Analysis of α-Tubulin in Yeast

by

Peter Joseph Schatz

Sc.B., Sc.M., Brown University (1982)

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at the
Massachusetts Institute of Technology
December 1987

© Peter Joseph Schatz 1987

Signature of Author _____

Department of Biology
November 1987

Certified by _____

David Botstein
Thesis Supervisor

Certified by _____

Frank Solomon
Thesis Supervisor

Accepted by _____

Robert T. Sauer
Chairperson, Departmental
Committee on Graduate Studies

Analysis of α-Tubulin in Yeast

by

Peter Joseph Schatz

Submitted to the Department of Biology
in November 1987 in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy
in Biology

ABSTRACT

Two α-tubulin genes from the budding yeast *Saccharomyces cerevisiae* were identified and cloned by cross-species DNA homology. Nucleotide sequencing studies revealed that the two genes, named *TUB1* and *TUB3*, encoded gene products of 447 and 445 amino acids, respectively, which were highly homologous to α-tubulins from other species. Comparison of the sequences of the two genes revealed a 19% divergence between the nucleotide sequences and a 10% divergence between the amino acid sequences. Each gene had a single intervening sequence, located at an identical position in codon 9. Cell fractionation studies showed that both gene products were present in yeast microtubules. These two genes, along with the *TUB2* β-tubulin gene, probably encode the complete complement of tubulin in budding yeast.

Microtubules in yeast are functional components of the mitotic and meiotic spindles and are essential for nuclear movement during cell division and mating. The relative importance in these processes of *TUB1* and *TUB3* was examined through the construction of null mutations and also by increasing their copy number on chromosomes and on plasmids. Experiments with null alleles of *TUB3* showed that *TUB3* was not essential for mitosis, meiosis or mating. Null alleles of *TUB3*, however, did cause several phenotypes including hypersensitivity to the antimicrotubule drug benomyl and poor spore viability. On the other hand, the *TUB1* gene was essential for growth of normal haploid cells. Even in diploids heterozygous for a *TUB1* null allele, several dominant phenotypes were evident including slow growth and poor sporulation. This functional difference between the two genes was apparently due to different levels of expression because extra copies of either gene could suppress the defects caused by a null mutation in the other. We concluded that in spite of the 10% divergence between the products of the two genes, there was no essential functional difference between them.

Comparisons of sequences from several species revealed the presence of a variable region near the amino terminus of α-tubulin proteins. We have perturbed the structure of this region in *TUB3* by inserting into it 3, 9, or 17 amino acids and have tested the ability of these altered proteins to function as the only α-tubulin protein in yeast cells. We found that each of these altered proteins was sufficient on its own for mitotic growth, mating, and meiosis of yeast. We concluded that this region could tolerate considerable variation without losing any of the highly conserved functions of α-tubulin. Our results suggested that variabiltiy in this region occurred because it could be tolerated, not because it specified an important function for the protein.

We have isolated 70 conditional-lethal mutations in *TUB1* using a plasmid replacement technique commonly called the plasmid shuffle. Of the 70 mutations isolated, 69 resulted in cold-sensitivity. We have mapped 31 of the mutations to deletion intervals within the *TUB1* gene. We characterized the phenotypes caused by 38 of the mutations after shifts of mutants

to the nonrestrictive temperature. Populations of temperature-shifted mutant cells contained an excess of large-budded cells with undivided nuclei, consistent with the previously determined role of microtubules in yeast mitosis. Several of the mutants arrested growth with a sufficiently uniform morphology to indicate that *TUB1* has a specific role in the progression of the yeast cell cycle. A number of the mutants had gross defects in microtubule assembly at the restrictive temperature, some with no microtubules and some with excess microtubules. Other mutants contained disorganized microtubules and nuclei. Greater than 90% of the mutants examined were hypersensitive to the antimicrotubule drug benomyl. None of the mutants were resistant to benomyl.

Thesis Supervisors: Dr. David Botstein, Professor of Biology
                        Dr. Frank Solomon, Professor of Biology

Acknowledgments:

Table of Contents

Chapter 1:

Microtubules, Mitosis, and Yeast

Microtubules are polymers of globular protein subunits which are present in most eukaryotic cells and function in a wide variety of roles in cellular motility and structure. The study of microtubules has been concerned with both the tubulin subunits that are their main structural element and on other components in cells that interact with them. In the first part of this introduction, I will discuss microtubule structure, assembly, and function. I will emphasize the surprising extent to which the properties of tubulin and microtubules *in vivo* can be explained by their behavior *in vitro*. The properties of tubulin alone, however, are not sufficient to explain the diverse functions of microtubules. Many other cellular constituents must be involved. The challenge of current microtubule research is to identify these other components and to determine their roles and the roles of tubulin in the mechanism of microtubule action.

This thesis is concerned with the study of microtubular function in the yeast *Saccharomyces cerevisiae*. The study of microtubules in yeast has been motivated by a desire to correlate biochemical and structural data with information about *in vivo* functions, obtained through the use of the sophisticated classical and molecular genetic system of yeast. Because the major role of microtubules in the yeast cell cycle is in mitosis, the second part of this introduction will consist of a brief discussion of mitosis in organisms other than yeast. Finally, I will discuss the function of microtubules in yeast mitosis, meiosis and mating.

Part 1: Microtubules:

Structure:

Microtubules are found in a very wide variety of cellular structures and, in the electron microscope, appear as hollow tubes about 24 nm in diameter (reviewed in Kirschner, 1978 and Dustin, 1984). Transverse sections usually reveal a tube consisting of 13 smaller

components called protofilaments, which are parallel to the long axis of the microtubule. The protein subunits are stacked in a regular helical array. The regular stacking of these subunits on top of one another leads to the parallel appearance of the protofilaments. Although 13 is the most common number of protofilaments observed, other numbers of protofilaments are found in cylindrical microtubules. In addition, irregular arrangements of protofilaments occur in specialized structures such as cilia and basal bodies.

Microtubules are most commonly found in structures that play a role in motility. These include mitotic and meiotic spindles, flagella, cilia and nerve cell processes. Other microtubules have a more obvious structural role, such as those in the marginal band of red blood cells in birds and cold-blooded vertebrates. Furthermore, most actively dividing eukaryotic cells display arrays of microtubules during interphase that originate from a single area (called the microtubule organizing center or MTOC). In higher cells, these microtubules form a lacy array which weaves throughout the cytoplasm and play a role in cell shape and motility.

Tubulin and Microtubule assembly *in vitro*:

Tubulin was originally identified as a soluble protein from eukaryotic cells that bound to the antimitotic drug colchicine (Borisy and Taylor, 1967). In its soluble form, tubulin probably exists as a 110 kd heterodimer of α– and β– subunits (Luduena et al., 1975). Under conditions of neutral pH, warm temperature, low Ca^{++}, sufficient concentrations of GTP, Mg^{++}, and tubulin dimers, the dimers will assemble into microtubules (Weisenberg, 1972).

There are two binding sites for GTP on a dimer. One is referred to as the E-site (exchangeable with the external medium) and the the other is the N-site (for nonexchangeable). GTP binding to these two sites is thought to have distinct functions. Since the N-site GTP has a half life comparable to that of the dimer, it probably functions as a stable structural part of the dimer (Spiegelman et al., 1977). In contrast, the E-site GTP is hydrolyzed to GDP during the microtubule assembly/disassembly process.

The fact that microtubule assembly/disassembly is coupled with the hydrolysis of GTP gives microtubules several interesting properties. If the assembly/disassembly process did not use energy, then microtubules would be equilibrium polymers, with their growth (in the absence of other factors) determined by the concentration of dimer at free ends and by the dissociation rate of dimers from the ends. The two ends of the polymer (commonly called "plus" and "minus" ends) could have different assembly rates, but the equilibrium constant for growth at each end would have to be identical (Asakura, 1968). Such a situation would place several constraints on the assembly process including: [1] relative insensitivity of the rate of assembly to monomer concentration, [2] slow rates of disassembly, and [3] static microtubule arrays (reviewed in Kirschner and Mitchison, 1986). As suggested by Wegner (1976) for the case of actin filaments and ATP, energy consumption allows differentiation between the ends of a linear polymer. At a steady state, net assembly could occur at one end accompanied by net disassembly at the other end, a process called "treadmilling". The plus end of a microtubule is defined as the favored end for assembly. Thus, treadmilling would involve addition of subunits at the plus end and loss of subunits at the minus end. Evidence for treadmilling has been reported by Margolis and Wilson (1978). It has been the basis of several models of microtubular organization and motility (Kirschner, 1980; Margolis and Wilson, 1981). Two crucial aspects of these models are: [1] selective stabilization of certain microtubules by capping of their minus ends by MTOC's and [2] production of force by treadmilling microtubules. Observations of microtubules *in vivo* and *in vitro*, however, led to a reexamination of the treadmilling models (see below).

More recent models of microtubule assembly also rely on the hydrolysis of GTP during the assembly/disassembly process, but they incorporate the possibility that the kinetics of assembly may not be the same as the kinetics of GTP hydrolysis. Two observations concerning *in vitro* assembly of microtubules are relevant to newer models. First, nonhydrolyzable analogues of GTP will support assembly of microtubules (Aria and Kaziro, 1976; Penningroth et al., 1976). Thus, the energy of GTP hydrolysis is not essential for

addition of subunits. Second, GTP hydrolysis may lag behind the assembly rate at high tubulin concentrations (Carlier and Pantaloni, 1981), although the extent and even the existence of this lag is disputed (O'Brien et al., 1987). Thus, a microtubule may be a mixed polymer of GDP-tubulin and GTP-tubulin. Theoretically, this situation gives the polymer several useful properties (Kirschner and Mitchison, 1986). In a pure tubulin polymer, there are three possible reactions: [1] the addition and loss of GTP-tubulin, [2] the addition and loss of GDP-tubulin, and [3] irreversible hydrolysis of GTP-tubulin to GDP-tubulin. If assembly of GTP-tubulin and disassembly of GDP-tubulin are strongly favored, then cells can regulate microtubule assembly by regulating GTP hydrolysis in microtubules. Alternatively, some other property of microtubule ends might distinguish the growing phase from the shrinking phase. Experimentally determined assembly and disassembly rates are consistent with such a phase transition and inconsistent with simple equilibrium models (Kirschner and Mitchison, 1986).

The existence of subpopulations of microtubules that could be distinguished on the basis of intrinsic assembly competence would predict that a population of microtubules would contain both growing and shrinking microtubules. This two-phase mode of microtubule polymerization has been called "dynamic instability" (Mitchison and Kirschner, 1984). In the most widely publicized version of the model, the distinguishing characteristic between growing and shrinking phases is the presence of GTP-tubulin at the ends of the microtubules. In the model, GTP is hydrolyzed to GDP with a constant probability per unit time. Therefore, faster growth leads to a larger GTP "cap" on the tubulin. Slow or stopped growth increases the probability that the GTP will be hydrolyzed to GDP, thus exposing GDP-tubulin ends and leading to rapid depolymerization. Support for the GTP cap model comes from theoretical studies (eg. Hill and Chen, 1984) and from the observation that nonhydrolyzable analogues of GTP stabilize the ends of microtubules (Weisenberg and Deery, 1976; Penningroth and Kirschner, 1977).

Alternatives to the GTP cap model explaining dynamic instability include binding of other

proteins or some conformational change in the microtubule after GTP hydrolysis. Without energy consumption, however, it is impossible for steadily growing and shrinking microtubules to coexist in the same population (Kirschner and Mitchison, 1986). The simplest version of the GTP cap model would predict that once a microtubule started shrinking, it would be very likely to disappear because of the low amount of GTP tubulin in the middle (the oldest part) of the polymer.

The behavior of microtubules *in vitro* is consistent with the dynamic instability model, although other explanations have been proposed (Farrell et al., 1987; Rothwell et al., 1987). Using light and electron microscopy, Mitchison and Kirschner (1984a,b) examined the growth of individual free microtubules and microtubules nucleated from purified centrosomes. At high concentrations of tubulin, microtubules grew steadily from centrosomes. When the tubulin concentration was lowered by dilution, however, some microtubules continued to grow while others disappeared quite rapidly. Clearly, there were two populations of microtubules distinguished by their assembly competence. Free microtubules were used as seeds for assembly to a steady level of polymer mass. Once the steady state of bulk assembly was reached, however, the number of microtubules declined while their average length increased. A more recent study using biotinylated tubulin as a marker has shown that both ends of the polymer grow steadily in most microtubules at steady state while a minority of microtubules are shrinking rapidly (Kristofferson et al., 1986). No evidence of treadmilling was observed. The length of microtubules in solution did not reach a stable steady state because: [1] growing microtubules were more likely to continue growing than shrinking ones and [2] shrinking microtubules were more likely to continue shrinking than growing ones.

The coexistence of growing and shrinking phases has reportedly been visualized *in vitro* by observation of individual microtubules using dark-field microscopy (Horio and Hotani, 1986). In these experiments, the ends of the microtubules apparently changed randomly between growing and shrinking phases, and the two ends behaved independently. In addition, the presumptive plus end grew and shrunk faster than the minus end and

interconverted between these phases more frequently. Horio and Hotani reported that growth phase interconversions were quite frequent and disappearance was rare. These frequent phase conversions are inconsistent with the simple version of the GTP cap model, which would predict that most microtubules that started to shrink would disappear. There are several possible explanations for this inconsistency. [1] The observed variations in length may have been due in part to movement of the microtubules into and out of the microscope's plane of focus. [2] The tubulin may have been contaminated with sufficient microtubule associated proteins to affect the phase transition frequency; this possibility is supported by the observation that added microtubule associated proteins suppressed the phase conversion and stabilized the growing phase. [3] The simple GTP cap model may be incorrect. Observations *in vivo* have cast doubts on the relevance of these frequent phase conversions (see below).

It is clear that dynamic instability of microtubule growth occurs *in vitro*, but the factor that determines the difference between growing and shrinking microtubules is as yet unknown.

Evidence for Dynamic Instability *in vivo:*

Microtubule dynamics have also been studied *in vivo*, using microinjection of labeled tubulin into cells. Bulk measurements of the rate of incorporation of tubulin into mitotic spindles and interphase cytoskeletons and rates of recovery of photobleached areas within cells demonstrated that microtubule arrays are extremely dynamic *in vivo* (Salmon et al., 1984, Saxton et al., 1984). Rates of incorporation were extremely high, particularly in mitotic spindles, which is consistent with dynamic instability and inconsistent with any simple equilibrium model. Observation at the level of individual microtubules indicated that most microtubules were continuously growing at their ends at a fairly steady rate (Soltys and Borisy, 1985; Schulze and Kirschner, 1986, Mitchison et al., 1986). These studies also showed that the unlabeled microtubules dissappeared quite rapidly, leading to rapid turnover of most microtubules in the cell. A few microtubules, however, were considerably more

stable than the average (Schulze and Kirschner, 1986). The rapid and complete turnover of most microtubules in the cells casts doubt on the relevance of the frequent *in vitro* growth phase transitions reported by Horio and Hotani (1986), although some *in vivo* phase transitions have been reported (Sammak and Borisy, 1987).

Theoretical Consequences of Dynamic Instability:

Dynamic instability is the basis for models of cellular regulation of microtubule organization and function (Kirschner and Mitchison, 1986): Due to the phase transition between growth states, microtubules can both grow and shrink quite rapidly from either end. Different populations of microtubules in a single cell can be growing and shrinking, as is often required in complicated functions such as mitosis (see below). Growth can occur below the critical concentration as long as there are nucleation sites available (eg. centrosomes). These nucleation sites will be greatly favored as sites of initiation because they give a kinetic advantage to assembly at low tubulin concentrations. Free microtubules that disappear during random fluctuations predicted by the dynamic instability model will be unable to regrow because of the lack of the ability to nucleate. Consistent with this, microtubules in most cell types appear to arise from organizing centers called MTOC's. Finally, the rapid turnover of microtubules gives the cell the opportunity to reorganize its cytoskeleton quickly by selectively stabilizing a subset of them in a region where they may be needed. In summary, it is clear that a number of the *in vivo* properties of microtubule assemblies can be explained as a consequence of fairly simple interactions between tubulin, GTP, and nucleation sites.

The current emphasis on dynamic instability is certainly appropriate when examining cells in mitosis or cells with dynamic cytoskeletons. Many cells, however, have very stable microtubular structures. Examples include neuronal cells and blood cells with marginal bands. In these cases, dynamic instability might explain very little about the *in vivo* behavior of microtubules.

Microtubule Function and Organization:

So far, this discussion has focused on the properties of tubulin and microtubules without consideration of many mechanisms that might regulate their function and organization in living cells. The need for such regulation is demonstrated most simply by considering the wide variety of structures of which microtubules are an integral part (Dustin, 1984). At the same time, the ultrastructure of microtubules is extremely conserved. Three possible sources for this functional diversity in the midst of structural conservation are: [1] variations in the primary structure of tubulins which form the microtubules in diverse structures, [2] specific modifications of tubulins to effect functional diversity, and [3] accessory proteins that regulate microtubular structures. The evidence for these three mechanisms will be discussed in the next three sections.

The Role of Tubulin Sequences:

While it is clear that tubulin sequences on the whole are highly conserved, there is abundant information about the existence of a variety of subtle structural variants of tubulins within species and within single cells (for reviews see Raff, 1984; Cleveland and Sullivan, 1985). Fulton and Simpson (1976) first proposed the hypothesis that different α- and β-tubulin molecules might form the basis for functionally distinct microtubules. This idea, called the "multitubulin hypothesis", gained momentum through the years from many observations of multiple tubulin proteins, some of which appear to segregate into different structures (Stephens, 1978). That some of this variation arises from the expression of different genes has been documented for a number of species (for reviews see Raff, 1984; Cleveland and Sullivan, 1985). Some of these genes encode proteins which can be classified into groups based on sequence similarity. These groups, called "isotypes", are often more conserved across species barriers than they are conserved compared to other isotypes in the same species. The isotypes also often share patterns of expression in the tissues of higher eukaryotes. This conservation of sequence and expression patterns has been interpreted to

indicate that each isotype familiy shares some functional specialization (see Cleveland and Sullivan, 1985).

The most extreme version of the multitubulin hypothesis would say that tubulins are segregated into different microtubular structures based on their primary sequence and that this segregation has some essential role in microtubule function. Such a model would predict that specific tubulins might have a restricted ability to function in a variety of contexts. Several observations, however, argue against the possibility that different tubulin gene products have restricted functions. In *Drosophila melanogaster* spermatogenesis, a sperm-specific β-tubulin gene has a role in the meiotic spindle, in nuclear shaping by cytoplasmic microtubules, and in the sperm flagella (Kemphues et al., 1982). In *Aspergillus nidulans*, the β-tubulin gene normally used for conidiation can be replaced by the divergent vegetative gene (May et al., 1985; Weatherbee et al., 1985). The two divergent α-tubulin proteins of each of the very distantly related yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* show a similar lack of functional specialization (Adachi et al., 1986; Chapter 3). In both species, either of the α-tubulin proteins is sufficient, on its own, for all known microtubule dependent processes (Chapter 3, M. Yanagida, personal communication).

Two studies of the function of divergent β-tubulins in animal cells have shown that their primary sequences placed no restrictions on their ability to assemble into all microtubules of the cells. Bond et al. (1986) showed that a chicken-yeast chimeric β-tubulin assembled efficiently into all microtubules of mouse fibroblasts and had no apparent effect on either growth rate or morphology. Lewis et al. (1987) showed that a variety of naturally occurring β-tubulin isotypes demonstrate neither complete nor partial segregation into different structures. Even a divergent isotype normally expressed only in hematopoietic cells was assembled into all microtubules in its normal context and in transfected human fibroblasts.

The above results suggest that any functional significance of divergent tubulin isotypes is likely to be subtle. The observation of the conservation of isotype sequences and expression patterns in vertebrates can be explained by a simple evolutionary argument. Specifically,

complex organisms have a variety of tissues with different quantitative requirements for tubulin. The presence of multiple genes could have arisen as an adaptation to this need for stage-specific regulation of tubulin synthesis during differentiation (Raff, 1984). Instead of evolving complicated promoters for a few genes, these organisms could have evolved simple promoters for many genes. Assuming that this duplication of genes occurred before the radiation of various vertebrate species, the expected result is a closer relationship between isotypes in a variety of species than between isotypes in the same species. The observed conservation of these isotypes over long periods of evolutionary time could be explained by tubulin's known resistance to divergence. Most random changes would be recessive lethals. Only genes expressed in specialized tissues would be free to diverge, as has been observed in a number of species (Villasante et al., 1986; Theurkauf et al., 1986; Pratt et al., 1987). In the absence of evidence that tubulin isotypes show structural or biochemical specialization, tests of subtle functions of these isotypes will necessarily involve gene replacement experiments. The most complex organism in which such studies are currently feasible is *Drosophila*.

The Role of Covalent Modifications of Tubulin:

A variety of covalent modifications of tubulin have been observed and have been suggested to play a role in microtubule function (for review see Cleveland and Sullivan, 1985). Gard and Kirschner (1985) observed phosphorylation of β-tubulin in mouse neuroblastoma cells and showed that levels of phosphorylation were correlated with levels of microtubule assembly. Two covalent modifications of α-tubulin have been observed. L'Hernault and Rosenbaum (1983) showed that the majority of α-tubulin in *Chlamydomonas* flagella was acetylated on the epsilon-amino group of a lysine residue. Although originally thought to be specific to cilia and flagella, acetylated α-tubulin was found in subsets of cytoplasmic microtubules in *Chlamydomonas* and *Physarum* (LeDizet and Piperno, 1986; Diggins and Dove, 1987). These studies used a monoclonal antibody that is probably specific for the acetylated form of α-tubulin (Piperno and Fuller, 1985). This antibody also recognized

microtubules in some but not all mammalian cells, including primary cilia, centrioles, mitotic spindles, and subsets of cytoplasmic microtubules (Piperno et al., 1987). The epitope also was detected in rat nerve cell processes (Cambray-Deakin and Burgoyne, 1987). One common feature of the microtubules containing acetylated α-tubulin is that they are generally more stable than other microtubules (eg. Piperno et al, 1987). Whether this relationship is cause or effect remains to be determined (see below).

