenotype (39, 51). For example, suppressors of a temperature-sensitive mutant may themselves cause cold sensitivity for growth. If the new phenotype is similar in detail to that of the mutant it suppresses, it could indicate that the suppressor gene product participates in the same functional pathway as the reference gene product. This criterion is the best way to avoid the possibility that some global alteration (such as a change in pH or intracellular ion concentration) is causing the restoration of a mutant's function.

The *sac* suppressors of the temperature-sensitive actin allele were chosen because they confer a cold-sensitive phenotype as well as suppression. When shifted to their nonpermissive temperature, the *sac* mutants yield phenotypes that resemble those caused by a defect in actin (see "Actin Mutants"; 86). These include a disruption of the normal pattern of actin assembly, a failure to deposit chitin properly in the growing cell wall, and, for *sac2* mutants, an aberrant accumulation of intracellular membrane-bound structures. In another example, Pringle and coworkers isolated pseudorevertants of the four *cdc* mutants defective in 10-nm neck filament assembly (*cdc3, cdc10, cdc11,* and *cdc12;* 62). Although most of the suppressor mutants selected do not confer an identifiable phenotype by themselves, further analysis revealed that they are alleles of genes that could mutate to cause the same phenotype: suppressors of *cdc3* were found to be alleles of *cdc10,* and suppressors of *cdc10* were found to be alleles of *cdc3*. Suppressors of a cold-sensitive β-tubulin mutant of *Saccharomyces* have been isolated that also confer a temperature-sensitive phenotype (83). One such suppressor mutant causes arrest of the cell division cycle at the nonpermissive temperature that resembles the phenotype of β-tubulin mutants.

Suppression is not restricted to a missense change in an interacting gene product. A mutationally impaired function may also be suppressible by an increase in concentration of another protein. Two simple mechanisms for this type of suppression can be suggested. First, a functionally homologous polypeptide may be able to substitute for the defective polypeptide if more of the former is made available. For example, complete deletions of *TUB1* are suppressed by overexpression of the functionally homologous *TUB3* gene product (71). Second, reduced affinity of one protein for another might be overcome by overproduction of one of the interacting partners.

These pseudoreversion analyses have limitations. Since the selection for pseudorevertants is commonly for viability, revertants may often have several genetic changes, each of which contributes slightly to the increased viability

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of the cell. The rare interactional suppressor may be difficult to recognize among this background.

SYNTHETIC PHENOTYPES A synthetic phenotype is one caused by the combination of mutant alleles of two different genes (24, 81), that is, a phenotype produced by the genotype *geneA*$^-$ *geneB*$^-$ but not by either *geneA*$^-$ or *geneB*$^-$ alone. The synthetic phenotype can be extreme; either lethality or conditional lethality. Synthetic phenotypes can provide good evidence for an interaction between gene products, especially when additional evidence suggests a relationship (i.e. when *geneA*$^-$ and *geneB*$^-$ share a common phenotype). The most obvious explanation of a synthetic phenotype is that the two proteins perform the same essential function. However, for functionally interacting proteins, other mechanisms might underlie synthetic phenotypes. The two proteins might both be components of a structure that is functional despite mutant forms (or the absence) of either protein alone, but not of both together. Alternatively, one protein might be structural, while the other is regulatory, determining the amount or activity of the structural protein. This regulation could be at the level of gene expression or at a posttranslational modification step. However, the inviability of a particular double mutant requires careful interpretation. Even though their gene products are not functionally related, if *geneA*$^-$ and *geneB*$^-$ cause a cell to grow poorly, then the double mutant may be inviable simply due to extremely poor growth.

A possible useful feature of the synthetic-phenotype approach becomes apparent when one considers the interactions required to maintain proper function of a complex cytoskeletal structure. If the structure is compromised by a mutant form of or a reduced activity in one of its components, then a second-site mutation is more likely to cause failure of the structure than specifically suppress the first defect. This probability suggests that it may be feasible to screen for synthetic phenotypes, as opposed to employing selective pressure typically required for isolating pseudorevertants. Screening can be accomplished in yeast by covering a chromosomal mutation in the gene of interest with a plasmid containing its wild-type allele. Following mutagenesis, one can screen for second-site mutants that no longer survive (or show conditional lethality) without the plasmid.

Synthetic phenotypes have been observed for mutant combinations in the actin system (P. Novick & D. Botstein, unpublished observations). Two *SAC1* alleles that suppress the temperature-sensitive *act1-1* allele cause inviability when combined with *act1-2*. However, a null allele of *SAC1* did not cause this synthetic phenotype, again demonstrating an allele-specific interaction between yeast actin and the *SAC1* gene product.