The second well-studied posttranslational modification of α-tubulin is the removal and replacement of the carboxy-terminal tyrosine residue (Barra et al., 1973a, 1973b, 1974). Most α-tubulin genes encode tyrosine as the last residue (Cleveland and Sullivan, 1985). This residue can be removed by a specific carboxypeptidase (Argarana et al., 1978; Kumar and Flavin, 1981) and replaced by the enzyme tubulin tyrosine ligase (Raybin and Flavin, 1977a; Murofushi, 1980). Although the absence of the terminal tyrosine did not seem to alter the *in vitro* assembly properties of tubulin (Raybin and Flavin, 1977b; Arce et al., 1978; Kumar and Flavin, 1982), the fraction of tyrosinated tubulin was different in the assembled and unassembled pools (Rodriguez and Borisy, 1979). Individual microtubules in mammalian cells could be distinguished on the basis of the relative presence or absence of this tyrosine (Gundersen et al., 1984).

The currently accepted model for tyrosination/detyrosination involves four steps: [1] assembly of Tyr-tubulin into microtubules, [2] detyrosination of Tyr-microtubules, [3] eventual disassembly of Glu-microtubules (detyrosinated), and [4] tyrosination of Glu-tubulin (Gunderson et al., 1987, Webster et al., 1987). The fact that Tyr-tubulin is the primary species which forms new microtubules is supported by the known preference of the ligase for unassembled tubulin *in vitro* (Arce et al., 1978) and the observation of very low amounts of Glu-tubulin in the unassembled pool (Gunderson et al., 1987). Step [2] is carried out by the carboxypeptidase, that has a preference for polymerized tubulin as a substrate (Kumar and Flavin, 1981; Arce and Barra, 1985). Support for this step also comes from experiments with agents that prevent tubulin depolymerization (Gunderson et al., 1987), that lead to an

accumulation of Glu-tubulin in microtubles. The efficiency of step [4] has been demonstrated by the drug-induced depolymerization of microtubules and the demonstration that >98% of the tubulin was present in the tyrosinated form (Gunderson et al., 1987).

The morphology of Glu-microtubules in interphase animal cells is substantially different from the majority Tyr-microtubules (Gunderson et al., 1984). Tyr-microtubules originate from a central area (MTOC), fill the whole cell, and tend to be fairly straight. In contrast, the Glu-microtubules are sinuous and restricted to the central part of the cell. This arrangement of Glu-microtubules is similar to that observed for acetylated microtubules (Piperno et al., 1987) and for a subset of stable microtubules, identified because of slow exchange rates with injected tubulin (Schulze and Kirschner, 1987). In spite of the similarity of appearance, the extent of overlap between these three sets of microtubules is unclear. Even if the three subsets are the same, it is not clear whether increased stability causes detyrosination and acetylation or vice versa. It is clear that these microtubules are biochemically and morphologically different and may be used for some specialized function, but the identity of this function remains obscure.

The Role of Other Proteins:

Due to the limited amount of variation in tubulins and the wide variety of structures of which microtubules are a part, investigators have focused much attention on other proteins in the cell that may have a role in regulating microtubule structure and function. Even "simple" microtubule structures, such as cilia and flagella, contain more than a hundred non-tubulin proteins (Lefebvre and Rosenbaum, 1986). The most notable protein identified by early experiments on cilia and flagella is dynein, an ATPase which is responsible for force generation during the beating of flagella and cilia.

One of the major criteria used in attempts to identify microtubule associated proteins (MAPs) has been the ability to bind to microtubules *in vitro* (for review see Olmsted, 1986). A variety of proteins have been identified that assemble with microtubules to a constant

specific activity through multiple cycles of polymerization and depolymerization. Although this assay is prone to artifactual binding by nonspecific "sticky" proteins, it has been successfully used to identify several proteins that probably have some role in microtubule function. A number of these proteins promote microtubule assembly *in vitro* and are associated with microtubular structures *in vivo* (Olmsted, 1986). Although many of these proteins have suggestive patterns of expression in the tissues of higher organisms and in differentiating cells, information about their *in vivo* function is scarce. One exception is a set of proteins called tau, which stabilized microtubules when injected into cells (Drubin and Kirschner, 1986).

A more rigorous criterion has been used to identify MAPs from a number of cell types (Solomon et al., 1979; Duerr et al., 1981; Pallas and Solomon, 1982) and specific cell stages (Zieve and Solomon, 1982). This technique utilizes extraction of cells in buffers that stabilize microtubules followed by depolymerization of the stabilized tubules in order to solubilize associated proteins. A parallel extraction with cells that have previously been treated with drugs or cold to depolymerize microtubules, defines the background proteins that are not specific to the microtubular structures. A set of proteins identified by this technique, called chartins, colocalized with microtubules *in vivo* (Magendantz and Solomon, 1985). Pillus and Solomon (1986) used a combination of this technique and copolymerization to identify MAPs from yeast.

MAPs have also been identified by functional criteria. Recently, a protein named kinesin has been identified by its ability to bind to microtubules in the presence of a nonhydrolyzable ATP analogue and to generate microtubule based motile events. Kinesin has been purified from a variety of species and tissues. *In vitro*, it can move microtubules along glass slides and synthetic beads along microtubules. It appears to have a role in fast axonal transport of vesicles towards the plus end of microtubules (for review see Vale et al., 1986). Kinesin has also been observed in mitotic spindles of dividing sea urchin eggs (Scholey et al., 1985), but its role in mitosis is unclear.

Genetic Analysis of Microtubular Systems:

The frustrations inherent in correlating biochemical and structural information with function have led a number of investigators to perform genetic analysis of microtubules. These studies represent an effort to generate more conclusive information about the functions of tubulins and MAPs *in vivo* (for review see Raff, 1984; Cleveland and Sullivan, 1985). In *Aspergillus nidulans*, mutations resulting in resistance to the anti-mitotic drug benomyl mapped to a β-tubulin gene (Sheir-Neiss et al., 1978). Reversion analysis of temperature-sensitive drug resistant mutations in this gene allowed identification of a gene for α-tubulin (Morris et al., 1979). Morris' group has shown that *Aspergillus* has two α- and two β-tubulin genes and has begun to analyze the relative functions of these genes (Weatherbee et al., 1985; May et al., 1985, see above). They have identified mutants defective in microtubule assembly that are defective in mitosis and nuclear migration. In addition, they have isolated mutants defective in microtubule disassembly which are defective in these processes (Oakley and Morris, 1981; Gambino et al., 1984). Mutations resulting in resistance to drugs related to benomyl (called benzimidazoles) have also been isolated in several tubulin genes of the slime mold *Physarum polycephalum* (Burland et al., 1984; Schedl et al., 1984).

The type of reversion analysis used by Morris et al. (1979) to identify a gene for α-tubulin by starting with a β-tubulin mutant had been previously used to study structural proteins important for bacteriophage morphogenesis (Jarvik and Botstein, 1975). It relies on suppression of mutations in genes encoding structural proteins by mutations in genes coding for proteins that physically interact with the original defective protein. It is one of the major genetic techniques used for identifying unknown components of complex structures such as microtubular and actin microfilament arrays (Huffaker et al., 1988a).

Drosophila melanogaster is the most complex organism whose tubulins have been analyzed genetically. These studies have been informative concerning the function of tubulin genes

expressed in specialized tissues and in introducing novel methods for identifying genes whose products interact with tubulin *in vivo* (for review see Raff, 1984). The β-2-tubulin gene was originally identified by the isolation of dominant male sterile mutants (Kemphues et al., 1979). Since this gene is only expressed in testes, the one tissue in *Drosophila* where flagellar axonemes are formed, these investigators speculated that the β-2 gene product might be specialized to construct flagella. Subsequent study of recessive alleles of this gene, however, have shown that it plays a role in a variety of microtubular processes during spermatogenesis. These include meiosis, shaping of the nucleus by cytoplasmic microtubules, and the construction of sperm flagellar axonemes (Kemphues et al., 1982). Mutations in more widely expressed tubulin genes in *Drosophila* have a variety of defects (Matthews and Kaufman, 1987). One of the most interesting results to arise from the *Drosophila* work was the isolation of recessive mutations that fail to complement recessive β-2 mutations, but map to distinct, nontubulin genes (Raff and Fuller, 1984; Fuller, 1986). This method is likely to lead to the identification of genes that code for proteins involved in the regulation of microtubular structure and function.

Another genetic approach to microtubule function in *Drosophila* has been the direct isolation of genes that code for microtubule associated proteins. Goldstein et al. (1986) isolated a gene for a 205 kD *Drosophila* MAP from an expression library and mapped the single copy gene to a region of a chromosome.

A second major genetic approach to the study of microtubules is the direct isolation of mutants defective in the function of specific microtubular structures. Studies of *Chlamydomonas* mutants defective in flagellar structure and/or motility have identified proteins that form various substructures and perform specific functions (for review see Luck, 1984). These experiments have also revealed various regulatory interactions that control flagellar function.

In the nematode *Caenorhabditis elegans*, a distinctive microtubular structure in a set of touch receptor neurons has been studied genetically. Normally, *C. elegans* microtubules have

11 protofilaments. In the touch receptors, however, the microtubules have 15 protofilaments and are packed into rigid hexagonal arrays (Chalfie and Thomson, 1982). Mutations in the mec-7 gene result in loss of touch sensitivity and the replacement of this set of 15 protofilament microtubules with 11 protofilament microtubules (Chalfie and Sulston, 1981; Chalfie and Thomson, 1982). Thus, the mec-7 gene product is involved in organizing a microtubular array whose function may rely on a specific microtubule structure.

Finally, extensive genetic studies of microtubule function have been carried out in the two distantly related yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These studies will be described below in the section on yeast microtubules.

Part 2: Mitosis:

Mitosis is the process by which dividing eukaryotic cells separate their genetic material. Although the morphology of the mitotic apparatus varies widely in divergent eukaryotes, microtubules are always a major structural component (for reviews see Dustin, 1984; Inoue, 1981; Pickett-Heaps et al., 1982). Mitosis is generally characterized by two poles. In animal cells, these poles are defined by centrosomes; in fungi and many other lower eukaryotes the poles are specific structures embedded in the nuclear envelope. Animal cells have what is called an "open" mitosis because the nuclear membrane breaks down and then reforms after chromosome separation. The "closed" mitosis of fungi like yeast is characterized by the persistence of the nuclear membrane throughout the cell cycle.

Description of Mitosis:

Despite wide variation in the morphology of mitosis in eukaryotes, several structural features are widely conserved. Although the existence of all of the features described below has not been confirmed in all organisms, I will assume that they are universal for the purposes of this discussion. In most cells, microtubules of the mitotic spindle form three distinct

structures: [1] Some microtubules connect the poles with specific structures, called kinetochores, located at the centromeres of the chromosomes. [2] Pole-to-pole microtubules originate at each pole and form a region of overlap in the center of the mitotic spindle. [3] The third set of microtubules emerges from the pole and projects away from the center of the cell towards the periphery. In cells with closed mitoses, the third set of microtubules are the only ones in the cytoplasm. All of the microtubules in the spindle appear to have the same polarity, with their plus ends distal from the pole (Haimo et al., 1979; Tippit et al., 1980; Euteneuer and McIntosh, 1981; Euteneuer et al., 1982). Once the spindle has formed, two kinds of motion occur, called anaphase A and anaphase B. Anaphase A consists of motion of chromosomes toward the poles along shortening kinetochore microtubules. Anaphase B consists of the motion of the poles away from each other while the pole-to-pole microtubules lengthen.

Mechanism of Mitosis:

In spite of an enormous amount of work on mitosis, the molecular basis of anaphase A and B movements remains obscure. Many models have been proposed, but strong evidence for any of them is lacking (for reviews see Dustin, 1984; Inoue, 1981; Pickett-Heaps et al., 1982). I will briefly discuss a few relevant experiments, making no effort at a complete review.

Anaphase B:

One question that has been addressed by several recent experiments concerns the sites of microtubule assembly and disassembly during anaphase movements. Using an *in vitro* system from diatoms, Masuda and Cande (1987) showed that tubulin could add to the plus ends of the pole-to-pole microtubules in the overlap zone. In the absence of this additional polymerization, ATP-dependent spindle elongation was limited to the length of the overlap zone. In the presence of tubulin, however, elongation was several times the length of the

original overlap zone. Experiments with labeled tubulin showed that it initially added to regions flanking this zone; as the spindle elongated (anaphase B), these two labeled zones moved together. These results suggest that anaphase B movements involve antiparallel sliding of the interdigitated midzone microtubules which grow at their plus ends distal to the poles. The results from this *in vitro* system are supported by *in vivo* experiments using microinjection of labeled tubulin and photobleaching techniques (Saxton and McIntosh, 1987). It is unclear whether the force for anaphase B is generated in this midzone region because the destruction of spindle microtubules with a UV microbeam has been observed to increase the rate of anaphase B (Aist and Berns, 1981).

Kinetochores:

Mitchison and Kirschner (1985a, 1985b) have studied the properties of kinetochores *in vitro* to determine how kinetochore microtubules assemble and act. They found that kinetochores can nucleate microtubules, but with mixed polarity. Previous results with cells recovering from treatment with depolymerizing drugs also showed that kinetochores can nucleate microtubule assembly *in vitro*, but in these experiments the microtubules were of the wrong polarity (that is plus end distal to the kinetochore, Bergen et al., 1980). When Mitchison and Kirschner (1985b) mixed kinetochores with microtubule assemblies previously nucleated by isolated centrosomes, they found that the kinetochores could capture the microtubule plus ends and stabilize them to depolymerization. They suggested that this observation, in conjunction with dynamic instability of microtubule growth, could explain formation of kinetochore microtubules *in vivo:* During the initial stages of mitosis, microtubules are continuously polymerizing from the centrosomes and then depolymerizing. Microtubules whose plus ends happen to bind to a kinetochore are selectively stabilized, leading to the correct polarity observed *in vivo*. The ability of kinetochores to nucleate microtubules under special conditions was explained as an artifact of their observed affinity

for tubulin.

Along with the ability to capture microtubule ends, the kinetochores studied by Mitchison and Kirschner (1985b) also had the ability to translocate along microtubules in an ATP-dependent fashion. When tubulin and ATP were added to kinetochore-microtubule complexes, the microtubules grew at the plus end attached to the kinetochore and the kinetochore moved with the growing plus end. If tubulin was added first and then ATP, the kinetochores could move along the preexisting microtubule lattice. Of course, the direction of this movement is opposite to that of kinetochore movement during anaphase A. During construction of the spindle, however, the chromosomes of animal cells move to the center of the spindle (the metaphase plate). Mitchison and Kirschner thus suggested that the ATPase may play a role in establishment of the metaphase plate through movement of chromosomes away from the pole.

Anaphase A:

The sites of microtubule disassembly during kinetochore fiber shortening in anaphase A have been investigated by two different methods. Mitchison et al. (1986) microinjected labeled tubulin into fibroblasts during metaphase and then investigated sites of incorporation at various times thereafter by electron microscopy. When the labeled tubulin was visualized at progressively later stages of metaphase, they observed increasing lengths of incorporation into individual kinetochore microtubules at the kinetochore ends. When labeled microtubules were examined in anaphase, 60% of the individual kinetochore fibers were unlabeled. Mitchison et al. interpreted these results as evidence of a slow poleward flux of tubulin subunits in kinetochore microtubules at metaphase, though it is also possible that this is a result of random chromosome oscillation around the metaphase plate. The incorporation at metaphase is followed by depolymerization at kinetochore ends during anaphase, presumably as the kinetochore lives up to its name and walks down the shrinking microtubules.

Gorbsky et al. (1987) also reached the conclusion that depolymerization takes place at

kinetochore ends during anaphase A. They microinjected fluoresceinated tubulin into cells before metaphase to allow uniform labeling of the spindle. When the cells entered anaphase, a band of labeled tubulin was bleached at varying distances from the chromosomes. Later in anaphase, the cells were fixed and examined with an antibody specific for unbleached fluorescein. In all cases, they observed that the chromosomes invaded the bleached region as they moved towards the pole. The problem with this experiment, however, is that it is difficult to distinguish kinetochore microtubules from pole-to-pole microtubules using light microscopy. Further studies will be required to prove that kinetochore microtubules depolymerize only at their plus (kinetochore) ends and not at the poles.

Part 3: Microtubule Function in Yeast:

This thesis is concerned with microtubule function in the budding yeast *Saccharomyces cerevisiae*. The study of cytoskeletal elements in yeast has been a growing field in recent years for a number of reasons. Yeast are simple, single-celled eukaryotes which contain a number of the proteins, like actin and tubulin, that have been studied for many years in other eukaryotes (see Huffaker et al., 1988a). Their cell cycle has been well-studied morphologically and genetically (see Byers, 1981; Pringle and Hartwell, 1981). Most importantly, classical and molecular genetic techniques have been developed that allow the detailed study of protein function unsurpassed in any other eukaryotic organism (Botstein and Davis, 1982). It is the power of the combined approaches of morphological analysis, biochemistry, and genetics which holds great promise for giving us a detailed understanding of the function of structural elements of the eukaryotic cell.

Microtubule Structures in Yeast:

Microtubules are one of the best understood structures with known, specific functions in the progression of the yeast cell cycle. Early microscopic studies revealed their presence in a relatively simple, "closed" intranuclear mitotic spindle (Matile et al., 1969). They are also integral parts of the meiotic spindle during sporulation of diploid yeast (Moens and Rapport, 1971). These spindles radiate from special structures, called spindle pole bodies (SPB's), embedded in the nuclear envelope (Byers and Goetsch, 1975; Moens and Rapport, 1971). Microtubules also radiate into the cytoplasm from the SPB. These cytoplasmic microtubules stretch toward the developing bud during mitotic growth and stretch between nuclei in the process of fusion during mating of haploid yeast (Byers and Goetsch, 1975; Kilmartin and Adams, 1984; Adams and Pringle, 1984). The central role of the SPB in organizing microtubules in yeast is generally accepted because all microtubules in wild-type yeast are thought to have one end associated with it (Byers and Goetsch, 1975; Peterson and Ris, 1976). SPB's can also nucleate microtubule assembly *in vitro* (Byers et al., 1978, Hyams and Borisy, 1978).

A Description of the Yeast Cell Cycle:

Microtubules persist throughout the mitotic cycle of yeast and undergo a characteristic series of morphological changes which have led to hypotheses (both correct and incorrect) about their function in growth. These observations have been made by electron microscopy (Byers and Goetsch, 1975; Peterson and Ris, 1976; King et al., 1982) and by indirect immunofluorescence light microscopy (Kilmartin and Adams, 1984; Adams and Pringle, 1984).

The start of the yeast cell cycle is defined as the stage with an unbudded cell with a single nucleus containing unreplicated DNA (for review see Pringle and Hartwell, 1981). At this stage, the SPB is a single densely stained disc embedded in the nuclear membrane with an adjacent densely stained portion called the half-bridge. Microtubules radiate from the SPB

into both the nucleus and the cytoplasm. As the cycle progresses, the SPB develops an amorphous "satellite" of dense material on the other side of the half-bridge. At the time of bud emergence, a double SPB forms, which consists of two single SPB's that share a bridge. Byers and Goetsch (1975) found that this double SPB was absent from unbudded cells and present in all budded cells examined. The cytoplasmic microtubules that radiate from the double SPB are preferentially oriented towards the emerging bud. The double SPB persists for about 30% of the budded portion of the cycle, coincident with the period of DNA replication (Byers and Goetsch, 1975).

At about the time of the completion of DNA replication, the double SPB splits into two single ones which move to opposite sides of the nucleus as the spindle forms between them. No intermediates in this process have been observed, so it must occur very rapidly. By this time, the nucleus has moved into the bud neck. The spindle at this stage is about 1 μm lo. ₃ and contains microtubules analogous to the three types typical of eukaryotic spindles (see above). The pole-to-pole or "continuous" microtubules emerge from either SPB and run straight towards the other pole. The "lateral" microtubules are shorter and splay out from the axis of the spindle. Since chromosomes do not condense in Saccharomyces, it is not clear if these microtubules correspond to the kinetochore microtubules in higher eukaryotes, though they sometimes appear to associate with chromatin fibers (Peterson and Ris, 1976). The number of intranuclear microtubules that issue from each SPB is slightly more than the number of chromosomes in yeast, which implies that there is only one per centromere/kinetochore plus only a few continuous pole-to-pole ones (Peterson and Ris, 1976). The cytoplasmic microtubules at this stage run from both SPB's and stretch towards the far ends of the mother and bud.

The spindle elongates with movements corresponding to anaphase B and A; the continuous microtubules grow until the spindle reaches a maximum length of 6 to 8 μm and the lateral microtubules shorten as the replicated chromosomes are separated into two distinct masses which move to opposite ends of the cell (Peterson and Ris, 1976). Finally, the

spindle breaks down from the middle and cytokinesis occurs. Thus, the movements of yeast mitosis are analogous to those in other eukaryotes. It is unclear if the sites of microtubule depolymerization in the yeast spindle correspond to those described earlier for higher cells. King et al. (1982) have even reported that only a single microtubule exists in the midzone of the yeast spindle during the later stages of elongation. Although this result could be due to microtubule instability during sample preparation, it would, if correct, challenge simple models of antipolar sliding of microtubules during anaphase B.

Functional Analysis of Microtubules in Yeast:

The role of microtubules in separation of chromosomes on mitotic and meiotic spindles and in nuclear movement during mitosis and mating has been confirmed using specific drugs and conditional lethal tubulin mutants. The drugs that have been used are of the benzimidazole class of compounds and include benomyl, methyl-benzimidazol-2-yl carbamate (MBC) and nocodazole (for structures see Davidse and Flach, 1977). Several lines of evidence suggest that these drugs specifically inhibit tubulin function. In *Aspergillus nidulans*, MBC has been shown to bind to tubulin purified from drug sensitive strains and not to that of drug resistant strains (Davidse and Flach, 1977). In addition, the *in vitro* assembly of purified yeast tubulin is inhibited by MBC (Kilmartin, 1981). The most convincing evidence, however, comes from mutations that result in resistance to very high levels of benomyl. These mutations are in the single β–tubulin gene of yeast (Thomas et al., 1985) and in one of the β–tubulin genes of *Aspergillus* (Sheir-Neiss et al., 1978).

The drugs described above inhibit the mitotic cell cycle of yeast subsequent to DNA replication and before nuclear division; the cells arrest with single large buds, though metabolism and cell growth continue (Quinlan et al., 1980; Wood and Hartwell, 1982). Treatment with MBC causes a high frequency of chromosome loss events in diploid cells (Wood, 1982). MBC also causes a high rate of failure of nuclear fusion, but not cell fusion, during mating of haploid yeast (Delgado and Conde, 1984). Nocodazole causes the

depolymerization of microtubules in yeast along with a mitotic block similar to that caused by MBC (Pringle et al., 1986). In addition, migration of the nucleus of growing yeast to the bud neck is inhibited by nocodazole (Pringle et al., 1986), consistent with previous results in *Aspergillus* (Oakley and Morris, 1980).

Experiments with conditional lethal tubulin mutants have confirmed the role of microtubules in nuclear migration and chromosome separation. The yeast *Saccharomyces cerevisiae* has a single β-tubulin gene, named TUB2, which is essential for growth (Neff et al., 1983). As mentioned above, mutations that result in resistance to high levels of benomyl occur exclusively in this gene (Thomas et al., 1985). Among a large set of benomyl resistant mutants, Thomas et al. (1985) found several that were also temperature-sensitive or cold-sensitive for growth. At the nonpermissive temperature, these mutants have a terminal arrest phenotype characteristic of a failure in mitosis, are defective in nuclear fusion during mating and are defective for meiosis during sporulation (Thomas, 1984). Additional alleles of TUB2 have been isolated by *in vitro* mutagenesis of the cloned gene (Huffaker et al., 1988b). Experiments with these mutants have shown a correlation of the loss of cytoplasmic microtubules at the nonpermissive temperature with a loss of nuclear migration to the bud neck (Huffaker et al., 1988b).

Functions Independent of Tubulin:

One of the more interesting aspects of the study of microtubules in yeast is the discovery of a number of processes in which they apparently have no role. Early electron microscopic observations that cytoplasmic microtubules extend towards or into the bud caused speculation that they were responsible for bud site selection and transport of vesicles into the bud (Byers and Goetsch, 1975). This hypothesis is consistent with the inhibition of secretion by antimicrotubule drugs which has been observed in many higher cells (Dustin, 1984). Two kinds of experimental data, however, argue against this hypothesis. First, nocodazole treated cells, which have no microtubules, arrest growth at the large budded stage. Even small

unbudded cells (isolated by differential centrifugation) produce large buds in the absence of detectable microtubules (Pringle et al., 1986). Second, conditional lethal tubulin mutants that rapidly lose all microtubules at the nonpermissive temperature arrest with large buds (Huffaker et al., 1988b; Chapter 5). Thus, intact microtubules are not essential for bud emergence and growth. Microtubules are also not required for secretion of the external enzyme invertase (Huffaker et al., 1988b).

Analysis of Microtubules in Fission Yeast:

The yeast *Schizosaccharomyces pombe* is related only very distantly to *Saccharomyces cerevisiae*, but the parallel study of tubulin in both has revealed many similarities. *S. pombe* has three large chromosomes which can be visualized in light microscopy under conditions of mitotic arrest produced by benzimidazole compounds or tubulin mutants (Umesono et al., 1983a; Hiraoka et al., 1984). This ability to examine chromosomes has allowed the production of many beautiful images of mitosis under various normal and disruptive conditions (Toda et al., 1981; Hiraoka et al., 1984; Uemura et al., 1987). Tubulin mutants of *S. pombe* have been isolated by direct screening for cold-sensitive nuclear division arrest (Toda et al., 1983) or by altered sensitivity to benzimidazole compounds (Yamamoto et al., 1980; Roy and Fantes, 1983; Umesono et al., 1983b). Two α-tubulin genes and one β-tubulin gene have been isolated and sequenced (Toda et al., 1984; Hiraoka et al., 1984). As is the case in *Saccharomyces cerevisiae*, these tubulin mutants show characteristic defects in mitotic and meiotic spindles and in nuclear migration (Toda et al., 1983; Umesono et al., 1983a,b; Toda et al., 1984; Hiraoka et al., 1984). The relative functions of the two α-tubulin genes have been explored genetically (Adachi et al., 1986; for a more complete discussion see Chapter 3).

Other Components of the Spindle:

Genes that code for proteins likely to be components of the *Saccharomyces cerevisiae*

spindle have been isolated by a number of methods (for review see Huffaker et al., 1988a).

Since tubulin is the only well-characterized component of spindles, most of these methods

have relied on finding mutants with phenotypes expected for defects in spindle function. The

NDC1 gene was originally identified using a cold-sensitive allele, ndc1-1, which causes a

weak cell cycle arrest at the large budded stage (Thomas and Botstein, 1986). At

nonpermissive temperature, ndc1-1 causes a failure of chromosome separation such that one

of the progeny receives a diploid chromosome complement and the other receives no nuclear

DNA. Despite the lack of chromosome separation, the SPB's are segregated to the two

progeny. Thus NDC1 may encode a component of the yeast mitotic apparatus necessary for

attachment of chromosomes to the SPB (Thomas and Botstein, 1986).

Several genes have been identified that may be functional components of the SPB (see

Baum et al., 1986b). The KAR1 gene was originally identified by mutations that affected

only the efficiency of nuclear fusion (karyogamy) during yeast mating (Conde and Fink,

1976). Subsequent construction of temperature-sensitive and null alleles has shown that

KAR1 is essential for mitotic growth (Rose and Fink, 1987). Both the temperature-sensitive

mutants and mutants conditional for overproduction of the KAR1 protein cause a conditional

cell cycle arrest as large budded cells with a single nucleus in the neck and an unduplicated

SPB. Aberrantly long extranuclear microtubules are produced by the temperature-sensitive

mutants at the nonpermissive temperature and by the original allele during mating. The

observation that KAR1-lacZ protein fusions localize to the SPB suggests that the KAR1 gene

product may be a part of the SPB (M. Rose, personal communication).

The cdc31-1 mutation causes temperature-sensitive cell cycle arrest as large budded cells

with a single nucleus (Pringle and Hartwell, 1981). The SPB fails to duplicate, but doubles

in size, leading to a doubling of the chromosome complement of transiently arrested cells

(Schild et al., 1981). The sequence of CDC31 shows similarity to that of several known

calcium binding proteins, suggesting that the CDC31 protein may regulate SPB duplication in response to calcium fluxes (Baum et al., 1986a). The SPA1 gene was identified because it encoded an antigen recognized by a human serum that reacts with mammalian spindle poles (Snyder and Davis, 1986). The 59 kD protein product of SPA1 copurifies with nuclei and is overproduced by a mutant that overproduces spindle pole bodies (Baum et al., 1986b). SPA1 is not essential for growth, but null alleles are slightly temperature-sensitive. At permissive temperature, null alleles result in a higher frequency of chromosome loss, a karyogamy defect, and abnormal numbers of nuclei in many cells, consistent with a role in SPB function (Snyder and Davis, 1986).

Genes that affect spindle function have also been identified by directly screening for mutants that lose chromosomes. The assays used have depended on colored sectoring of colonies that lose marked chromosomes at an elevated rate (Heiter et al., 1985; Koshland et al., 1985; Meeks-Wagner and Hartwell, 1986; Meeks-Wagner et al., 1986; M.A. Hoyt, personal communication). Many of these mutants are also hypersensitive to benomyl. One example is the CIN1 gene, originally identified both by chromosome loss screening and by direct screening for benomyl hyper-sensitivity (M.A. Hoyt, T. Stearns, and D. Botstein, personal communication). CIN1 null strains are viable, but benomyl hyper-sensitive and slightly cold-sensitive. They have reduced amounts of microtubules in the cold, have a weak karyogamy defect, and show lethality when they are crossed to make double mutants with certain tubulin alleles. Thus, the CIN1 gene product may function to stabilize microtubules by some mechanism.

As was mentioned above, Pillus and Solomon (1986) have identified several microtubule-associated proteins from yeast using a fractionation procedure followed by copolymerization with mammalian microtubules. Analysis of these proteins will have to await purification of reasonable amounts of proteins or the identification of the genes that encode them.

The challenge remains to integrate all of these suggestive results into a detailed molecular mechanism of the yeast spindle. In addition to genetic analysis, the problem will have to be examined through improved techniques for localization of gene products and improved biochemical analysis of the functions of interesting proteins.

This thesis describes the characterization and mutational analysis of two α-tubulin genes in yeast. Chapters 2 and 3 describe the isolation of these genes, the demonstration of their presence in y st microtubules, and analysis of null mutations. Chapter 4 describes a brief foray into functional analysis of one region of one of the molecules. Finally, Chapter 5 presents the isolation and characterization of a large set of conditional lethal α-tubulin mutations, that I hope will be used in the future to help answer more of the mechanistic questions about microtubules in yeast.

References:

Adachi, Y., T. Toda, O. Niwa, and M. Yanagida. 1986. Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol. Cell. Biol. 6: 2168-2178.

Adams, A.E.M., and J.R. Pringle. 1984. Localization of actin and tubulin in wild-type and morphogenetic mutants of *Saccharomyces cerevisiae*. J. Cell Biol. 98: 934-945.

Aist, J.R., and M.W. Berns. 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.

Arce, C.A., and H.S. Barra. 1985. Release of C-terminal tyrosine from tubulin and microtubules at steady state. Biochem. J. 226: 311-317.

Arce, C.A., M.E. Hallak, J.A. Rodriguez, H.S. Barra, and R. Caputto. 1978. Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino acids on tubulin assembly. J. Neurochem. 31: 205-210.

Argarana, C.E., H.S. Barra, and R. Caputto. 1978. Release of [^{14}C] tyrosine from tubulinyl-[^{14}C] tyrosine by brain extract. Separation of a carboxy peptidase from tubulin tyrosine ligase. Mol. Cell Biochem. 19: 17-22.

Aria, T., and Y. Kaziro. 1976. Effect of guanine nucleotides on the assembly of brain microtubules: ability of 5'-guanlyl imidodiphosphate to replace GTP in promoting the

polymerization of microtubules *in vitro*. Biochem. Biophys. Res. Commun. 69: 369-376.

Asakura, S. 1968. A kinetic study of the *in vitro* polymerization of flagellin. J. Mol. Biol. 35: 237-239.

Barra, H.S., C.A. Arce, J.A. Rodriguez, and R. Caputto. 1973. Incorporation of phenylalanine as a single unit into rat brain protein: reciprocal inhibition by phenylalanine and tyrosine of their respective incorporations. J. Neurochem. 21: 1241-1251.

Barra, H.S., C.A. Arce, J.A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins. Biochem. Biophys. Res. Commun. 60: 1384-1390.

Barra, H.S., J.A. Rodriguez, C.A. Arce, and R. Caputto. 1973. A soluble preparation from rat brain that incorporates into its own proteins [^{14}C] arginine by a ribonuclease-sensitive system and [^{14}C] tyrosine by a ribonuclease-insensitive system. J. Neurochem. 20: 97-108.

Baum, P., C. Furlong, and B. Byers. 1986a. Yeast gene required for spindle pole body duplication: homology of its product with Ca^{2+}-binding proteins. Proc. Natl. Acad. Sci. USA 83: 5512-5516.

Baum, P., L. Goetsch, and B. Byers. 1986b. Genetics of spindle pole body regulation. p. 155-158., in Yeast Cell Biology, J. Hicks, ed., Alan R. Liss, Inc., NY.

Bergen, L.G., R. Kuriyama, and G.G. Borisy. 1980. Polarity of microtubules nucleated by centrosomes and chromosomes of Chinese hamster ovary cells *in vitro*. J. Cell Biol. 84: 151-159.

Bond, J.F., J.L. Fridovich-Keil, L. Pillus, R.C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules *in vivo*. Cell 44: 461-468.

Borisy, G.G., and E.W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. J. Cell Biol. 34: 525-534.

Botstein, D., and R.W. Davis. 1982. Principles and practice of recombinant DNA research with yeast. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 607-638.

Burland, T.G., T. Schedl, K. Gull, and W.F. Dove. 1984. Genetic analysis of resistance to benzimidazoles in *Physarum:* differential expression of β-tubulin genes. Genetics 108: 123-141.

Byers, B. 1981. Cytology of the yeast life cycle. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 59-96.

Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. J. Bacteriol. 124: 511-523.

Byers, B., K. Shriver, and L. Goetsch. 1978. The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in *Saccharomyces cerevisiae*. J. Cell Sci. 30: 331-352.

Cambray-Deakin, M.A., and R.D. Burgoyne. 1987. Posttranslational modifications of α-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. J. Cell Biol. 104: 1569-1574.

Carlier, M.F., and D. Pantaloni. 1981. Kinetic analysis of guanosine 5'-triphosphate hydrolysis associated with tubulin polymerization. Biochemistry 20: 1918-1924.

Chalfie, M., and J. Sulston. 1981. Developmental genetics of the mechanosensory neurons of *Caenorhabditis elegans*. Dev. Biol. 82: 358-370.

Chalfie, M., and J.N. Thomson. 1982. Structural and functional diversity in the neuronal microtubules of *Caenorhabditis elegans*. J. Cell Biol. 93: 15-23.

Cleveland, D.W., and K.F. Sullivan. 1985. Molecular biology and genetics of tubulin. Ann. Rev. Biochem. 54: 331-365.

Conde, J., and G.R. Fink. 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA 73: 3651-3655.

Davidse, L., and W. Flach. 1977. Differential binding of methyl-benzimidazol-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in strains of *Aspergillus nidulans*. J. Cell. Biol. 72: 174-193.

Delgado, M.A., and J. Conde. 1984. Benomyl prevents nuclear fusion in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 193: 188-89.

Diggins, M.A., and W.F. Dove. 1987. Distribution of acetylated α-tubulin in *Physarum*

polycephalum. J. Cell Biol. 104: 303-309.

Drubin, D.G., and M.W. Kirschner. 1986. Tau protein function in living cells. J. Cell Biol. 103: 2739-2746.

Duerr, A., D. Pallas, and F. Solomon. 1981. Molecular analysis of of cytoplasmic microtubules in situ: Identification of both widespread and specific proteins. Cell 24: 203-211

Dustin, P. 1984. Microtubules. Springer-Verlag, New York.

Euteneuer, U., W.T. Jackson, and J.R. McIntosh. 1982. Polarity of spindle microtubules in *Haemanthus* endosperm. J. Cell Biol. 94: 644-653.

Euteneuer, U., and J.R. McIntosh. 1981. Structural polarity of kinetochore microtubules in PtK1 cells. J. Cell Biol. 89: 338-345.

Farrell, K.W., M.A. Jordan, H.P. Miller, and L. Wilson. 1987. Phase dynamics at microtubule ends: the coexistence of microtubule length changes and treadmilling. J. Cell Biol. 104: 1035-1046.

Fuller, M.T. 1986. Genetic analysis of spermatogenesis in *Drosophila:* the role of the testis-specific β-tubulin and interacting genes in cellular morphogenesis. p. 19-41. In: Gametogenesis and the early embryo. 44th Symposium of the Society for Developmental Biology. Ed. J. Gall. Alan R. Liss, Inc., New York, NY.

Fulton, C., and P.A. Simpson. 1976. Selective synthesis and utilization of flagellar tubulin.

In The Multi-Tubulin Hypothesis in Cell Motility, R. Goldman, I. Pollard, and J. Rosenbaum, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., p. 987-1005.

Gambino, J., L.G. Bergen, and N.R. Morris. 1984. Effects of mitotic and tubulin mutations on microtubule architecture in actively growing protoplasts of *Aspergillus nidulans*. J. Cell Biol. 99: 830-838.

Gard, D.L., and M.W. Kirschner. 1985. A polymer-dependent increase in phosphorylation of β-tubulin accompanies differentiation of a mouse neuroblastoma cell line. J. Cell Biol. 100: 764-774.

Goldstein, L.S.B., R.A. Laymon, and J.R. McIntosh. 1986. A microtubule-associated protein in *Drosophila melanogaster:* identification, characterization, and isolation of coding sequences. J. Cell Biol. 102: 2076-2087.

Gorbsky, G.J., P.J. Sammak, and G.G. Borisy. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. J. Cell Biol. 104: 9-18.

Gundersen, G.G., M.H. Kalnoski, and J.C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and nontyrosinated α-tubulin are distributed differently *in vivo*. Cell 38: 779-789.

Gundersen, G.G., S. Khawaja, and J.C. Bulinski. 1987. Postpolymerization detyrosination of α-tubulin: a mechanism for subcellular differentiation of microtubules. J. Cell Biol. 105: 251-264.

Haimo, L.T., B.R. Telzer, and J.L. Rosenbaum. 1979. Dynein binds to and crossbridges cytoplasmic microtubules. Proc. Natl. Acad. Sci. USA 76: 5759-5763.

Heiter, P., C. Mann, M. Snyder, and R.W. Davis. 1985. Mitotic stability of yeast chromosomes: A colony color assay that measures nondisjunction and chromosome loss. Cell 40: 381-392.

Hill, T.L., and Y.-D. Chen. 1984. Phase changes at the end of a microtubule with a GTP cap. Proc. Natl. Acad. Sci. USA 81: 5772-5776.

Hiraoka, Y., T. Toda, and M. Yanagida. 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.

Horio, T., and H. Hotani. 1986. Visualization of the dynamic instability of individual microtubules by dark-field microscopy. Nature 321: 605-607.

Huffaker, T.C., M.A. Hoyt, and D. Botstein. 1988a. Genetic analysis of the cytoskeleton of yeast. Ann. Rev. Genet. (in press).

Huffaker, T.C., J.H. Thomas, and D. Botstein. 1988b. Diverse effects of β-tubulin mutations on microtubule assembly and function J. Cell Biol. (submitted).

Hyams, J.S., and G.G. Borisy. 1978. Nucleation of microtubules in vitro by isolated spindle pole bodies of the yeast Saccharomyces cerevisiae. J. Cell Biol. 78: 401-414.

Inoue, S. 1981. Cell division and the mitotic spindle. J. Cell Biol. 91: 131s-147s.

Jarvik, J., and D. Botstein. 1975. Conditional lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. Proc. Natl. Acad. Sci. USA 72: 2738-2742.

Kemphues, K.J., T.C. Kaufman, R.A. Raff, E.C. Raff. 1982. The testis-specific β-tubulin subunit in Drosophila melanogaster has multiple functions in spermatogenesis. Cell 31: 655-670.

Kemphues, K.J., R.A. Raff, T.C. Kaufman, and E.C. Raff. 1979. Mutation in a structural gene for a β-tubulin specific to testis in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 76: 3991-3995.

Kilmartin, J. 1981. Purification of yeast tubulin by self-assembly in vitro. Biochemistry 20: 3629-3633.

Kilmartin, J., and A. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. J. Cell Biol. 98: 922-933.

King, S.M., and J.S. Hyams. 1982. The mitotic spindle of Saccharomyces cerevisiae: assembly, structure, and function. Micron 13: 93-117.

King, S.M., J.S. Hyams, and A. Luba. 1982. Absence of microtubule sliding and an analysis of spindle formation and elongation in isolated mitotic spindles from the yeast Saccharomyces cerevisiae. J. Cell Biol. 94: 341-349.

Kirschner, M.W. 1978. Microtubule assembly and nucleation. Int. Rev. Cytol. 54: 1-71.

Kirschner, M.W. 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers *in vivo*. J. Cell Biol. 86: 330-334.

Kirschner, M.W., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. Cell 45: 329-342.

Koshland, D., J.C. Kent, and L.H. Hartwell. 1985. Genetic analysis of the mitotic transmission of minichromosomes. Cell 40: 393-403.

Kristofferson, D., T. Mitchison, M.W. Kirschner. 1986. Direct observation of steady-state microtubule dynamics. J. Cell Biol. 102: 1007-1019.

Kumar, N., and M. Flavin. 1981. Preferential action of a brain detyrosinating carboxypeptidase on polymerized tubulin. J. Biol. Chem. 256: 7678-7686.

Kumar, N., and M. Flavin. 1982. Modulation of some parameters of assembly of microtubules *in vitro* by tyrosination of tubulin. Eur. J. Biochem. 128: 215-222.

LeDizet, M., and G. Piperno. 1986. Cytoplasmic microtubules containing acetylated α-tubulin in *Chlamydomonas reinhardtii*: spacial arrangement and properties. J. Cell Biol. 103: 13-22.

Lefebvre, P., and J.L. Rosenbaum. 1986. Ann. Rev. Cell Biol. 2: 517-46.