Numerous examples of synthetic phenotypes involving pairwise com-

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binations of mutations in the microtubule system have recently been observed (T. Stearns, M. A. Hoyt, D. Botstein, unpublished observations). The *tub1-1* mutation is a cold-sensitive allele of the major α-tubulin gene of *Saccharomyces* that has no effect on growth at the permissive temperature. However, double mutants constructed between *tub1-1* and mutant alleles of *TUB3* or *TUB2* are often inviable at all temperatures. Strong synthetic phenotypes also suggest an interaction between the protein encoded by the *CIN1* locus and microtubules. All mutant alleles of *CIN1* tested to date show synthetic lethality when combined with a number of *tub1* alleles. In addition, the combination of a nonconditional mutant *TUB2* allele and *CIN1* missense alleles causes cold sensitivity for growth. At the nonpermissive temperature, these double mutants arrest the cell cycle, as if blocked in mitosis.

In some instances, supersensitivity to a drug can be compared to a synthetic phenotype. For example, many of the *CIN* loci can mutate to yield a benomyl supersensitive phenotype. This phenotype is analogous to the synthetic phenotype caused by certain double-mutant combinations, except that instead of mutations compromising the activity of both proteins, one of the proteins, tubulin, is compromised by a sublethal dose of the drug.

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UNLINKED NONCOMPLEMENTATION A gene is traditionally defined by a collection of linked, noncomplementing mutant alleles. When two haploid yeast strains, each containing a recessive mutation in a different gene, are crossed, the resulting doubly heterozygous diploid usually displays a wild-type phenotype. Each parental genotype provides the function that is defective in the other parent. However, numerous examples of unlinked mutations that fail to complement have been reported (3, 8, 66), and are usually interpreted as being indicative of an interaction between the gene products involved. Raff & Fuller (28a, 65) have described unlinked mutations that fail to complement alleles of a testis-specific β-tubulin gene in *Drosophila*. One such unlinked noncomplementing mutation has been found to be tightly linked to an α-tubulin gene (28a).

T. Stearns & D. Botstein (unpublished observations) have extended this type of analysis to *Saccharomyces*. Starting with a cold-sensitive *tub2* haploid, they screened for mutants that, when crossed to the *tub2cs* strain, yielded cold-sensitive diploids. This screen netted two new alleles of *TUB2*, the expected class, but also an allele of *TUB1* (*tub1-1*). The *tub1-1* allele caused a cold-sensitive phenotype in haploids, making it possible to repeat this regimen, this time screening for noncomplementers of *tub1-1*. This screen yielded one new allele each of *TUB2* and *TUB3* as well as two alleles of *TUB1*. Since the products of the *TUB* genes are known to interact physically, these results validate this type of approach.

Mechanistically, the failure of unlinked mutant alleles to complement may be complex. Noncomplementation of *tub2cs* by *tub1-1* shows allele specific-

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ity: *tub1-1* fails to complement two *tub2cs* alleles but does complement four others. The differences in complementation in this case do not appear to be related to the residual activity ("leakiness") of the *TUB2* mutants, but may reflect specific defects in the α-tubulin–β-tubulin interaction. This situation is difficult to interpret, however, as *tub1-1/+*, *tub2cs/+* diploids contain five different tubulin proteins: the gene products of *tub1-1* and *tub2cs,* as well as the wild-type gene products of *TUB1*, *TUB2*, and *TUB3*.

In contrast, noncomplementation of *tub1-1* by *tub3* may simply be due to a reduction in α-tubulin levels past a point that can be tolerated at colder temperatures. Indeed, this noncomplementation is not allele specific; a null allele of *TUB3* also fails to complement *tub1-1*.

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PHENOTYPES OF CYTOSKELETAL MUTATIONS

Tubulin Mutants

Cold-sensitive mutations in *TUB1* (major α-tubulin) and *TUB2* (β-tubulin) have been obtained by several methods. The *tub2cs* mutations were isolated by selecting cells resistant to benomyl (85) and by in vitro mutagenesis of the cloned gene (T. C. Huffaker & D. Botstein, unpublished observations). The *tub1cs* mutations were obtained by screening for supersensitivity to benomyl and for noncomplementation of mutations in *TUB2* (T. Stearns & D. Botstein, unpublished observations).

The phenotypes of six β-tubulin mutants and three α-tubulin mutants were examined in detail. When these strains are shifted to the restrictive temperature, they arrest mitotic growth at a specific stage of the cell cycle and accumulate as large-budded cells containing a single undivided nucleus. None of the β-tubulin mutations interfere with DNA replication. Therefore, the block in nuclear division is not due to failure to produce two sets of chromosomes but rather to failure of the mitotic spindle to segregate these chromosomes.