Lewis, S.A., W. Gu, and N.J. Cowan. 1987. Free intermingling of mammalian β-tubulin isotypes among functionally distinct microtubules. Cell 49: 539-548.

L'Hernault, S.W., and J.L. Rosenbaum. 1983. *Chlamydomonas* α-tubulin is posttranslationally modified by acetylation on the epsilon-amino group of a lysine. Biochemistry 24: 473-478.

Luck, D.J.L. 1984. Genetic and biochemical dissection of the eucaryotic flagellum. J. Cell Biol. 98: 789-794.

Luduena, R., L. Wilson, and E.M. Shooter. 1975. In Microtubules and Microtubule Inhibitors. M. Burgers and M. deBrabendes, eds., p. 47-58. North-Holland, Amsterdam.

Magendantz, M., and F. Solomon. 1985. Analyzing the components of microtubules: Antibodies against chartins, associated proteins from cultured cells. Proc. Natl. Acad. Sci. USA 82: 6581-6585.

Margolis, R.L., and L. Wilson. 1978. Opposite end assembly and disassembly of microtubules at steady state *in vitro*. Cell 13: 1-8.

Margolis, R.L., and L. Wilson. 1981. Microtubule treadmilling - possible molecular machinery. Nature 293: 705-711.

Masuda, H., and W.Z. Cande. 1987. The role of tubulin polymerization during spindle elongation *in vitro*. Cell 49: 193-202.

Matile, P., H. Moor, and C.F. Robinow. 1969. Yeast Cytology. p. 219-302. in The Yeasts, vol. I, A.H. Rose and J.S. Harrison., Academic Press, NY.

Matthews, K.A., and T.C. Kaufman. 1987. Developmental consequences of mutations in the 84B α-tubulin gene of *Drosophila melanogaster*. Dev. Biol. 119: 100-114.

May, G.S., J. Gambino, J.A. Weatherbee, N.R. Morris. 1985. Identification and functional analysis of β-tubulin genes by site specific integrative transformation in *Aspergillus nidulans*. J. Cell Biol. 101: 712-719.

Meeks-Wagner, D., and L.H. Hartwell. 1986. Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. Cell 44: 43-52.

Meeks-Wagner, D., J.S. Wood, B. Garvik, and L.H. Hartwell. 1986. Isolation of genes that affect mitotic chromosome transmission in *S. cerevisiae*. Cell 44: 53-63.

Mitchison, T., L. Evans, E. Schulze, and M.W. Kirschner. 1986. Sites of microtubule assembly and disassembly in the mitotic spindle. Cell 45: 515-527.

Mitchison, T., and M.W. Kirschner. 1984a. Microtubule assembly nucleated by isolated centrosomes. Nature 312: 232-237.

Mitchison, T., and M.W. Kirschner. 1984b. Dynamic instability of microtubule growth. Nature 312: 237-242.

Mitchison, T., and M.W. Kirschner. 1985a. Properties of the kinetochore *in vitro*. I. Microtubule nucleation and tubulin binding. J. Cell Biol. 101: 755-765.

Mitchison, T., and M.W. Kirschner. 1985b. Properties of the kinetochore *in vitro*. II. Microtubule capture and ATP-dependent translocation. J. Cell Biol. 101: 766-777.

Moens, P.B., and E. Rapport. 1971. Spindles, spindle plaques, and meiosis, in the yeast *Saccharomyces cerevisiae* (Hansen). J. Cell. Biol. 50: 344-361.

Morris, N.R., M.H. Lai, and C.E. Oakley. 1979. Identification of a gene for α-tubulin in *Aspergillus nidulans*. Cell 16: 437-442.

Murofushi, H. 1980. Purification and characterization of tubulin-tyrosine ligase from porcine brain. J. Biochem. 87: 979-984.

Neff, N., J.H. Thomas, P. Grisafi and D. Botstein. 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function in vivo. Cell 33: 211-219.

Oakley, B., and N.R. Morris. 1980. Nuclear movement is β-tubulin dependent in *Aspergillus nidulans*. Cell 19: 255-262.

O'Brien, E.T., W.A. Voter, and H.P. Erickson. 1987. GTP hydrolysis during microtubule assembly. Biochemistry 26: 4148-4156.

Olmsted, J.B. 1986. Microtubule-associated proteins. Ann. Rev. Cell Biol. 2: 421-457.

Pallas, D., and F. Solomon. 1982. Cytoplasmic microtubule-associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. Cell 30: 407-414.

Penningroth, S.M., D.W. Cleveland, and M.W. Kirschner. 1976. *In vitro* studies of the regulation of microtubule assembly. In Cell Motility, R. Goldman, T. Pollard, and J.

Rosenbaum, eds.,Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., p.1233-1257.

Penningroth, S.M., and M.W. Kirschner. 1977. Nucleotide binding and phosphorylation in microtubule assembly *in vitro*. J. Mol. Biol. 115: 643-673.

Peterson, J.B., and H. Ris. 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. J. Cell Sci. 22: 219-242.

Pickett-Heaps, J.D., D.H. Tippit, and K.R. Porter. Rethinking mitosis. Cell 29: 729-744.

Pillus, L., and F. Solomon. 1986. Components of microtubular structures in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 83: 2468-2472.

Piperno, G., and M.T. Fuller. 1985. Monoclonal antibodies specific for an acetylated form of α–tubulin recognize the antigen in cilia and flagella from a variety of organisms. J. Cell Biol. 101: 2085-2094.

Piperno, G., M. LeDizet, and X.-j. Chang. 1987. Microtubules containing acetylated α–tubulin in mammalian cells in culture. J. Cell Biol. 104: 289-302.

Pratt, L.F., S. Okamura, and D.W. Cleveland. 1987. A divergent testis-specific α–tubulin isotype that does not contain a coded C-terminal tyrosine. Mol. Cell. Biol. 7: 552-555.

Pringle, J. R., and L. H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 97-142.

Pringle, J.R., S.H. Lillie, A.E.M. Adams, C.W. Jacobs, B.K. Haarer, K.G. Coleman, J.S. Robinson, L. Bloom, and R.A. Preston. 1986. Cellular morphogenesis in the yeast cell cycle. In Yeast Cell Biology, J. Hicks, ed., Alan R. Liss, New York.

Quinlan, R.A., C.I. Pogson and K. Gull. 1980. The influence of the microtubule inhibitor, methyl benzimidazol-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. J. Cell Sci. 46: 341-352.

Raff, E.C. 1984. Genetics of microtubule systems. J. Cell Biol. 99: 1-10.

Raff, E.C., and M.T. Fuller. 1984. Genetic analysis of microtubule function in *Drosophila*. In Molecular Biology of the Cytoskeleton, Cold Spring Harbor Press, Cold Spring Harbor, NY., p. 293-304.

Raybin, D., and M. Flavin. 1977a. Enzyme which specifically adds tyrosine to the α–chain of tubulin. Biochemistry 16: 2189-2194.

Raybin, D., and M. Flavin. 1977b. Modification of tubulin by tyrosylation in cells and extracts and its effect on assembly *in vitro*. J. Cell Biol. 73: 492-504.

Rodriguez, J.A., and G.G. Borisy. 1979. Tyrosination state of free tubulin subunits and tubulin disassembled from microtubules of rat brain tissue. Biochem. Biophys. Res. Commun. 89: 893-899.

Rose, M.D., and G.R. Fink. 1987. KAR1, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell 48: 1047-1060.

Rothwell, S.W., W.A. Grasser, H.N. Baker, and D.B. Murphy. 1987. The relative contributions of polymer annealing and subunit exchange to microtubule dynamics *in vitro*. J. Cell Biol. 105: 863-874.

Roy, D., and P.A. Fantes. 1983. Benomyl-resistant mutants of *Schizosaccharomyces pombe* cold-sensitive for mitosis. Curr. Genet. 6: 195-202.

Salmon, E.D., R.J. Leslie, W.M. Saxton, M.L. Karow, and J.R. McIntosh. 1984. Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurement of fluorescence redistribution after laser photobleaching. J. Cell Biol. 99: 2165-2174.

Sammak, P.J., and G.G Borisy. 1987. Microtubule dynamics: direct observation of fluorescent microtubules in living cells. J. Cell Biol. 105: 90a.

Saxton, W.M., and J.R. McIntosh. 1987. Interzone microtubule behavior in late anaphase and telophase spindles. J. Cell Biol. 105: 875-886.

Saxton, W.M, D.L. Stemple, R.J. Leslie, E.D. Salmon, M. Zavortink, and J.R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. J. Cell Biol. 99: 2175-2186.

Schedl, T., J. Owens, W.F. Dove, and T.G. Burland. 1984. Genetics of the tubulin gene families of *Physarum*. Genetics 108: 143-164.

Schild, D., H.N. Ananthaswamy, and R.K. Mortimer. 1981. An endomitotic effect of a cell cycle mutation of *Saccharomyces cerevisiae*. Genetics 97: 551-562.

Scholey, J.M., M.S. Porter, P.M. Grissom, and J.R. McIntosh. 1985. Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. Nature 318: 483-486.

Schulze, E., and M.W. Kirschner. 1986. Microtubule dynamics in interphase cells. J. Cell Biol. 102: 1020-1031.

Schulze, E., and M.W. Kirschner. 1987. Dynamic and stable populations of microtubules in cells. J. Cell Biol. 104: 277-288.

Sheirr-Neiss, G., M. Lai, and N. Morris. 1978. Identification of a gene for β–tubulin in *Aspergillus nidulans*. Cell 15: 639-647.

Snyder, M., and R.W. Davis. 1986. Molecular analysis of chromosome segregation in yeast. Yeast 2: s363.

Solomon, F., M. Magendantz, and A. Salzman. 1979. Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. Cell 18: 431-438.

Soltys, B.J., and G.G. Borisy. 1985. Polymerization of tubulin *in vivo:* direct evidence for assembly onto microtubule ends and from centrosomes. J. Cell Biol. 100: 1682-1689.

Spiegelman, B.M., S.M. Penningroth, and M.W. Kirschner. 1977. Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. Cell 12: 587-600.

Stephens, R.E. 1978. Primary structural differences among tubulin subunits from flagella,

cilia, and the cytoplasm. Biochemistry 17: 2882-2891.

Theurkauf, W.E., H. Baum, J. Bo, and P.C. Wensink. 1986. Tissue-specific and constitutive α-tubulin genes of *Drosophila melanogaster* code for structurally distinct proteins. Proc. Natl. Acad. Sci. USA 83: 8477-8481.

Thomas, J.H. 1984. Genes controlling the mitotic spindle and chromosome segregation in yeast. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.

Thomas, J.H., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. Cell 44: 65-76.

Thomas, J.H., N.F. Neff and D. Botstein. 1985. Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 112: 715-734.

Tippet, D.H., L. Pillus, J.D. Pickett-Heaps. 1980. Organization of spindle microtubules in Ochromonas danica. J. Cell Biol. 87: 531-545.

Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida. 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.

Toda, T., K. Umesono, A. Hirata, and M. Yanagida. 1983. Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. J. Mol. Biol. 168: 251-270.

Toda, T., M. Yamamoto, and M. Yanagida. 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.

Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. Cell 50: 917-925.

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

Umesono, K., T. Toda, S. Hayashi, and M. Yanagida. 1983b. Two cell division cycle genes NDA2 and NDA3 of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. J. Mol. Biol. 168: 271-284.

Vale, R.D., Scholey, J.M., and M.P. Sheetz. 1986. Kinesin: possible biological roles for a new molecular motor. Trends Biochem. Sci. 11: 464-468.

Villasante, A., D. Wang, P. Dobner, P. Dolph, S.A. Lewis, and N.J. Cowan. 1986. Six mouse α-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. Mol. Cell. Biol. 6: 2409-2419.

Weatherbee, J.A., G.S. May, J. Gambino, N.R. Morris. 1985. Involvement of a particular species of β-tubulin (β-3) in conidial development in *Aspergillus nidulans*. J. Cell Biol. 101: 706-711.

Webster, D.R., G.G. Gundersen, J.C. Bulinski, and G.G. Borisy. 1987. Assembly and turnover of detyrosinated tubulin *in vivo*. J. Cell Biol. 105: 265-276.

Wegner, A. 1976. Head to tail polymerization of actin. J. Mol. Biol. 108: 139-150.

Weisernberg, R.C., and W.J. Deery. 1976. Role of nucleotide hydrolysis in microtubule assembly. Nature 263: 792-793.

Weisenberg, R.C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. Science 177: 1104-1105.

Wood, J.S. 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae*. Mol. Cell Biol. 2: 1064-1079.

Wood, J.S. and L.H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *S. cerevisiae*. J. Cell Biol. 94: 718-726.

Yamamoto, M. 1981. Genetic analysis of resistant mutants to antimitotic benzimidazole compounds in *Schizosaccharomyces pombe*. Mol. Gen. Genet. 180: 231-234.

Zieve, G., and F. Solomon. 1982. Proteins specifically associated with the microtubules of the mammalian mitotic spindle. Cell 28: 233-242.

Chapter 2:

Two functional α-Tubulin Genes of the Yeast
Saccharomyces cerevisiae Encode Divergent Proteins

Two Functional α-Tubulin Genes of the Yeast *Saccharomyces cerevisiae* Encode Divergent Proteins

PETER J. SCHATZ,[1] LORRAINE PILLUS,[1,2] PAULA GRISAFI,[1] FRANK SOLOMON,[1,2] AND DAVID BOTSTEIN[1*]

Department of Biology[1] and Center for Cancer Research,[2] Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Two α-tubulin genes from the budding yeast *Saccharomyces cerevisiae* were identified and cloned by cross-species DNA homology. Nucleotide sequencing studies revealed that the two genes, named *TUB1* and *TUB3*, encoded gene products of 447 and 445 amino acids, respectively, that are highly homologous to α-tubulins from other species. Comparison of the sequences of the two genes revealed a 19% divergence between the nucleotide sequences and a 10% divergence between the amino acid sequences. Each gene had a single intervening sequence, located at an identical position in codon 9. Cell fractionation studies showed that both gene products were present in yeast microtubules. These two genes, along with the *TUB2* β-tubulin gene, probably encode the entire complement of tubulin in budding yeast cells.

The process of cell division has been studied extensively in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. A particularly useful technique has been the isolation of conditional lethal mutants that arrest at specific morphological stages of the cell cycle (23, 35, 38, 39, 47, 56, 70). The normal function of the mutant gene product in such cell cycle mutants has, unfortunately, been discovered in relatively few instances. To define the role of these gene products in the mechanism of cell division, it will be essential to combine the genetic approach with biochemical and morphological analysis. Yeasts have become favorite organisms for such studies because of ease of growth and manipulation, because of the sophisticated recombinant DNA and classic genetic techniques that have been developed (11, 61), and because of the successful application of such techniques as electron microscopy and immunofluorescence (1, 13, 26, 27, 34, 42).

One of the proteins whose function in the cell division cycle is best understood is the α,β-tubulin heterodimer, which polymerizes to form the microtubules found in most eucaryotic cells (63). By electron and light microscopy, microtubules in yeast have been observed to be elements of structures involved in chromosome and nuclear movement (1, 13, 26, 27, 34, 42). Evidence obtained with antimicrotubule drugs (16, 40, 59) has suggested that microtubules in yeast have essential functions in the mitotic and meiotic spindles and in nuclear movement during cell division and mating (20, 48, 49, 73, 74). Conditional lethal mutations in tubulin genes have cell cycle defects, indicating a failure of the mitotic spindle (24, 56, 68–71), consistent with previous results with *Aspergillus nidulans* (40, 59). These mutants also show defects in meiosis and in nuclear fusion during mating (24, 69; J. Thomas, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984). Tubulin has been purified from yeast and shown to have biochemical properties similar to tubulin from higher organisms (25).

The single β-tubulin gene of *S. cerevisiae* has been isolated, sequenced, and shown to be essential for growth (36). All of the tubulin genes of *Schizosaccharomyces pombe* have been isolated and sequenced, including one β-tubulin gene (24) and two functional α-tubulin genes (69). All of

these genes show striking homologies to tubulin genes from other species (for reviews see references 15 and 50). In this report we complete the identification and sequence analysis of tubulin genes from *S. cerevisiae*. We report the isolation of the two functional α-tubulin genes, the complete sequence of both genes, and the identification of their protein products in yeast microtubules. In an accompanying paper (57), we report on the genetic analysis of null mutations in these two genes, which we have named *TUB1* and *TUB3*.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* HB101 (12) was used for bacterial transformation and plasmid growth. Strain BNN45 (65) was used to grow λ phage vectors. Bacterial media were made as described by Davis et al. (17) except for NZC medium (8), used for λ phage growth in liquid culture. *S. cerevisiae* strains PT6-2D (DBY1087) *MATa rna2 rna8 ura1* (from Jim Haber) and DBY1389 *MATa ade2* were used to prepare RNA and were grown in YPD medium (60).

Gel electrophoresis and nucleic acid preparation. Restriction enzymes, DNA polymerase I, DNA polymerase I large fragment, polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs and used in the buffers described by Maniatis et al. (32). Agarose gel electrophoresis and plasmid DNA isolation were performed as described by Davis et al. (17). Total cellular RNA was isolated from yeast cells by glass bead lysis in the presence of phenol and chloroform followed by ethanol precipitation, as described by Carlson and Botstein (14), except that the lysis buffer also contained 1% sodium dodecyl sulfate (SDS). The RNA was electrophoresed in formaldehyde-agarose gels as described in the GeneScreen manual (New England Nuclear Corp.).

Hybridization methods. DNA fragments were transferred from agarose gels to Zetapor membrane (AMF Inc.) by the method of Southern (64) with 20× SSPE (32). Hybridizations were done at 42°C in the buffer described by Wahl et al. (72) with nick-translated probes (52). The stringency of hybridization was lowered by decreasing the amount of formamide from 50 to 20% in steps of 10%. Genomic libraries were screened in hybridization buffer containing 30% formamide. Low-stringency washes were carried out in 2× SSPE–0.5% SDS at 50°C, while high-stringency washes were done in

---

* Corresponding author.

0.1× SSPE–0.1% SDS at 50°C. RNA gel transfer hybridizations were performed with GeneScreen hybridization membrane (New England Nuclear) by the methods described in the GeneScreen manual. A modification of the method was used that reduced the background (suggested by K. Durbin), which consisted of boiling the membrane for 5 min in distilled water immediately before prehybridization.

Plasmid and λ clones. The phage λ304, containing the *TUB3* gene, was isolated by plaque hybridization (7) at low stringency with two probes containing the two *Hind*III fragments of the *Schizosaccharomyces pombe NDA2* α-tubulin gene (69) from a library of partially *Sau*3A-digested strain S288C DNA in the vector λBF101 (36). An internal *Bgl*II fragment was then subcloned into the pBR322-derived polylinker vector pPL7 (J. Mullins, personal communication) to obtain pRB300 (insert shown in Fig. 1). The vector pPL7 was constructed by inserting a 95-base-pair (bp) polylinker containing *Eco*RI, *Cla*I, *Hind*III, *Xba*I, *Bgl*II, *Mbo*II, *Pst*I, and *Bam*HI sites (58) between the *Eco*RI and *Bam*HI sites of pBR322 (9).

A partial fragment of the *TUB1* gene was similarly isolated from a library of *Eco*RI-digested strain FL100 DNA in the vector λgt7 (55). The insert from this phage (λ305) was then used as a probe to isolate plasmids from a library of partially *Sau*3A-digested strain S288C DNA (M. Rose, J. Thomas, and P. Novick, personal communication) in the vector YCP50 (C. Mann, personal communication; see reference 29 for map) by colony hybridization (22). Part of the insert from one *TUB1*-containing plasmid was subsequently subcloned into pBR322 (9) to obtain pRB306 (see Fig. 1).

DNA sequence analysis. The sequences of the *TUB1* and *TUB3* genes were determined by the method of Maxam and Gilbert (33) with plasmids pRB300 and pRB306, respectively. Restriction fragments were labeled at their 3' ends by filling 5' overhangs with the appropriate α-^{32}P-labeled nucleotide with the Klenow fragment of DNA polymerase I. Fragments were labeled at their 5' ends with γ-^{32}P-labeled ATP and polynucleotide kinase (32). Computer analysis of DNA sequences was performed on a VAX 11-780 computer with programs written by the National Biomedical Research Foundation, by the University of Wisconsin Genetics Computer Group, and by the Whitaker College Computer Facility.

Cell fractionation and protein gels. Strains DBY1375 (*MATα ade2*) and DBY 1703 (*MATα ade2 ura3-52 tub3::URA3*) were grown in SD medium (60) supplemented with adenine sulfate (20 mg/liter) to a density of 5 × 10^6 cells/ml at 26°C. One millicurie of [^{35}S]methionine was added to 500 ml of cells, and growth was continued for 90 min. The cells were harvested, and the tubulin was fractionated into assembled and unassembled pools (44). The assembled pool was mixed with calf brain tubulin, taken through two cycles of temperature-dependent assembly and disassembly, and then run on two-dimensional gels as described previously (44). The gels were stained with Coomassie blue to visualize the carrier tubulin and were then treated with En^{3}Hance (New England Nuclear). The dried gels were marked for orientation with radioactive ink and exposed to preflashed Kodak XAR-5 film for 5 months.

RESULTS

Isolation of two α-tubulin genes. To identify the α-tubulin genes of *S. cerevisiae*, two fragments of the *Schizosaccharomyces pombe NDA2* α-tubulin gene (69) (generously provided by T. Toda and M. Yanagida) were used as

hybridization probes. Gel transfer hybridization experiments with genomic *S. cerevisiae* DNA (64) with these probes at low stringency showed two independent sets of strongly hybridizing bands (data not shown). Molecular cloning experiments yielded two genes, henceforth called *TUB1* and *TUB3* (the *TUB2* gene encodes β-tubulin). The *TUB3* gene was isolated from a library of partially *Sau*3A-digested genomic DNA in the vector λBF101 by plaque hybridization (7) and subcloned into the vector pPL7 to make plasmid pRB300. The *TUB1* gene was isolated as described in Materials and Methods and subcloned into pBR322 to make plasmid pRB306. The restriction maps of the yeast DNA inserts of pRB300 and pRB306 are shown in Fig. 1.

One additional sequence in the yeast genome hybridized weakly to the probe from the 5' half of the *NDA2* gene. This sequence was cloned, and the homologous fragment was found to contain the yeast *TUB2* gene, which encodes β-tubulin (36). This cross-hybridization of α- and β-tubulins was not altogether unexpected since the two proteins are closely related in primary structure (50). To search for additional sequences in the yeast genome with homology to α-tubulins, the *TUB1* and *TUB3* genes were used as probes in gel transfer hybridization experiments with genomic yeast DNA. The two genes cross-hybridized quite strongly, but did not hybridize with any other yeast sequences (see, for example, Fig. 2 in the accompanying paper [57]).

Nucleotide sequence of *TUB1* and *TUB3*. The complete sequences of the *TUB1* and *TUB3* genes were determined by the method of Maxam and Gilbert (33). The restriction map, sequencing strategy, and open reading frames are shown in Fig. 1. Each gene encodes an amino acid sequence highly homologous to those of α-tubulins from other species. The nucleotide sequences are shown in Fig. 2 and 3, along with the predicted amino acid sequences.

The open reading frames of each gene were interrupted by sequences presumed to be intervening sequences because they resembled other yeast introns: each had a perfect consensus 5' splice site (GTATGT) and 3' splice site (CAG), and each had the consensus TACTAAC internal site that has been shown to be necessary for efficient splicing (30, 31, 37, 41, 43, 66). Each intron was located in the identical position in codon 9 of the open reading frame. The *TUB1* intron was 115 bases long, and the *TUB3* intron was 298 bases long.

The presumptive spliced transcripts of the *TUB1* and *TUB3* genes encoded products of 447 and 445 amino acids, respectively, with calculated molecular weights of 49,701 and 49,694. The protein sequences were compared with those of porcine, *Schizosaccharomyces pombe*, and *Chlamydomonas reinhardi* α-tubulins (45, 62, 69) (Fig. 4); comparisons among sequences from these species are summarized in Table 1. There was one region, spanning amino acids 35 to

TABLE 1. α-Tubulin protein sequence comparisons

Sequence	% Identical amino acids residues[a]					
	TUB1	*TUB3*	Porcine	*S. pombe* 1	*S. pombe* 2	*C. reinhardi*
TUB1	100	90	74	75	74	69
TUB3		100	72	73	73	69
Porcine			100	76	76	85
S. pombe 1				100	86	70
S. pombe 2					100	71
C. reinhardi						100

[a] Percentages of identical amino acid residues are given based on the alignments shown in Fig. 4 of the porcine (45), *Schizosaccharomyces pombe* (69), and *Chlamydomonas reinhardi* α1 (62) α-tubulin sequences.
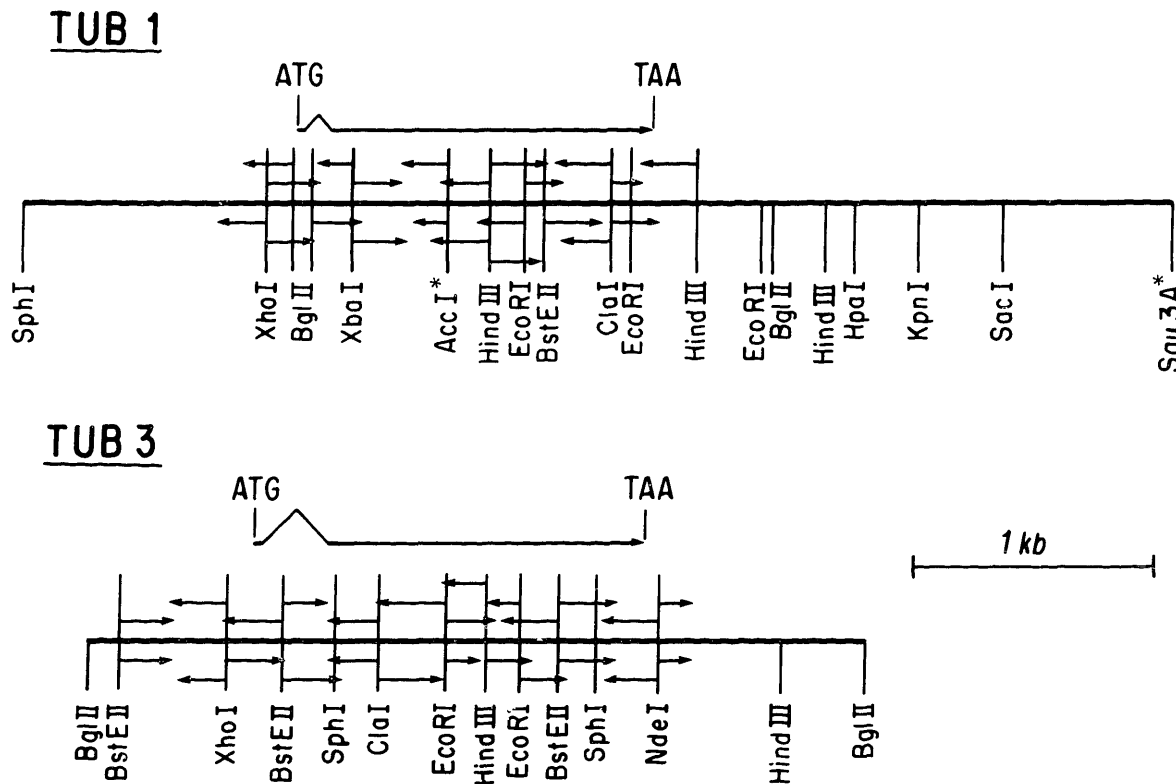
FIG. 1. Restriction enzyme maps, sequencing strategies, and open reading frames in the α-tubulin gene regions. Fragments of *S. cerevisiae* DNA containing the *TUB1* and *TUB3* genes are shown. The *TUB1* fragment was cloned between the *Sph*I and *Bam*HI sites of pBR322 to make plasmid pRB306. The *TUB3 Bgl*II fragment was cloned into the *Bgl*II site of the pBR322-based polylinker vector pPL7 to make plasmid pRB300. Short arrows above the heavy line indicate sequence determined from the top DNA strand (running 5' to 3' from left to right). Arrows below the line indicate sequencing from the other strand. The long arrows above the lines show the positions of the open reading frames encoding the α-tubulin sequences, with the introns indicated. Asterisks indicate restriction enzymes whose sites of cleavage were not mapped in the whole insert.

54, of considerable divergence near the amino terminus, including several deletions. This divergence was noted previously by Toda et al. (69) among other α-tubulins. Interestingly, *TUB1* and *TUB3* were identical in this region. The sequences aligned without insertions or deletions for the remainder of their length until the carboxy terminus; then there was again considerable divergence. The carboxy-terminal amino acid in both of the *S. cerevisiae* proteins was phenylalanine instead of tyrosine, which has been found in all other α-tubulins sequenced to date (15).

The *TUB1* and *TUB3* nucleotide sequences were compared in the coding regions, the noncoding flanking regions, and the introns. The coding regions showed 81% homology, with most of the differences not affecting the amino acid sequence. The differences were fairly evenly spread throughout the genes. The differences in coding sequence argue for a considerable evolutionary time of divergence between the two genes. The spatial distribution of these substitutions suggests an absence of recent gene conversions between them. The 5' and 3' noncoding regions showed very little detectable homology. The introns also showed very little homology except for the consensus sequences and for a region rich in thymidine near the 3' splice junction.

Expression of *TUB1* and *TUB3*. Gel transfer hybridization with whole-cell RNA from exponentially growing cultures of haploid cells showed that both genes gave rise to an mRNA of approximately 1.6 kilobases (kb). Transfers were also done with RNA from an *rna2 rna8* temperature-sensitive

splicing mutant (54, 67) at the permissive and restrictive temperatures. The results of the analysis with a *TUB3* probe are shown in Fig. 5. At the restrictive temperature (35°C), putative mRNA precursors to both the *TUB1* and *TUB3* sequences were observed, each larger by approximately the amount predicted from the size of the proposed introns. The *TUB1* gene appeared to be expressed at a higher level than the *TUB3* gene, but this could not be estimated quantitatively because of cross-hybridization of the probes from each gene with the mRNA from the other. The fact that both genes expressed a 1.6-kb mRNA was confirmed by using RNA from null mutants of each gene, which are described in the accompanying paper (57). When the entire *TUB1* region shown in Fig. 1 was used as a probe, a transcript of 1.9 kb also hybridized. The *TUB3* fragment shown in Fig. 1 hybridized to additional transcripts of 1.1 and 2.2 kb. We have not investigated further the origin of these transcripts.

Both gene products are present in yeast microtubules. Previous analyses of yeast tubulin demonstrated the existence of two species in the α-tubulin region of two-dimensional gels (25, 44). Using a *TUB3* null mutant described in the accompanying paper (57), we were in a position to examine the origin of these two proteins. Since the predicted sequence of *TUB1* contained two more amino acids and three more negative charges than that of *TUB3*, we expected that the smaller, more basic protein was the *TUB3* gene product and therefore would not be present in extracts from the *TUB3* null mutant. Wild-type cells were labeled

TUB1
TUB3   GGTNACCTCACGTCATGGAAATTTCGCCTTATTCATGTTGTTGGGTATCTTCACAACCTTGTTGATCCCAGAAACTAA   −481

GAGAAAGACTCTAGAAGAGAAATTAACGAGCTATACCACGATGAAATGACTACCGCTAAACTTCAGTGATCTTCAACTGA   −361

CACAATTGCATACTTTCCATTATAACTGACTGTTCAGATCCTGCAATAGAAATGTTTTTAAGTAATAGACGATCGGAAA
AGCATAAAAGCCTCAAAGATGCACTCAAACTAGAACAAATAACAAATATTTTTATCAAAACCCCTTACATATCTCAGGTTACATAT   −241

CTATTAATAATAAGTAGTTTACATATATATATATATTTCCTCACTCTCTTCCTTCCATTTCATTTCGATATTTCTTTTAATTTCATTATTTCGGCTGCCCGGGTATGCCACGGTTCCGGCGAAAACGCCTCGCAGCCAAGGAAAAACTAAA   −121

CTACAAAACAAAACAAAACAAAACAAAACATCTACTACTATATGCGTTTACCCAATTCAGTATTCGTTTACAAAGAATATCCCTCTACAACAGTTCTCGCCCACCCAAGATCTGTAAACTGCAAACAAACA
CATTTCTCGAGCCCTCTGGAAACCGCAAACGTTTTACAATTGCACATCTAAAGAAATATAAACATCAGTATCTCATAAAGTACAATAATCGACTAAGCAAGCCACTGAGACA   −1

Intron
                  10   20   30
ATG AGA GAA GTT ATT AGT ATT AAT GCT CAA ATT GGT TGT CAG ATT GGT TGT TGG GAA TTA TAT TCC CTT GAG CAC GGT ATT   90
Met Arg Glu Val Ile Ser Ile Asn Ala Gln Ile Gly Cys Gln Ile Gly Cys Trp Glu Leu Tyr Ser Leu Glu His Gly Ile
ATG AGA GAG GTC ATT AGT ATT AAT GCA CAA GGT GGT TGT ATA GGT GGA GAA GGA TGC TGG GAA TTG TAC TCC CTA GAG CAT GGC ATC
Intron

                          40   60
AAG CCG GAT CAT CTA GAA GAT GGC CTT TTT TTC ACG TCC TCA AAG GGA GGA GAG GAA GGT TTT TTC CAT GGA CAC GGT TAC GGT   180
Lys Pro Asp His Leu Glu Asp Gly Leu Phe Phe Thr Ser Ser Lys Gly Gly Glu Glu Gly Phe Phe His Gly His Gly Tyr Gly
AAG GAA GAC GGC TTG CTG ATT GGC TTG TCA TCT TTT TCT ACA TCT AAA GGT GGA GAA GAG GGA TTC TTC CAT GGA CAT GGA

                          70   80   90
AAG TTC GTT CCA AGG GCT ATT TAC GTG GAT TTA GAG CCC CGT AAC GTC ATT GAC GAA TAC AAG CCT TAC GGT ATT CCA   270
Lys Phe Val Pro Arg Ala Ile Tyr Val Asp Leu Glu Pro Arg Asn Val Ile Asp Glu Tyr Lys Pro Tyr Gly Ile Pro
AAA TTC TTC GTC CCA AGA GCA ATC TAC GTG GAC TTA GAT TTA GAG CAG GTA GTT ATC GAT CAT GAA TTC TTC AAG CTT TTC CAT CCA
                                                              Arg Phe
                                                              Thr

                                  110   120
GAA CAG TTG CTC AGT GGT AAG GAG GAC GCT AAT AAT TAT GCA AGA GGC CAT TAC ACC GGT GAT GTT TTG GGC GAT GTT GTT CTG   360
Glu Gln Leu Leu Ser Gly Lys Glu Asp Ala Asn Asn Tyr Ala Arg Gly His Tyr Thr Thr Gly Asp Val Leu Gly Asp Val Val Leu
GAA GAA GAC GGT AAC GGT AAG AAG GAC GCC GCC AAT AAC AAC TAC TAT ACA AGA GGC CAT GGC TAT TAC ATA GGT GAT GAA GTT GAA
                                                                                                    Val Asp Glu Glu

                  130   140   150
GAT AGG ATT AGA AGA ATT ATG GCA CAA GAC TGT TGT CAG GGG TTA CAA GGG TTT TTT CTT TCT GGT ACT GGT TCC GGT CTA   450
Asp Arg Ile Arg Arg Ile Met Ala Gln Asp Cys Cys Gln Gly Leu Gln Gly Phe Phe Leu Ser Gly Thr Gly Ser Gly Leu
GAA AGA ATT AGA AGA ATT AAG ATG GAC GAC GAC CAA CAA TGT TGT CAA CAA GGG TTC TTG CAT TCT CTT GGT GGT TCC GGT TTA
Glu   Met

                  160   170   180
GGG TCC TTA CTG TTG TTG GAA CTA TCC GCT CTG GAT AGG ATT AGG GAA TAC CTG AAG AAA TCC GGT TAC GTA TAC CCT GCT GTG TCT ACT   540
Gly Ser Leu Leu Leu Glu Leu Ser Ala Leu Asp Arg Ile Arg Glu Tyr Leu Lys Lys Ser Gly Tyr Val Tyr Pro Ala Val Ser Thr
GGT TCC CTG TTA TTA GAA AAC TCG GAA GCC ATG ATT AGG GAG TAC TAC GGG AAG AAA TCC AAA GGG GAA CTG CAA CGT CCT CCG CAA TTG TCT ACT
Asn   Met   Leu

                  190   200   210
TCT GTG GTT ACT AAC ACA GTT TTA GAA CAT ACA CAT GCA GAT CCT AGA TTC ATG GTC GAT AAT GAG GCT ATT   630
Ser Val Val Thr Asn Thr Val Leu Glu His Thr His Ala Asp Pro Arg Phe Met Val Asp Asn Glu Ala Ile
TCC GTC GTG ACG AAC AAC ACG CTG GAA CAC ACC CAC CAC GAC CTG CCT ACG TTT ATG GTC GAC AAC GAA ATT

FIG. 2. Sequences of coding and flanking regions. The nucleotide sequence of the *TUB3* gene is shown above the *TUB3* sequence, numbered to the right of each line. Translation of the *TUB3* coding sequence is shown below its sequence line. Only the differences in the *TUB3* amino acid sequence from that of *TUB1* are shown. Amino acids are numbered above the coding sequence lines. The positions of the introns (see Fig. 3) are shown by arrowheads.

TUB1 INTRON:

```
  1  GTATGT TCGA  TTTGCCCGTC  CAGGCTAGAT  CTTTTTTTGT  TAGTTCATTT

 51  TGGCGTTTCA  TCTTTCATTA  CTAACTTCAA  CGCAAAATTT  TTTTTTTTT

101  GATTTCTCTT  TACAG
```

TUB3 INTRON:

```
  1  GTATGT ATGC  GTTCCTTTTT  TTGTTCAATA  TTCGCAACCA  ATGGCACCTG

 51  TGGGACAGGG  AAAGAAGTTT  GATCTGATCT  GGTTTGATTC  ATTCCCAATT

101  GGTCACCATC  TGGTTGATTT  ACGGCAAATA  ATTTGACTTG  TACCAGCACA

151  GTT TACTAAC  AGTTTCTTTT  TCTCCATTTT  TTCTGGGCAT  ACTCGGACGA

201  AAAAGCTCAT  AATTGACCTC  ATTACATGGG  GAGTGATTTT  TGTGTCTTCT

251  TCTTCGGAGG  ATTGCTGGAA  CTTTTGTTAT  TTTTCTTTTT  TACAA CAG
```

FIG. 3. Intron sequences. The nucleotide sequences of · ie putative *TUB1* and *TUB3* introns are shown. Consensus 5', internal, and 3' splicing sequences are enclosed by boxes.

with [^{35}S]methionine, and the yeast cellular tubulin was fractionated into unassembled and assembled pools (44). These extracts contained many protein species, but the microtubule components could be enriched greatly by coassembly with unlabeled carrier calf brain microtubule protein. The assembled-pool fraction was mixed with the carrier, and after two cycles of temperature-dependent assembly, the proteins were analyzed by two-dimensional isoelectric focusing (IEF)-SDS-polyacrylamide gel electrophoresis (PAGE). The radioactive proteins were visualized by autoradiography, and the carrier proteins were stained (Fig. 6a). The spot previously identified as β-tubulin is indicated (44). The pair of proteins with similar mobility in SDS-PAGE gels and more basic pIs were identified as α-tubulins by three criteria. First, they disappeared from the assembled-pool fraction when the cells were pretreated with the microtubule-depolymerizing drug nocodazole (44). Second, they coassembled with the carrier tubulin to constant specific activity through up to four cycles of assembly and disassembly, while other proteins in this region of the gel were progressively lost in successive cycles (L. Pillus and F. Solomon, unpublished data). Finally, the darker spot comigrated with the carrier α-tubulin.

To determine the identity of the gene products, extracts containing assembled microtubule components were also made from a strain which carried a *TUB3* null mutation and analyzed by the same procedure (Fig. 6b). The smaller, more basic spot disappeared in the strain that lacked functional *TUB3*. Panel b is overexposed relative to panel a to emphasize the disappearance of the *TUB3* spot. The larger, more acidic spot was assumed to represent the *TUB1* gene product. The proposed *TUB1* spot was considerably darker than the *TUB3* spot. This observation is consistent with the results of the accompanying paper (57), which indicate that the *TUB1* gene may be expressed at a higher level than *TUB3*. The fact that both of these proteins were present in the cell fraction containing components of assembled

microtubules strengthens the conclusion that both *TUB1* and *TUB3* encode functional α-tubulins.

DISCUSSION

The budding yeast *S. cerevisiae* has a single essential β-tubulin gene (*TUB2*), whose complete structure is known (36). Here we report the isolation and sequence of two functional α-tubulin genes from *S. cerevisiae*, which we have named *TUB1* and *TUB3*. Our identification of *TUB1* and *TUB3* as α-tubulin genes is based on sequence homology. The conclusion that both genes encode functional α-tubulins is based on the observed incorporation of both gene products into yeast cell microtubules and on the phenotypes of mutations in the two genes (see accompanying paper [57]). We have not detected any other sequences in the yeast genome with significant homology to either of the α-tubulin genes or to the single β-tubulin gene even under very low stringency hybridization conditions (G. E. Georges, P. J. Schatz, and D. Botstein, unpublished data). Therefore, these three genes probably represent the entire complement of tubulin genes for this organism.