Immunofluorescence studies of cellular microtubules reveal that the *tub2* mutations have different effects on the microtubule composition of arrested cells. Because of the variety of phenotypes demonstrated by these mutants, it has been possible to infer that cytoplasmic microtubules are responsible for migration of the nucleus to the bud neck during the mitotic cell cycle and for nuclear movement and fusion during conjugation. Both of these processes are inhibited in four of the mutants, indicating that microtubule function is required. The two mutants that are not blocked in these processes are the only ones that contain prominent cytoplasmic microtubules. These results agree with the observations that nocodazole blocks nuclear migration during mitosis (62) and that benomyl interferes with nuclear fusion during mating (23). In addition, a cold-sensitive mutation in one of the α-tubulin genes of *Schizosac-*

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charomyces causes aberrant nuclear localization at restrictive temperature (87, 88). In *Aspergillus,* nuclear migration during hyphal growth is also inhibited by benomyl and cold-sensitive mutations in the gene encoding β-tubulin (58).

In yeast, both cell surface growth and protein secretion are mediated by vesicles, and both processes are confined primarily to the growing bud. The observation that cytoplasmic microtubules extend into the cell bud has led to the suggestion that these microtubules are responsible for the transport of secretory vesicles (13, 15, 16). The *tub2* mutations demonstrate that cytoplasmic microtubules do not play an essential role in the transport of secretory vesicles in yeast. Bud formation is not inhibited by any of the *tub2* mutations, including two mutations that eliminate all cellular microtubules in the cold. In addition, protein secretion is not affected by these mutations.

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Nontubulin Mutants That Affect Spindle Function

The *ndc1-1* mutant was initially isolated as a cold-sensitive strain that exhibited a weak cell division cycle arrest similar to the cold-sensitive β-tubulin mutants (84). At the nonpermissive temperature, *ndc1-1* causes failure of chromosome separation but does not block the cell cycle. This defect results in an asymmetric cell division in which one daughter cell doubles in ploidy and the other inherits no chromosomes. Tubulin staining by immunofluorescence shows that the spindle poles are properly segregated to the two daughter cells. However, the chromosomes are associated with only one pole and are thus delivered to one daughter cell. The *ndc1-1* mutation appears to define a gene required for the attachment of chromosomes to the spindle pole and may encode a component of the spindle pole body or a centromere-binding protein in yeast.

The *KAR1* gene was identified through a mutation, *kar1-1,* that causes a defect in nuclear fusion during conjugation (21). This allele was selected under conditions that required growth, so it has no effect on the mitotic cell cycle. The *KAR1* gene has been cloned by complementation of the *kar1-1* mutation (68). Temperature-sensitive alleles of *KAR1,* produced by in vitro mutagenesis and gene replacement, demonstrate that *KAR1* is essential for mitosis. At the nonpermissive temperature these strains arrest the cell cycle and accumulate as large-budded cells containing an undivided nucleus in the bud neck. Electron microscopic examination has shown that these cells arrest with an unduplicated spindle pole body, much like the *cdc31* mutants. At semipermissive temperatures the mutants become polyploid, presumably owing to defects in chromosome segregation. Recent experiments demonstrate that a *KAR1–lacZ* fusion protein localizes to the spindle pole bodies as judged by immunofluorescent staining with anti-β-galactosidase antibody (M. Rose, personal communication). These results imply that the product of *KAR1* is a component of the spindle pole body in yeast.

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The first mutation in the *CDC31* gene, *cdc31-1*, was identified as a temperature-sensitive cell division cycle mutation. At the nonpermissive temperature, *cdc31-1* mutants arrest as uninucleate, large-budded cells (61). DNA replication occurs, but the spindle pole body fails to duplicate. Consequently, diploid cells arise from the transient arrest of a haploid strain (73). Although the spindle pole body fails to double, its dimensions increase, suggesting that the principal constituents of the daughter spindle pole body have been assembled. This enlarged spindle pole body nucleates about twice the number of microtubules as the normal spindle pole body (14). *CDC31* has been cloned by complementation of its temperature-sensitive phenotype (5). Its amino acid sequence, derived from the nucleotide sequence, displays significant homology to calmodulin and several other members of the eukaryotic Ca^{2+}-binding family of proteins. This similarity suggests that the *CDC31* product regulates spindle pole body duplication in response to a flux of calcium ions at a specific stage of the cell cycle.