Of the many organisms whose tubulin genes have been studied (reviewed in references 15 and 50), the yeast genes show the most striking similarities to those found in the fission yeast *Schizosaccharomyces pombe*. *Schizosaccharomyces pombe* also has a single β-tubulin gene and two functional α-tubulin genes (24, 69). Its two α-tubulins have 86% amino acid homology, while *TUB1* and *TUB3* have 90% homology. This level of divergence is much higher than has been found between tubulin isotypes in other species examined, raising the possibility that the α-tubulins within each yeast species might be functionally distinct. If a common progenitor of these quite different yeasts had evolved two distinct α-tubulin genes, one might expect that the interspecies homologies between appropriate pairs of proteins might be greater than the intraspecies homologies. At the least,

```
                10                30                  50                  70                  90
TUB1     MREVISINVGQAGCQIGNACWELYSLEHGIKPDG-HLEDGLSKPKGGEEGFS--TFFHETGYGKFVPRAIYVDLEPNVIDEVRNGPYKDLFHPEQLLSGK
TUB3                                      E-                                                          T       E     IN
PORCINE  C        H            V          C  Q  --QMPSDKTIG DDS N--          VF      T   T   RF  E        IT
POMBE 1           VH           V          C  G  FPT NSEVHKNNSYLNDGFG         T    Q      T   RQ           MVT
POMBE 2  I        H            T          C  N- --YMNPDTASQNSDG  --          Q        TD  R             IT
CHLAMY            HI           I    V     C  Q  --QMPSDKTIG DDA N--          C FL    T V RQ             I

                110               130                150                170                190
TUB1     EDAANNYARGHYTVGREILGDVLDRIRKLADQCDGLQGFLFTHSLGGGTGSGLGSLLLEELSAEYGKKSKLEFAVYPAPQVSTSVVEPYNTVLTTHTTLE
TUB3                            VDE EE                                                 L
PORCINE          I K   IDL     M                SVF  F    FT  MR VD       N  Y       SI    A      SI        S
POMBE 1          K MIDS     E      RM  NS        VF  F     T   RNM           NQ S               A        S
POMBE 2   S      K LVDE T K    RIG NS        FA  VF  F     A   RAM  T                 GI T                A    D
CHLAMY       F   I K   VDLA     N T          R   VFNAV                      RVD       GT       S           SL

                210               230                250                270                290
TUB1     HADCTFMVDNEAIYDMCKRNLDIPRPSFANLNNLIAQVVSSVTASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVSYSPVLSKSKAFHESNSVSEITNACF
TUB3                  I   G S  S  G             I                            A  AT A I  KR T
PORCINE   S   A     I R     TYT   R G I   A     T          A   AT A  AEY  QL       S     S
POMBE 1   NS       C I R    TYE     I     I                 T  TA IV AA              Q          S         A
POMBE 2   L      SC I R     YE      I     L            M S  A  TA IV AA                  Q                 A    D
CHLAMY    T  VAV L   IRS  E TYT   R I     A     IT             A II AE  Q  QL A          Q           A

                310               330                350                370                390
TUB1     EPGNQMVKCDPRDGKYMATCLLYRGDVVTGDVQRAVEQVKNKKTVQLVDWCPTGFKIGICYEPPTATPNSQLATVDRAVCMLSNTTSIAEAWKRIDRKFD
TUB3              TK    N     R           M                    SVI S E N              A    D   Q
PORCINE   A      H  C         PK      NA IATI T R I F     V N   VV GGD  KQ            A    L   H
POMBE 1   Y      T R         IPR     TSI SRR I F               QHV G GI KN          A      S   L   H
POMBE 2   Y      A R         IPR     TTI A R I F          DR   QHIEG EI K           I        S   L   H
CHLAMY    ASM    H    C  M   PK      NAS ATI T R I F      C NQ  VV GGD  KQ                   S           L   H

                410                    430                      450
TUB1     LMYAKRAFVHWYVGEGMEEGEFTEAREDLAALERDYIEVGADS------YAEEEF
TUB3                                ----    ---- --
PORCINE                  S      M     K  E  V  VEGEGEE G  Y      S
POMBE 1   S              S      K  E     Q  MDNEM EAD  Y         S
POMBE 2   S              S      E  Q     MEVDYME--      Y         S
CHLAMY                   S      K FE     E  AEGAGEG  G  Y
```

FIG. 4. α-Tubulin sequence comparisons. The *TUB1* amino acid sequence is shown in one-letter code and compared with that of *TUB3*, porcine (45), *Shizosaccharomyces pombe* (69), and *C. reinhardi* (62) α-tubulins. Since the two *C. reinhardi* proteins differ at only two residues, only the α1 gene product is shown. The numbers correspond to the sequence of *Schizosaccharomyces pombe* α1 tubulin, with dashes inserted into the other sequences to bring them into register. Positions of identity to the *TUB1* sequence are left blank.
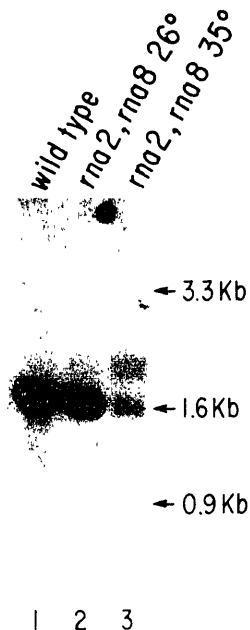
FIG. 5. RNA gel transfer hybridization analysis of the *TUB3* transcripts. Total cellular RNA was made from exponentially growing wild-type cells or an *rna2 rna8* mutant grown at the permissive temperature (26°C) or grown at the permissive temperature and then shifted to the restrictive temperature (35°C) for 30 min. The RNA was run on a formaldehyde-agarose gel, transferred to a GeneScreen membrane, and analyzed with a nick-translated probe made from the *XhoI-NdeI* fragment containing the *TUB3* coding sequence. The numbers to the right indicate the positions of size markers of the larger RNA (3.3 kb) and the *URA3* transcript (0.9 kb) along with the calculated size of the *TUB1* and *TUB3* transcripts (1.6 kb).

functionally significant pairings might be suggested by the different interspecies comparisons. As shown in Table 1, this was not the case. The intraspecies homologies were greater than the interspecies homologies, and all interspecies comparisons gave about the same percent identity value. This result suggests that each species evolved a second α-tubulin gene after they diverged. The positions of introns in the *S. cerevisiae* and *Schizosaccharomyces pombe* genes reinforce this hypothesis. Both *S. cerevisiae* genes have an intron in the same place in codon 9. One of the *Schizosaccharomyces pombe* genes has an intron in codon 19, while the other is free of introns (69). An alternative hypothesis to explain these observations is that the progenitor of these yeasts had two α genes, but that gene conversion events in both species, after their divergence, led to higher levels of intraspecies homology. The high level of divergence between the *S. cervisiae* and *Schizosaccharomyces pombe* proteins (as great as that between either and the porcine proteins) reinforces the vast evolutionary distance between these two yeasts.

As shown in Fig. 6, the *TUB1* gene product was apparently incorporated into assembled structures at a higher level than that of *TUB3* in exponentially growing haploid cells (assuming equal efficiency of coassembly of each heterodimer with carrier tubulin). This difference was also reflected in the mRNA levels. These results suggest that the *TUB1* gene may be more important for haploid cell growth. Among the range of hypotheses that can be used to explain the presence of two α genes are two extremes. One is that the two proteins are functionally different. One protein might preferentially function in specific populations of microtubules in the same cell or during specific stages of cell growth or differentiation (e.g., during meiosis). At the other extreme, the proteins might be functionally interchangeable, and any differences between the function of the two genes could be explained by different levels of expression. We address the question of whether there are major functional
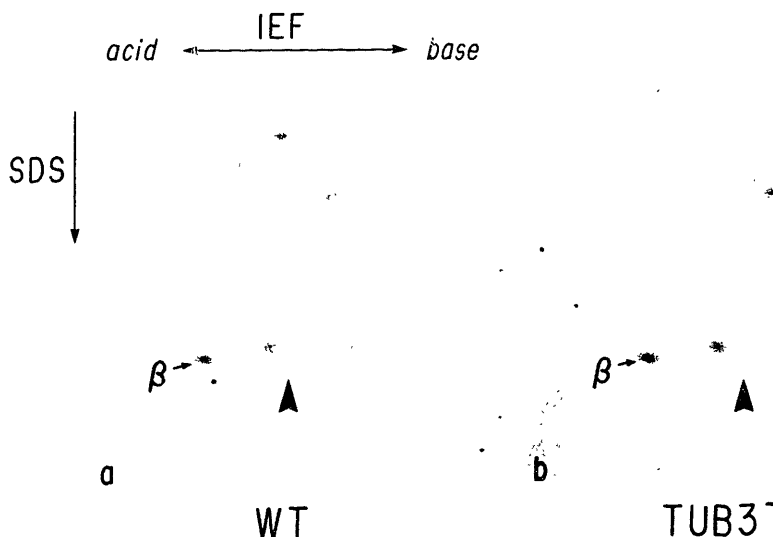

FIG. 6. Two-dimensional gel analysis of yeast tubulin. Yeast cells were labeled with [^{35}S]methionine, and the tubulin was fractionated into unassembled and assembled pools (44). The assembled fraction was mixed with unlabeled carrier calf brain microtubule protein and carried through two cycles of temperature-dependent assembly. The proteins were analyzed on two-dimensional IEF/SDS-PAGE gels, and the radioactive proteins were visualized by autoradiography. Only the region of the gel near the tubulins is shown. (a) Proteins from wild-type (WT) cells. (b) Proteins from a *TUB3* null mutant. The spot previously identified as β-tubulin is indicated (β). The arrowhead points to the *TUB3* gene product in panel a that was not present in panel b. The protein with a slightly more acidic pI and slightly lower mobility in SDS-PAGE than the *TUB3* gene product was assumed to be the *TUB1* gene product.

differences between the proteins in an accompanying paper (57).

The *TUB1* and *TUB3* genes code for proteins of 447 and 445 amino acids, respectively, and the *TUB2* β-tubulin gene product is 457 amino acids long (36). *S. cerevisiae* therefore has the longest known β-tubulin and the shortest known α-tubulins, mostly due to divergence at the carboxy termini of the subunits. When the α- and β-tubulins of different species were compared, the carboxy terminus was consistently one of the regions of greatest variability (15) (Fig. 4). This divergence may be responsible for functional differences between tubulins. Alternatively, it may occur because differences near the carboxy termini do not affect function. Results from the accompanying paper (57) and from another study of the role of sequence diversity in specifying tubulin function (10) indicate that the latter interpretation is more likely to be correct.

An interesting difference found between the *S. cerevisiae* genes and all other known α-tubulin genes was the occurrence of phenylalanine instead of tyrosine as the carboxy-terminal amino acid. The terminal tyrosine on α-tubulin can be both removed and replaced by enzymes originally found in rat brain (3, 5, 6) and since found in many other tissues and species (18, 46, 51). Although the presence or absence of the terminal tyrosine does not seem to alter the in vitro assembly properties of tubulin (2), the fraction of tyrosinated tubulin is different in assembled and unassembled pools (53). Individual microtubules within the same cell can be distinguished on the basis of the presence or absence of this tyrosine (21). Thus, tyrosination has been suggested to be important in microtubule function (19, 28). Since the tubulin:tyrosine ligase and the carboxypeptidase enzymes can also use phenylalanine as a substrate in vitro (2, 4, 18), the *S. cerevisiae* residues may be functionally homologous to the tyrosine residues found in other tubulins. The conservative nature of this change is consistent with an important functional role for the terminal aromatic residue.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Adams, A. E. M., and J. R. Pringle. 1984. Localization of actin and tubulin in wild-type and morphogenetic mutants of *Saccharomyces cerevisiae*. J. Cell Biol. 98:934–945.
2. Arce, C. A., M. E. Hallak, J. A. Rodriguez, H. S. Barra, and R. Caputto. 1978. Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino acids on tubulin assembly. J. Neurochem. 31:205–210.
3. Argarana, C. E., H. S. Barra, and R. Caputto. 1978. Release of [14C]tyrosine from tubulinyl-[14C]tyrosine by brain extract. Separation of a carboxy peptidase from tubulin tyrosine ligase. Mol. Cell. Biochem. 19:17–22.
4. Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1973. Incorporation of phenylalanine as a single unit into rat brain protein: reciprocal inhibition by phenylalanine and tyrosine of

their respective incorporations. J. Neurochem. 21:1241–1251.
5. Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins. Biochem. Biophys. Res. Commun. 60:1384–1390.
6. Barra, H. S., J. A. Rodriguez, C. A. Arce, and R. Caputto. 1973. A soluble preparation from rat brain that incorporates into its own proteins [14C]arginine by a ribonuclease-sensitive system and [14C]tyrosine by a ribonuclease-insensitive system. J. Neurochem. 20:97–108.
7. Benton, W., and R. Davis. 1977. Screening of λ-gt recombinant clones by hybridization to single plaques in situ. Science 196:180–182.
8. Blattner, F., B. Williams, A. Bulechl, K. Thompson, H. Faber, L. Furlong, D. Grunwald, D. Kiefer, D. Moore, J. Schumm, E. Sheldon, and O. Smithies. 1977. Charon phages: safer derivatives of bacteriophage λ for DNA cloning. Science 196:161–169.
9. Bolivar, F., R. Rodriguez, P. Green, M. Betlach, H. Heynecker, H. Boyer, J. Crossa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
10. Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. Cell 44:500–509.
11. Botstein, D., and R. W. Davis. 1982. Principles and practice of recombinant DNA research with yeast, p. 607–638. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*, vol. 2. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
12. Boyer, H., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. 41:459–472.
13. Byers, B. 1981. Cytology of the yeast life cycle. p. 59–96. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The Molecular Biology of the Yeast *Saccharomyces*, vol. 1. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
14. Carlson M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28:145–154.
15. Cleveland, D. W., and K. F. Sullivan. 1985. Molecular biology and genetics of tubulin. Annu. Rev. Biochem. 54:331–365.
16. Davidse, L., and W. Flach. 1977. Differential binding of methylbenzimidazol-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in strains of *Aspergillus nidulans*. J. Cell. Biol. 72:174–193.
17. Davis, R., D. Botstein, and J. Roth. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Deanin, G. G., and M. W. Gordon. 1976. The distribution of tyrosyltubulin ligase in brain and other tissues. Biochem. Biophys. Res. Commun. 71:676–683.
19. Deanin, G. G., S. F. Preston, R. K. Hanson, and M. W. Gordon. 1980. Mechanism of turnover of the carboxy terminal tyrosine of α-tubulin. Eur. J. Biochem. 109:207–216.
20. Delgado, M. A., and J. Conde. 1984. Benomyl prevents nuclear fusion in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 193:188–189.
21. Gundersen, G. G., M. H. Kalnoski, and J. C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and nontyrosinated α-tubulin are distributed differently in vivo. Cell 38:779–789.
22. Hanahan, D., and M. Meselson. 1980. Plasmid screening at high colony density. Gene 10:63–67.
23. Hartwell, L. H. 1974. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. 38:164–198.
24. Hiraoka, Y., T. Toda, and M. Yanagida. 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.
25. Kilmartin, J. 1981. Purification of yeast tubulin by self-assembly in vitro. Biochemistry 20:3629–3633.
26. Kilmartin, J., and A. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomy-*

64

ces. J. Cell. Biol. **98:**922–933.

27. **King, S. M., and J. S. Hyams.** 1982. The mitotic spindle of *Saccharomyces cerevisiae*: assembly, structure, and function. Micron **13:**93–117.

28. **Kumar, N., and M. Flavin.** 1981. Preferential action of a brain detyrosinating carboxypeptidase on polymerized tubulin. J. Biol. Chem. **256:**7678–7686.

29. **Kuo, C., and J. L. Campbell.** 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. **3:**1730–1737.

30. **Langford, C. J., and D. Gallwitz.** 1983. Evidence for an intron-containing sequence required for the splicing of yeast RNA polymerase II transcripts. Cell **33:**519–527.

31. **Langford, C. J., F.-J. Klinz, C. Donath, and D. Gallwitz.** 1984. Point mutations identify the conserved, intron-contained TACTAAC box as an essential splicing signal sequence in yeast. Cell **36:**645–653.

32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

33. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.

34. **Moens, P. B., and E. Rapport.** 1971. Spindles, spindle plaques, and meiosis, in the yeast *Saccharomyces cerevisiae* (Hansen). J. Cell. Biol. **50:**344–361.

35. **Nasmyth, K., and P. Nurse.** 1981. Cell division cycle mutants altered in DNA replication and mitosis in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **182:**119–124.

36. **Neff, N., J. H. Thomas, P. Grisafi, and D. Botstein.** 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function in vivo. Cell **33:**211–219.

37. **Newman, A. J., R. Lin, S. Cheng, and J. Abelson.** 1985. Molecular consequences of specific intron mutations on yeast mRNA splicing *in vivo* and *in vitro*. Cell **42:**335–344.

38. **Nurse P., and P. Thuriaux.** 1980. Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. Genetics **96:**627–637.

39. **Nurse, P., P. Thuriaux, and K. Nasmyth.** 1976. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **146:**167–178.

40. **Oakley, B., and N. R. Morris.** 1980. Nuclear movement is β-tubulin dependent in *Aspergillus nidulans*. Cell **19:**255–262.

41. **Parker, R., and C. Guthrie.** 1985. A point mutation in the conserved hexanucleotide at a yeast 5′ splice junction uncouples recognition, cleavage, and ligation. Cell **41:**107–118.

42. **Peterson, J. B., and H. Ris.** 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. J. Cell Sci. **22:**219–242.

43. **Pikielny, C. W., J. L. Teem, and M. Rosbash.** 1983. Evidence for the biochemical role of an internal sequence in yeast nuclear mRNA introns: implications for U1 RNA and metazoan mRNA splicing. Cell **34:**395–403.

44. **Pillus, L., and F. Solomon.** 1986. Components of microtubular structures in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83:**2468–2472.

45. **Ponstingl, H., E. Krauhs, M. Little, and T. Kempf.** 1981. Complete amino acid sequence of α-tubulin from porcine brain. Proc. Natl. Acad. Sci. USA **78:**2757–2761.

46. **Preston, S. F., G. G. Deanin, R. K. Hanson, and M. W. Gordon.** 1979. The phylogenetic distribution of tubulin: tyrosine ligase. J. Mol. Evol. **13:**233–244.

47. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

48. **Pringle, J. R., S. H. Lillie, A. E. M. Adams, C. W. Jacobs, B. K. Haarer, K. G. Coleman, J. S. Robinson, L. Bloom, and R. A. Preston.** 1986. Cellular morphogenesis in the yeast cell cycle, p. 193–209. *In* J. Hicks (ed.), Yeast cell biology. Alan R. Liss, New York.

49. **Quinlan, R. A., C. I. Pogson, and K. Gull.** 1980. The influence of the microtubule inhibitor methyl benzimidazol-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. J. Cell Sci. **46:**341–352.

50. **Raff, E. C.** 1984. Genetics of microtubule systems. J. Cell Biol. **99:**1–10.

51. **Raybin, D., and M. Flavin.** 1977. Enzyme which specifically adds tyrosine to the α-chain of tubulin. Biochemistry **16:**2189–2194.

52. **Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113:**237–251.

53. **Rodriguez, J. A., and G. G. Borisy.** 1979. Tyrosination state of free tubulin subunits and tubulin disassembled from microtubules of rat brain tissue. Biochem. Biophys. Res. Commun. **89:**893–899.

54. **Rosbash, M., P. K. W. Harris, J. L. Woolford, and J. L. Teem.** 1981. The effects of temperature-sensitive RNA mutants on the transcription products from cloned ribosomal protein genes of yeast. Cell **24:**679–686.

55. **Rose, M., P. Grisafi, and D. Botstein.** 1984. Structure and function of the yeast URA3 gene: expression in *Escherichia coli*. Gene **29:**113–124.

56. **Roy, D., and P. A. Fantes.** 1983. Benomyl-resistant mutants of *Schizosaccharomyces pombe* cold-sensitive for mitosis. Curr. Genet. **6:**195–202.

57. **Schatz, P. J., F. Solomon, and D. Botstein.** 1986. Genetically essential and nonessential α-tubulin genes specify functionally interchangeable proteins. Mol. Cell. Biol. **6:**3722–3733.

58. **Seed, B.** 1983. Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*. Nucleic Acids Res. **11:**2427–2445.

59. **Sheirr-Neiss, G., M. Lai, and N. Morris.** 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell **15:**639–647.

60. **Sherman, F., G. Fink, and C. Lawrence.** 1974. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

61. **Shortle, D., P. Novick, and D. Botstein.** 1984. Construction and genetic characterization of temperature-sensitive alleles of the yeast actin gene. Proc. Natl. Acad. Sci. USA **81:**4889–4893.

62. **Silflow, C. D., R. L. Chisholm, T. W. Conner, and L. P. W. Ranum.** 1985. The two α-tubulin genes of *Chlamydomonas reinhardi* code for slightly different proteins. Mol. Cell. Biol. **5:**2389–2398.

63. **Snyder, J., and J. McIntosh.** 1976. Biochemistry and physiology of microtubules. Annu. Rev. Biochem. **45:**699–720.

64. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. **98:**503–517.

65. **St. John, T., and R. Davis.** 1981. The organization and transcription of the galactose gene cluster of *Saccharomyces*. J. Mol. Biol. **152:**285–315.

66. **Teem, J. L., N. Abovich, N. F. Kaufer, W. F. Schwindinger, J. R. Warner, A. Levy, J. Woolford, R. J. Leer, M. M. C. van Raamsdonk-Duin, W. H. Mager, R. J. Planta, L. Schultz, J. D. Friesen, H. Fried, and M. Rosbash.** 1984. A comparison of yeast ribosomal protein gene DNA sequences. Nucleic Acids Res. **12:**8295–8312.

67. **Teem, J. L., J. R. Rodriguez, L. Tung, and M. Rosbash.** 1983. The rna2 mutation of yeast affects the processing of actin mRNA as well as ribosomal protein mRNA's. Mol. Gen. Genet. **192:**101–103.

68. **Thomas, J. H., N. F. Neff, and D. Botstein.** 1985. Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics **112:**715–734.

69. **Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida.** 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.

70. **Toda, T., K. Umesono, A. Hirata, and M. Yanagida.** 1983. Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. J. Mol. Biol. **168:**251–

270.

71. **Umesono, K., T. Toda, S. Hayashi, and M. Yanagida.** 1983. Two cell division cycle genes NDA2 and NDA3 of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. J. Mol. Biol. **168:**271–284.

72. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxy-methyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. USA **76:**3683–3687.

73. **Wood, J. S.** 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae.* Mol. Cell. Biol. **2:**1064–1079.

74. **Wood, J. S., and L. H. Hartwell.** 1982. A dependent pathway of gene functions leading to chromosome segregation in *S. cerevisiae.* J. Cell Biol. **94:**718–726.

Chapter 3:

Genetically Essential and Nonessential α-Tubulin Genes Specify Functionally Interchangeable Proteins

67

Genetically Essential and Nonessential α-Tubulin Genes Specify Functionally Interchangeable Proteins

PETER J. SCHATZ,[1] FRANK SOLOMON[1,2] AND DAVID BOTSTEIN[1]*

Department of Biology[1] and Center for Cancer Research,[2] Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Microtubules in yeast are essential components of the mitotic and meiotic spindles and are essential for nuclear movement during cell division and mating. The relative importance in these processes of the two divergent α-tubulin genes of the budding yeast *Saccharomyces cerevisiae*, *TUB1* and *TUB3*, was examined through the construction of null mutations and by increasing their copy number on chromosomes and on plasmids. Experiments with null alleles of *TUB3* showed that *TUB3* was not essential for mitosis, meiosis, or mating. Null alleles of *TUB3*, however, did cause several phenotypes, including hypersensitivity to the antimicrotubule drug benomyl and poor spore viability. On the other hand, the *TUB1* gene was essential for growth of normal haploid cells. Even in diploids heterozygous for a *TUB1* null allele, several dominant phenotypes were evident, including slow growth and poor sporulation. This functional difference between the two genes is apparently due to different levels of expression, because extra copies of either gene could suppress the defects caused by a null mutation in the other. We conclude that in spite of the 10% divergence between the products of the two genes, there is no essential qualitative functional difference between them.

The α,β-tubulin heterodimer polymerizes into microtubules, which are functional components of many structures involved in eucaryotic cell motility (42). We have chosen to study the regulation of microtubule assembly and function through the genetic analysis of tubulin in yeast. Microtubules in yeast are essential components of the mitotic and meiotic spindles and are essential for nuclear movement during cell division and mating (12, 13, 27, 28, 34, 49–51, 53, 55, 56). The budding yeast *Saccharomyces cerevisiae* has one essential β-tubulin gene, named *TUB2* (23), and two functional α-tubulin genes, named *TUB1* and *TUB3*. In the preceding paper (35), we described the isolation and sequence of *TUB1* and *TUB3* and the identification of both gene products in yeast microtubules.

The observation of multiple genes that encode α- or β-tubulin has been made previously in many species (for reviews, see references 10 and 29). For example, the fission yeast *Schizosaccharomyces pombe* also has a single β-tubulin gene and two α-tubulin genes (13, 50). The frequent occurrence of such families of closely related genes leads to questions about the functions of the individual members of these groups. Among a wide variety of hypotheses that can explain the presence of multiple genes are two extremes. One is that the gene products have distinguishable functions. Multiple related products might preferentially function in different reactions or locations in the same cell or during specific stages of cell growth or differentiation. At the other extreme, the products might be functionally interchangeable, and any differences between the genes could be explained by different levels of expression. Such hypotheses have stimulated interest in the tubulin field because of the extreme diversity of microtubular structures and the identification of differences in the primary structure of tubulin proteins in the same species (10).

Using the molecular clones of the *S. cerevisiae* α-tubulin genes, *TUB1* and *TUB3*, we tested the relative function of two members of this simple gene family by manipulating their copy numbers in vivo. Given the level of divergence (10%) between the *TUB1* and *TUB3* gene products, one might predict that they have different functions. We show, however, that although the two genes differ markedly in their importance for normal cell growth, either one alone can perform all the functions normally performed by the pair if present at a high enough copy number.

MATERIALS AND METHODS

Bacterial strains and media, electrophoresis, DNA preparations, and hybridizations were as described in the accompanying paper (35), except as noted below.

Strains and media. Media for yeast growth and sporulation were made as described by Sherman et al. (39), except that adenine, uracil, and tryptophan were routinely added to YPD medium after autoclaving. Benomyl, 98.6%, was a gift from O. Zoebisch, E. I. duPont de Nemours and Co., Inc. It was kept as a 10-mg/ml stock in dimethyl sulfoxide at 4°C and was added to warm YPD medium with swirling immediately before the plates were poured. The yeast strains used in this paper were derived from a set of essentially isogenic S288C strains provided by G. R. Fink. The strains used are listed in Table 1.

Preparation of yeast DNA. Small-scale preparations of yeast DNA were routinely prepared by the method of Holm et al. (13a).

Genetic techniques and transformation. Methods of yeast mating, sporulation, and tetrad analysis were as described by Sherman et al. (39). Yeast cells were transformed by the lithium acetate method of Ito et al. (14) as modified by Kuo and Campbell (19) with 1 to 4 μg of plasmid DNA and 50 μg of sonicated chicken blood DNA (Sigma Chemical Co.) as carrier. Transformants were plated on SD medium supplemented with the appropriate nutrients to select cells with the

---

* Corresponding author.

TABLE 1. Yeast strains and plasmids used in this study

Strain[a] or plasmid	Genotype of strains or yeast genes on plasmid
Strains	
DBY2291	*MATα ade2 his3-Δ200 leu2-3,112 ura3-52 gal80::HIS3 (hxk2-Δ202 gal10-Δ120)?*
DBY2292	*MATa his3-Δ200 leu2 lys2-801 trp1-1 ura3-52 ndc1-1 TUB1-LEU2-TUB1*(pRB334) *TUB3-URA3-TUB3*(pRB336)
DBY1811	*MATa ade2 his3-Δ200 leu2-3,112 lys2-801 trp1-Δ1 ura3-52*
DBY1812	*MATα his3-Δ200 leu2-3,112 trp1-Δ1 ura3-52*
DBY1813	DBY1811 × DBY1812
DBY1828	*MATa ade2 his3-Δ200 leu2-3,112 trp1-1 ura3-52*
DBY1829	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52*
DBY1830	DBY1828 × DBY1829
DBY2254	*MATa/MATα ade2/+ his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/+ trp1-1/ trp1-1 ura3-52/ura3-52 TUB1/tub1::HIS3*
DBY2282	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 TUB1-LEU2-TUB1*(pRB334)
DBY2283	*MATa ade2 his3-Δ200 leu2-3,112 trp1-1 ura3-52 TUB3-URA3-TUB3*(pRB336)
DBY2284	*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 TUB3-URA3-TUB3*(pRB336)
DBY2287	*MATa his3-Δ200 leu2-3,112 trp1-1 ura3-52 tub3::TRP1*
DBY1508[b]	*MATa can1-51 hom3 leu2 lys2-801 ura3-52 tub2-104*
DBY1520[b]	*MATα lys2-801 trp1-Δ1 ura3-52 tub2-104*
Plasmids	
pRB306	*TUB1*
pRB300	*TUB3*
pRB334	*TUB1 LEU2*
pRB336	*TUB3 URA3*
pRB298	Codons 169–266 of *TUB3, URA3*
pRB315	*TRP1*
pRB333	*tub3::TRP1*
pRB318	Codons 36–266 of *TUB1, URA3*
pRB328	*HIS3*
pRB332	*tub1::HIS3*
pRB326	*TUB1, URA3, CEN4, ARS1*
pRB327	*TUB1, LEU2,* 2μm plasmid origin of replication
pRB325	*TUB3, URA3, CEN4, ARS1*
pRB316	*TUB3, URA3,* 2μm plasmid origin of replication

[a] Except as noted, all strains listed were constructed for this study.

[b] Source: J. H. Thomas, Massachusetts Institute of Technology.

plasmid. After 3 to 5 days of growth on SD, cells were purified by streaking on YPD and then checked for the presence of the plasmid marker. Cells transformed with centromeric plasmids, which are somewhat unstable (4), or 2μm plasmids, which are very unstable (7), were routinely propagated after transformation on SD with selection for the plasmid marker. Before transfer to sporulation medium, such strains were grown overnight on YPD. Because some tetrads from these strains did not carry the plasmid, larger numbers of tetrads were dissected.

Plasmid constructions. The plasmids pRB306 and pRB300, which are pBR322 (5) derivatives carrying the *TUB1* and *TUB3* genes, respectively, are described in the preceding paper (35; see Fig. 1 therein for restriction map). They were used as the starting materials for all of the plasmid construc-

tions described below. The properties of the plasmids are summarized in Table 1.

The plasmid pRB334 was constructed by inserting a *Sal*I-*Xho*I fragment containing the yeast *LEU2* gene (1a, 2, 30), into the *Sal*I site of pRB306. The resulting *LEU2*- and *TUB1*-containing integrating plasmid was cut with *Xba*I to direct integration (26) to the *TUB1* locus. The plasmid pRB336 was constructed by inserting the *TUB3*-containing *Bgl*II fragment from pRB300 into the *Bam*HI site of the vector YIp5 (8), which contains the yeast *URA3* gene (3, 31, 32). The resulting plasmid could be cut at a unique *Nco*I site to direct integration to the *URA3* locus or at a unique *Kpn*I site to direct integration to the *TUB3* locus.

The *TUB3* partial duplication disruption plasmid pRB298 was made by inserting a small internal *Eco*RI fragment of *TUB3* (codons 169 to 266) into pRB290, a derivative of YIp5 with no *Hin*dIII site (made by filling in the 5' overhangs of *Hin*dIII-cut YIp5 with Klenow enzyme followed by blunt end ligation). This plasmid was cut with *Hin*dIII to direct integration to the *TUB3* locus and with *Nco*I to direct integration to the *URA3* locus. To construct the *tub3::TRP1* insertion-deletion, an *Eco*RI-*Pst*I fragment containing the *TRP1* gene (16, 47, 52) was first ligated into the pBR322-derived polylinker vector pPL7 (J. Mullins, personal communication) to produce pRB315. This step was necessary to separate the nearby replicator *ARS1* (16, 47, 52) from *TRP1* (so that plasmids containing the fragment would be incapable of autonomous replication) and also to provide convenient sites for the next step. The *TRP1* gene was removed from pRB315 with *Sal*I and *Eco*RI and inserted between the *Xho*I site (110 base pairs [bp] before the *TUB3* start codon) and the downstream *Eco*RI site (codon 265) of *TUB3* to produce the plasmid pRB333. To obviate the need for partial digestion, this step was done with a derivative of pRB300 in which the *Eco*RI site of the plasmid vector had been destroyed with Klenow enzyme as above. Digestion of pRB333 with *Bgl*II produced a fragment that was used to replace (33) the normal *TUB3* gene with the version carrying the *tub3::TRP1* insertion-deletion.

To construct the *TUB1* partial duplication disruption plasmid, an internal *Xba*I-to-*Eco*RI fragment (codons 36 to 266) was first inserted between the *Xba*I and *Eco*RI sites of pPL7. A slightly larger piece containing this fragment was removed with *Eco*RI and *Bam*HI and ligated into the YIp5 derivative mentioned above. The resulting plasmid, pRB318, was cut with *Hin*dIII to direct integration to the *TUB1* locus and with *Nco*I to direct integration to the *URA3* locus. To construct the *TUB1* replacement plasmid, a *Bam*HI fragment containing the *HIS3* gene (45, 46) was first ligated into pPL7 to produce pRB328. *HIS3* was excised with *Xho*I and *Cla*I and used to replace the *Xho*I (138 bp before the initiation codon) to *Cla*I (codon 391) fragment of the *TUB1* gene. This plasmid, pRB332, was digested with *Sph*I and *Sac*I to produce a fragment that was gel purified and used to replace intact *TUB1* in the yeast genome.

The *TUB1* centromere plasmid (pRB326) was constructed by ligating a *TUB1* fragment from the *Sph*I site (1.1 kilobases [kb] before the start codon) to a *Bgl*II site (0.5 kb beyond the stop codon) in place of the small *Sph*I-to-*Bam*HI fragment of YCp50 (C. Mann, personal communication; see reference 19 for map). The same *TUB1* fragment was ligated into the analogous sites of the vector YEp21 (8), which carries *LEU2* and the 2μm plasmid origin of replication, to produce pRB327. The *TUB3* centromere plasmid (pRB325) consists of the *TUB3*-containing *Bgl*II fragment from pRB300 ligated into the *Bam*HI site of YCp50. The same *TUB3* fragment

was ligated into the BamHI site of YEp24 (8), which carries URA3 and the 2μm origin, to produce pRB316.

RESULTS

Both α-tubulin genes map to chromosome 13. Some of the most extensively studied gene families in higher organisms, such as histones and globins, are clustered. In yeast, linkage of related genes occurs only occasionally (e.g., the genes involved in galactose metabolism). Both TUB1 and TUB3 were mapped not only so that we could determine whether they were linked but also because knowledge of the map positions of both genes was essential to the genetic analysis described in later sections. Both of these genes were assigned to chromosome 13 by blot hybridization of gels of intact yeast chromosomes (9) (blots kindly provided by T. Stearns).

To allow more precise localization of the genes, both were marked with integrating plasmids, as follows. First, each gene was cloned onto a plasmid that contained a selectable yeast gene but no yeast origin of replication (see Materials and Methods). These plasmids were then cut in the TUB1 or TUB3 sequences to direct integration to these loci (26) and were transformed into yeast cells with selection for the plasmid marker. The plasmid pRB334 was integrated at the TUB1 locus, placing the yeast LEU2 gene (1a, 2, 30) between duplicated copies of TUB1. Similarly, the plasmid pRB336 was integrated at the TUB3 locus, creating a TUB3 duplication marked by the yeast URA3 gene (3, 31, 32). Each of these integration events was confirmed by gel transfer hybridization analysis with restriction enzymes that did not cut in the TUB1 or TUB3 sequence (data not shown). Both events caused the disappearance of the wild-type chromosomal band and the appearance of bands consistent with a single integration event. Since both markers then segregated 2:2 in crosses, we concluded that TUB1 and TUB3 were single genes.

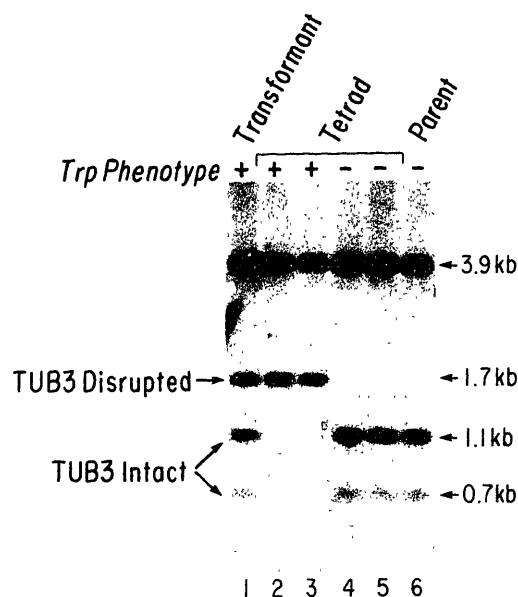Crosses to markers on chromosome 13 revealed linkage of TUB3 and TUB1 to each other and to the NDC1 gene, which

FIG. 1. Gel transfer hybridization of total yeast DNA from strains transformed with the tub3::TRP1 insertion-deletion construction. Total yeast DNA was purified from a diploid transformant (lane 1), from four progeny spores from the diploid (lanes 2 through 5), and from the untransformed parent diploid (lane 6). The DNA was digested with BstEII, and the fragments were separated on an agarose gel. The DNA was transferred to a Zetapor membrane and analyzed with a probe made from a 3.2-kb BglII fragment containing the complete TUB3 gene region. The size and origin of the hybridizing fragments are indicated in the margins. The Trp phenotype is shown above the lanes.

TABLE 2a. Mapping data

Gene	Total no. of tetrads	Percent TT[a]	Distance to CEN13[b]
NDC1	108	38	21
GAL80	127	54	32
TUB1	185	74	
TUB3	188	72	

[a] Crosses were done with a strain carrying a trp1 marker, which is assumed to be completely centromere linked. TT, Tetratype.

[b] Values were determined from the graph in Fig. 2 of Mortimer and Schild (21), assuming a chiasma interference value of 0.3. TUB1 and TUB3 show centromere superrecombination (21).

TABLE 2b. Mapping data

Gene pair	Segregation (no. of tetrads)[a]			Map distance[b]
	PD	NPD	TT	
NDC1-GAL80	78	0	18	9
NDC1-TUB1	54	6	98	42
NDC1-TUB3	14	16	95	
GAL80-TUB1	65	0	77	27
GAL80-TUB3	24	13	87	67
TUB1-TUB3	102	7	131	36

[a] PD, Parental ditype; NPD, nonparental ditype; TT, tetratype.

[b] The map distances were calculated by using equation 3 of Mortimer and Schild (21). No corrections were made to the long map distances.

is on the left arm closely linked to the RAD52 gene (48). Further crosses were carried out with a disruption of the nearby GAL80 gene (24, 58). This disruption was made by replacing the internal BglII fragment in the GAL80 gene with a BamHI fragment containing the HIS3 gene (45, 46) and using the construction to replace (33) (see below for a description of this technique) the chromosomal copy of GAL80 (yeast strain kindly provided by H. Ma). Data from tetrad analysis of these crosses (mostly from DBY2291 × DBY2292) are shown in Table 2. Since neither TUB1 nor TUB3 is closely linked to the centromere, these data unambiguously establish the gene order and map distances (centimorgans) as follows: CEN13–21 cM–NDC1–9 cM–GAL80–27 cM–TUB1–36 cM–TUB3 and extend the map of chromosome 13 by about 30 cM. This increase in the mapped length of chromosome 13 was predicted from estimates of the physical size of the chromosome in the most recent version of the yeast genetic map (22). We have not mapped TUB1 relative to the nearby genes arg81 and SUP79.

Disruption of the two α-tubulin genes. To study the function of these two genes, we made null mutations in each of them. Two methods are commonly used to construct such null mutations in yeast. In the method of Shortle et al. (40), a DNA fragment whose ends are both within the coding sequence of the gene is subcloned into a yeast integrating plasmid (containing no yeast origin of replication). This plasmid carries a selectable yeast gene, which we will refer to as the disruption marker. The plasmid is used to transform a strain that carries a stable chromosomal mutation in the disruption marker gene, with selection for the plasmid-borne

TABLE 3. Tetrad viability from *TUB1* disruption heterozygotes

Disruption marker	Spore viability in tetrads					Total no. of tetrads examined
	$4^+:0^0$	$3^+:1^0$	$2^+:2^0$	$1^+:3^0$	$0^+:4^0$	
URA3	7	8	24	21	14	74
HIS3	8	4	10	32	10	64

copy of this gene. If the plasmid integrates by a single homologous recombination event at the locus of the gene of interest, the result is a disrupted gene which consists of two partial copies of the gene flanking the plasmid sequences containing the disruption marker. Two other common types of recombination event will lead to a strain that can grow under the selection. One is the integration of the plasmid at the locus of the disruption marker. The other is the gene conversion of the chromosomal mutation in the disruption marker gene by the copy of that gene on the plasmid. Neither of these two events leads to a disruption of the gene in question. Integration of the plasmid at a particular locus can be favored greatly by cutting the plasmid with a restriction enzyme in the sequences homologous to that locus (26).

The second method, reported by Rothstein (33), can be used to create a simultaneous insertion and deletion in the gene of interest. An internal fragment of the gene is removed and replaced with a selectable yeast gene, which we will also refer to as the disruption marker, leaving intact the sequences flanking the gene. This insertion-deletion construction is digested with restriction enzymes that cut in the flanking sequences and then purified on gels. This fragment is then used to transform yeast cells that carry a mutation in the disruption marker gene, with selection for expression of the copy on the fragment. Because of the recombinogenic nature of free ends of yeast DNA, the most common integration event is replacement of the chromosomal copy of the gene in question with the insertion-deletion version. Two other events can give rise to a strain that will grow under selection. As above, the disruption marker gene on the DNA fragment can gene convert the chromosomal mutation. The fragment also can circularize (17, 18) and integrate by a single homologous event at either the disruption marker locus or the locus of the gene in question, in neither case disrupting the gene.

Because the potential problems associated with each method are largely independent of those of the other, a very high degree of confidence can be obtained if both methods yield similar results. We used both methods to disrupt *TUB1* and *TUB3*, and in each case the two methods created mutations with indistinguishable phenotypes. Because of the uncertainties mentioned above about integration events from such gene disruption experiments, all events have to be confirmed by the gel transfer hybridization technique developed by Southern (43). From the known restriction maps of the plasmids and genes involved, predictions can be made about the sizes of restriction fragments that will arise from each of the possible integration events. These experiments will also reveal the presence of extra unmarked copies of the disrupted gene that may arise from any of a number of gene duplication events. As shown below, such experiments proved essential to the analysis of disruptions of the *TUB1* gene.

To allow for cases in which the null phenotype of a gene is death, these experiments were done by transforming a diploid strain (homozygous for mutations in the disruption

marker) with the disruption construction. The phenotype of the null mutant can be examined in haploids by sporulating the diploid and dissecting tetrads. The spores carrying the disruption can be identified by testing for the presence of the disruption marker. Two possible results are commonly found from such an experiment. If the gene is essential we expect 2:2 segregation of a recessive lethal linked to the disruption marker. If the gene is not essential, we expect 4:0 viability of many tetrads. Since null mutants are usually recessive, we expect no observable phenotype in the transformed diploid. As described below, *TUB3* null mutations gave the fairly simple result of nonlethality in progeny spores, showing that *TUB3* is not essential for growth. On the other hand, the *TUB1* null allele gave unpredicted results.

Disruption of the *TUB3* gene. The *TUB3* gene was disrupted by both methods described above. In the first method, an internal fragment containing the coding sequence for amino acids 169 to 266 was subcloned into the integrating vector YIp5. Integration of this construction at the *TUB3* locus should produce a partial duplication that contains the yeast *URA3* gene between two incomplete copies of the *TUB3* gene. In the second method, a fragment of the gene from 110 bp before the initiation codon to codon 265 was replaced by the yeast *TRP1* gene (16, 47, 52). This *tub3::TRP1* construction was used to replace the wild-type gene by cutting in the sequences flanking the *TUB3* gene and selecting for *TRP1* gene expression.