Human autoantibodies that react with the mammalian spindle pole were isolated from scleroderma patients and used to identify related antigens in yeast (79). A 59-kd yeast protein, identified by three of these antisera, may be a component of the spindle pole body. It copurifes with a yeast nuclear fraction and is enriched in *esp1* mutant cells that overproduce spindle pole bodies. The *SPA1* gene encoding this protein was cloned by immunoscreening a yeast genomic DNA expression library. Disruption of *SPA1* demonstrated that it is not essential for growth at 30°C but is essential at 38°C. Cells containing *SPA1* disruptions display a number of phenotypes that are consistent with an involvement in mitosis: they lose chromosomes at a 10–50-fold higher frequency than wild-type cells, show a karyogamy defect, and produce abnormal numbers of nuclei in 15–30% of the cells.

Six mutant alleles of *CIN1* were identified by their elevated rates of loss of a marked chromosome III (M. A. Hoyt & D. Botstein, unpublished observations). All are extremely supersensitive to benomyl. In addition, nine other alleles were isolated in a benomyl supersensitive screen (T. Stearns & D. Botstein, unpublished observations). None of these mutants shows significant conditional lethality. The *CIN1* gene was cloned by complementation of the benomyl supersensitive phenotype. Haploid cells with a *cin1* disruption grow at wild-type rates at 26°C but are inhibited for growth, relative to wild-type, at 11°C. When grown at 11°C, these cells contain reduced microtubule structures, as determined by immunofluorescence. The *cin1* mutants also have a weak karyogamy defect at all temperatures and show synthetic phenotypes when combined with tubulin mutants. The *CIN1* protein is, therefore, predicted to be required for maximal microtubule stability. It may accomplish this function by either regulating the synthesis or activity of a microtubule component (i.e. tubulin) or by actually participating in the microtubule structure.

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Actin Mutants

In vitro mutagenesis and gene replacement techniques were used to produce three temperature-sensitive alleles of *ACT1* (77). Both *act1-1* and *act1-3* changed pro_{31} to leucine, while *act1-2* changed ala_{57} to threonine.

These mutations in actin affect its localization in the cell (56). After two hours at the nonpermissive temperature, *act1-3* cells contain only randomly distributed dots of actin, and *act1-2* cells contain a mesh of fine filaments and randomly distributed dots near the cell surface.

The actin mutants affect other cell processes as well (56). The percentage of budded cells drops to about 30% after 2 hours at nonpermissive temperature; a wild-type culture contains 50–60% budded cells. The mutants also appear considerably larger and more rounded than wild-type cells. The vacuole swells, filling much of the cytoplasm, and lysis becomes prevalent. Chitin is no longer confined to the bud neck but is delocalized throughout the cell surface. In addition, both actin mutants show a pronounced osmotic sensitivity. These observations suggest at least two possible roles for actin in yeast. Actin may play a role in bud emergence, as suggested by the formation of actin rings near the neck in wild-type cells and by the reduction in the number of budded cells and delocalization of chitin in the mutants. Actin may also play a role in osmotic regulation of the cell, as suggested by the cell lysis, osmotic sensitivity, and swollen vacuoles of the actin mutants.

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Ten-Nanometer Filament Mutants

Budding yeast cells contain a highly ordered ring of 10-nm filaments, of unknown biochemical composition, that lie under the inner surface of the plasma membrane within the bud neck (18). At the nonpermissive temperature, temperature-sensitive *CDC3*, *CDC10*, *CDC11*, and *CDC12* mutants lack these filaments (17) and display a pleiotropic phenotype that includes abnormal bud growth and an inability to complete cytokinesis (34, 62). These genes have been cloned by complementation of their temperature-sensitive phenotypes (62). Immunofluorescence studies, using antibodies raised against *CDC3* and *CDC12* fusion proteins, suggest that both of these gene products are associated with 10 nm filaments (31a; see Fig. 1 for *CDC12* staining). DNA sequencing of the *CDC3*, *CDC10*, *CDC11* and *CDC12* genes has shown striking homologies among the four predicted amino acid sequences (B. Haarer, S. Ford, S. Ketcham, D. Ashcroft, J. Pringle, personal communication). Thus, it seems virtually certain that all four gene products are components of or closely associated with the 10-nm filaments.

CONCLUSIONS

It now seems possible, using a combination of genetic and biochemical approaches, to identify most of the components of the yeast cytoskeleton, to

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determine their roles in vivo, and to obtain a description of the molecular interactions among these proteins. The techniques we have described should be applicable, with suitable modification, to a number of other systems.

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