Each of these constructions was used to disrupt one copy

FIG. 2. Gel transfer hybridization of total yeast DNA from strains transformed with the *tub1::HIS3* insertion-deletion construction. Total yeast DNA was purified from the untransformed parent diploid (lane 1), from a diploid transformant (lane 2), and from four progeny spores from each of two tetrads from the diploid (lanes 3 through 10). The DNA was digested with *Bgl*II and analyzed with a probe made from a 1.9-kb *Bgl*II fragment containing most of the *TUB1* coding sequence. The size and origin of the hybridizing fragments are indicated in the margins. The His phenotype is shown above the lanes.

of the *TUB3* gene in a diploid homozygous for the *ura3* or *trp1* disruption marker (strain DBY1813). The diploids were then sporulated and tetrads were analyzed. In both cases, many tetrads had all four spores viable, and the disruption marker segregated 2:2. As described above, such apparently simple results must be confirmed by restriction analysis of genomic DNA. Figure 1 shows gel transfer hybridization experiments that demonstrate the disruption of the *TUB3* gene by the *TRP1* construction. DNA was prepared from a Trp+ diploid and four spore colonies from a progeny tetrad, digested with *Bst*EII, run on an agarose gel, and analyzed with a *TUB3* probe. The wild-type gene yielded fragments of 0.7, 1.1, and 3.9 kb, while the disruption was expected to yield bands of 1.7 and 3.9 kb. A 4.0-kb band due to cross-hybridization with *TUB1* was obscured by the 3.9-kb *TUB3* band. As expected, the diploid showed both sets of bands, the Trp⁻ spores and the pretransformation diploid showed only wild-type bands, and the Trp⁺ spores showed only the disruption-specific bands. Similar experiments with the partial duplication disruption gave analogous results. From these results we conclude that the *TUB3* gene is not required for germination and mitotic growth. Strains containing the disruption were able to mate, and the resulting diploids, although they grew more slowly than wild type or *TUB3⁻* heterozygotes, sporulated efficiently. These results indicate that a functional copy of *TUB3* is not essential for any of the known microtubule-dependent processes in yeast.

The spores with the *TUB3* null mutations were examined for possible nonlethal phenotypes. Based on observations of colony size after 2 days of growth at 30°C on rich plates, spores carrying either of the two disruptions grew at about the same rate as the wild type (in liquid medium, the mutant cells grew more slowly; see Discussion). They also grew at a wide variety of temperatures, from 11 to 37°C. Nevertheless, both disruptions showed two phenotypes, linked, in each case, to the disrupted gene. First, as shown in Table 5, lines 9 and 10, these strains were hypersensitive to the antimicrotubule drug benomyl (11, 25, 38), as are some α-tubulin mutants of *Schizosaccharomyces pombe* (50, 53). Second, spores that lacked *TUB3* had lower viability than their wild-type sisters. Of spores carrying a null allele, 64% were viable compared with 97% of the wild-type spores (based on counts from 125 tetrads from strains heterozygous for the disruption). One possible explanation for the poor viability of *TUB3⁻* spores would be that these spores could only germinate and grow in the presence of some suppressor gene that arose in the diploid transformant. Thus, the apparently random lethality would be explained by the segregation of the unlinked suppressor gene. This possibility was ruled out by two experiments. First, the *TUB3* gene disruption could be made by direct transformation of haploid cells. Second, a cross was carried out between a *TUB3⁻* strain (postulated genotype *TUB3⁻ SUP*) and a *TUB3⁺* strain predicted to contain the suppressor (postulated genotype *TUB3⁺ SUP*). This strain should have contained the hypothetical suppressor because it arose from a tetrad from the same diploid as the *TUB3⁻* strain, but one in which both *TUB3⁻* spores died. Even though the suppressor should be homozygous in this diploid, the *TUB3* null still gave similar levels of inviable spores. Thus, if the suppressor exists, we can at least say that it does not segregate in a Mendelian fashion.

Although *TUB3* is not essential for any known microtubule-dependent process, the phenotype associated with the disruptions along with the presence of the *TUB3*

TABLE 4. Segregation of Ade4 in crosses to potentially disomic strains

Cross	Segregation[a] of Ade4⁺:Ade4⁻ in:					
	Complete tetrads[b]			Tetrads with 3 viable spores		
	4:0	3:1	2:2	3:0	2:1	1:2
1	5	2	0	4	1	0
2	1	5	0	4	3	0
3	0	1	0	7	4	0
4	3	3	1	2	1	0
5	0	0	0	3	2	0
6	0	2	0	3	4	0
7	0	0	8	0	0	0
8	0	0	7	0	1	0

[a] Crosses 1 through 6 were done between normal haploid strains carrying an *ade4* allele and *ADE4⁺* strains that carried both normal and disrupted *TUB1* (as determined by gel transfer hybridization analysis). Crosses 7 and 8 were control crosses of the *ade4* strains to normal *ADE4⁺* haploids. Progeny tetrads were scored for the segregation of the Ade4 phenotype and other markers.

[b] In all of the crosses except 6, greater than 75% of the tetrads contained at least three viable spores. In cross 6, only 50% of the tetrads contained at least three viable spores.

gene product in microtubules (35) show that the *TUB3* gene is expressed.

Disruption of the *TUB1* gene. The *TUB1* gene was disrupted by the same two methods. A fragment containing the coding sequence from amino acid 36 to 266 was subcloned into YIp5 to produce the partial duplication disruption construction carrying the *URA3* gene. The second disruption was produced by replacing the sequences from 138 bp before the initiation codon to codon 391 with the yeast *HIS3* gene (45, 46) and integrating the construction (denoted *tub1::HIS3*) so that it replaced the wild-type copy of the *TUB1* gene. As before, both types of disruptions produced similar results.

Both constructions were introduced by DNA transformation into the diploid strain DBY1830 with selection for the *URA3* or *HIS3* disruption marker. As a control, the partial duplication disruption plasmid was also transformed into the same strain after being cut in the *URA3* sequence to direct integration to the *URA3* locus. Transformants were purified by streaking on rich (YEPD) medium and sporulated, and tetrads were dissected.

We were surprised to find that the heterozygous null mutations showed two dominant effects in the diploid transformants. First, these strains grew more slowly than the control *URA3* integrants after being streaked onto rich plates. Second, they sporulated at a significantly lower efficiency than the controls (14% recognizable tetrads or triads compared with 49% in control transformants). When tetrads from these strains were dissected, the spores showed very poor viability. As shown in Table 3, the viability was worse than 50% in many tetrads. Most recovered spores (71%) did not contain the disruption marker. A significant fraction (29%), however, did contain the *URA3* or *HIS3* marker, and a large number of complete tetrads were recovered. All of the spores which contained the disruption marker grew more slowly than their sisters without the marker.

At first glance, this result might be taken to indicate that *TUB1*, like *TUB3*, is partially dispensable in normal haploid strains. This was not the case. The experiments described below showed that all strains that contained the disruption marker had chromosomal abnormalities that suppressed the lethality caused by the *TUB1* null mutation. The conclusion
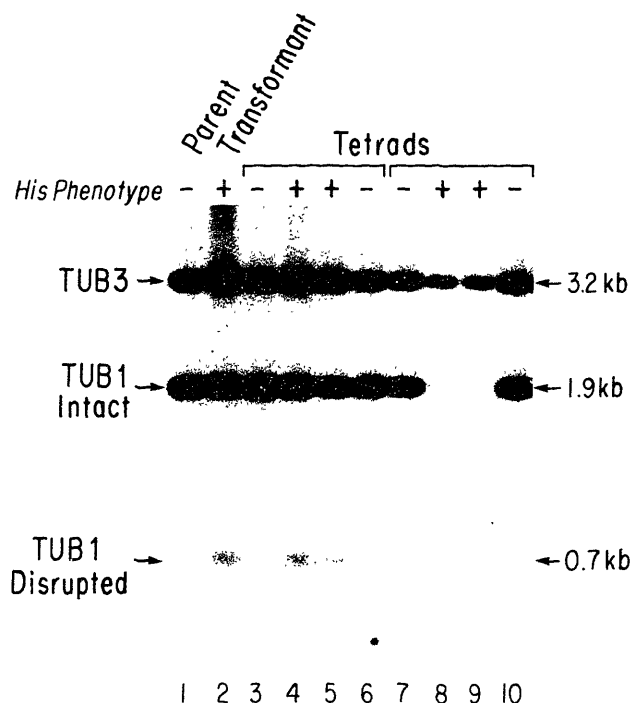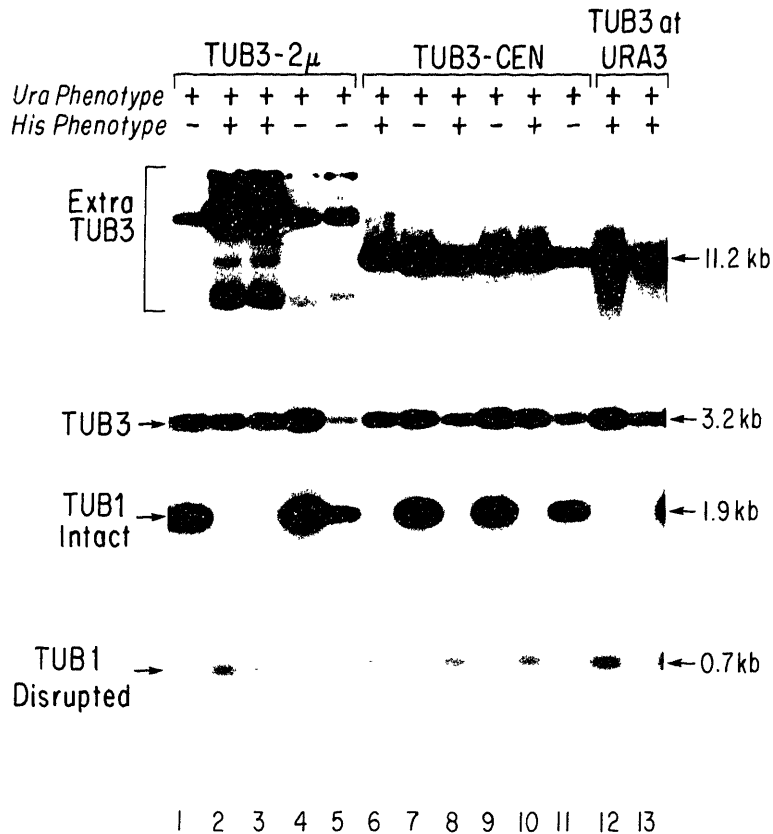
FIG. 3. Gel transfer hybridization of total yeast DNA from strains heterozygous for the *tub1::HIS3* insertion-deletion construction transformed with plasmids containing extra copies of *TUB3*. Total yeast DNA was purified from four spores of a tetrad from the diploid transformed with a plasmid (pRB316) containing *TUB3*, *URA3*, and the 2μm plasmid origin of replication (lanes 1 through 4) and from a wild-type strain transformed with the plasmid (lane 5). Lanes 6 through 10 show DNA purified from spores from the heterozygous diploid transformed with a plasmid (pRB325) containing *TUB3*, *URA3*, *CEN4*, and *ARS1*, and lane 11 shows DNA from a wild-type strain transformed with pRB325. Lanes 12 and 13 show DNA from spores from the heterozygous diploid transformed with an integrating plasmid (pRB336) containing *TUB3* and *URA3* and cut to direct integration to the *URA3* locus. The DNA was digested with *Bgl*II and analyzed with a probe made from a 1.9-kb *Bgl*II fragment containing most of the *TUB1* coding sequence. The size and origin of the hybridizing fragments are indicated in the margins. The Ura and His phenotypes are shown above the lanes.

we reached is that *TUB1* is essential in normal haploid strains.

To investigate the genotype of the spores containing the disruption marker, gel transfer hybridization experiments were performed on genomic DNA made from these strains. Figure 2 shows representative blots of DNA from the *HIS3* replacement experiment digested with *Bgl*II and probed with the 1.9-kb *Bgl*II fragment containing most of the *TUB1* coding sequence. The pretransformation diploid contained the expected 1.9-kb band from *TUB1* and also a 3.2-kb band from *TUB3* due to cross-hybridization between the sequences (Fig. 2, lane 1). The replacement in a single copy of the gene is expected to cause the disappearance of the 1.9-kb band from that gene and the appearance of a 0.7-kb band (due to a *Bgl*II site in the insert) homologous to the probe. Lane 2 shows a transformed diploid with bands representing both intact and replaced *TUB1*. Lanes 3 through 6 show DNA from a complete tetrad that arose from the diploid. As expected, His3⁻ spores showed the wild-type pattern. Surprisingly, the His3⁺ spores showed bands corresponding to both intact and replaced *TUB1*. Such a result can be explained by an event that caused a duplication of the *TUB1* gene so that the His3⁺ spores received two copies of the

information in the region of *TUB1*. Possible causes for such an event include translocation, duplication of the *TUB1* region through unequal mitotic crossing over, or the gain of an extra chromosome by nondisjunction. Since tubulin is intimately involved in chromosome segregation and the diploid had less than the normal number of α-tubulin genes, we considered the last possibility most likely.

To test the hypothesis of nondisjunction, these two His3⁺ strains, along with four analogous strains from the *URA3* partial duplication disruption experiment, were crossed to a strain containing an *ade4* marker (located 280 cM from *TUB1* at the other end of chromosome 13). If the aberrant strains were disomic for chromosome 13, the expectation would be that the Ade4⁺ phenotype would frequently segregate 4:0 or 3:1 in tetrads from the trisomic +/+/− diploid. Since *ADE4* is distant from its centromere, the expected phenotypic segregation ratios for Ade4⁺:Ade⁻ from such a diploid are 4 of 15 4:0, 10 of 15 3:1, and 1 of 15 2:2. This calculation is based on two assumptions. First, we assume that all three homologous chromosomes of the trisome pair together at meiosis I, as shown by Shaffer et al. (37). Second, unlike Shaffer et al. (37), we assume that all three chromosomes take part in recombination in the trivalent complex. If the

TABLE 5. Growth of tubulin mutants on benomyl

Haploid genotype[a]			Growth rate[b] at benomyl concn (μg/ml):							
α		β (TUB2)	5	15	25	35	45	55	65	75
TUB1	TUB3									
2×	1×	tub2-104 ⎫								
1×	2×	tub2-104 ⎬	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
1×	1×	tub2-104 ⎭								
1×	0	tub2-104	+ + +	+ + +	+ +	+	+	p	p	p
2×	2×	1× ⎫								
2×	1×	1× ⎬	+ + +	+ + +	+ +	+	+/−	+/−	−	−
2×	0	1× ⎬								
1×	2×	1× ⎭								
1×	1×	1×	+ + +	+ +	+	+/−	−	−	−	−[i]
1×	0	1×	+/−	−	−	−	−	−	−	−

[a] Genotypes are identified as follows: 2×, chromosomal duplication of gene; 1×, wild-type gene; 0, null allele; tub2-104, allele of TUB2 isolated for resistance to benomyl (49).

[b] Cells were suspended in water and transferred to YPD plates containing various concentrations of benomyl with a multipronged innoculating device. Growth rates at 30°C were scored as follows: + + +, growth into a thick, uniform patch of cells after 1 day; + +, growth into a thick, uniform patch of cells after 2 days; +, growth into a thick, uniform patch of cells after 3 days; +/−, slow, nonuniform growth after 3 days; p, heavy papillated growth after 2 days; −, no growth of lawn but some light papillated growth in most cases.

Ade4+ parent were trisomic for chromosome 13, the expected segregation ratios from the +/+/+/− diploid, based on analogous assumptions, would be 6 of 7 4:0, 1 of 7 3:1, and 0 of 7 2:2. As shown in Table 4, all six strains showed a high frequency of 4:0 and 3:1 segregation for Ade4+, consistent with the disome model but (except possibly for cross 1) not the trisome model. Markers on several other chromosomes segregated 2:2. We conclude that these strains were disomic for chromosome 13 but probably normal for most of the rest of the yeast genome. Of the 20 marker-positive spores that were analyzed in Southern blot experiments, 16 showed bands corresponding to both intact and disrupted TUB1 genes and probably represented additional examples of the disomic genotype. The slow growth rate of these spores was presumably caused by the gene imbalances caused by the disomic genotype.

The fact that any complete tetrads were recovered suggested that the diploid that gave rise to the type of tetrad shown in Fig. 2, lanes 3 through 6, was trisomic for chromosome 13, containing two copies with intact TUB1 and one copy with disrupted TUB1. The frequent recovery of such tetrads could be explained by an increased rate of nondisjunction events in the heterozygous diploid transformant (see below). Because of the observed slow growth rate of the transformants, presumably caused by insufficient amounts of α-tubulin, clones of cells that acquired an extra wild-type copy of chromosome 13 might grow faster and take over the population. This hypothesis was supported by our observation that disomic spores were recovered more frequently from strains that were grown for longer periods of time after transformation. Tetrads from such diploids might also be preferentially selected for dissection because of increased sporulation efficiency in a background of poor sporulation of the heterozygote. Nondisjunction events involving other chromosomes might not have been observed because of the absence of selective pressure or because of the lack of diagnostic markers in crosses.

So far, we have explained 16 of the 20 spores with the disruption marker that were examined by gel transfer hybridization analysis. The four spores that remained, including two from a complete tetrad, showed the complete absence of an intact TUB1 gene. Southern blots of DNA from the spores of this tetrad are shown in Fig. 2, lanes 7 through 10. The intact gene and the HIS3 replacement appeared to segregate normally in this tetrad. Because of the rarity of such spores, however, we were suspicious that they might not be normal haploids. Since TUB3 is on chromosome 13, one likely mechanism for suppression of the lethality associated with the TUB1 null allele would be the accumulation of additional copies of that chromosome and therefore additional copies of TUB3. To test this model, the two TUB1− spores from the complete tetrad were crossed to the ade4 strains used above. One of the crosses gave normal segregation of non-chromosome 13 markers, while chromosome 13 markers segregated aberrantly. All spores recovered, including ones from complete tetrads, were both Ade4+ and His3+. These results suggest that the original strain was haploid but had more than two copies of chromosome 13. The other cross gave tetrads with very poor viability and very slow growth. Non-chromosome 13 markers segregated aberrantly. As before, all recovered spores were both Ade4+ and His3+. These results suggest that in addition to more than two copies of chromosome 13, the strain contained other chromosomes in multiple copies. Since these two spores were recovered as sister spores from a complete tetrad, it is likely that the parent diploid was polysomic for chromosome 13, containing at least two copies with replaced TUB1 and at least one copy with intact TUB1. Further aberrations in chromosome number may have arisen during the growth of the spores into colonies.

The other two TUB1− spores, which we assume contained multiple copies of TUB3, were recovered from tetrads in which the other three spores were dead and may have arisen by nondisjunction events during meiosis in the absence of

sufficient amounts of α-tubulin. The high rate of spore death in these and other tetrads from the *TUB1⁻* heterozygotes could be explained by chromosomal imbalances in many spores (21). This model is supported by the observed defect in sporulation of the heterozygous *TUB1* null strains. Alternatively, excess spore death could be caused by sporulation of diploids/monosomic for any one of the chromosomes. This model is supported by the increased rate of chromosome loss in *TUB1⁻* heterozygotes (see discussion).

The above results suggest that the *TUB1* gene is essential either for germination or for growth of normal haploid strains. To determine whether the defect was only in germination, we tested for lethality of the *TUB1* null mutation by direct transformation of haploid strains. The two haploid parents of DBY1830 (strains DBY1828 and DBY1829) were transformed in parallel with DBY1830 with the two disruption plasmids. As a control, the partial duplication disruption plasmid was also transformed after having been cut to direct integration to the *URA3* locus. While the control gave approximately equal numbers of transformants in both the haploids and the diploid, the disrupting plasmids gave about 20-fold fewer transformants in the haploid strains. All 12 haploid transformants recovered from transformations with the disrupting plasmids were subjected to the type of gel transfer hybridization analysis shown in Fig. 2, and all showed bands representing intact *TUB1* and some showed additional bands. These transformants therefore represent gene conversions of the *HIS3* or *URA3* locus or integration of the plasmid so that the *TUB1* gene was not disrupted. We conclude that the *TUB1* gene is essential under our conditions for the growth of normal haploid strains.

Dosage relationships between *TUB1* and *TUB3*. The results above show that while the *TUB1* gene alone is able to perform all functions necessary for known microtubule-dependent processes in yeast, a single copy of *TUB3* cannot. The *TUB1* null mutation causes death, while the *TUB3* null mutation causes several relatively minor defects, including benomyl hypersensitivity and poor spore viability. The question remains whether these facts are the result of unequal expression of the two genes or of functional differences between the products of the two genes. This question was addressed by asking whether extra copies of either gene could complement the deficiency produced by a null mutation in the other. If the difference between the genes simply reflected differences in the amount of protein, one might expect extra copies of one gene to suppress a null mutation in the other. This was possibly the case above, when extra copies of chromosome 13 suppressed the lethality of a *TUB1* null allele, perhaps due to the increased gene dosage of *TUB3*. We show below that extra copies of either gene can suppress all of the obvious defects produced by a null mutation in the other.

Extra copies of the two genes were supplied in three different ways. The first was to integrate one extra copy of a gene into another chromosome marked by a nutritional prototrophy. The *TUB3* gene was subcloned onto the integrating vector YIp5 and was directed to integrate at the *URA3* locus by cutting the plasmid (pRB336) in the *URA3* sequence, transforming *ura3* yeast cells, and selecting for uracil prototrophy. The second method of supplying extra copies of the two genes was to subclone them onto a plasmid containing a functional yeast centromere and an origin of replication; these plasmids are fairly stable in yeast (about 1% loss per cell division) and maintain low copy number (4). Both genes were inserted into the vector YCp50 (C. Mann, personal communication; see reference 19 for map) contain-

ing *CEN4*, *ARS1*, and the *URA3* gene as the selectable marker. Finally, both genes were inserted into vectors containing the 2μm plasmid origin of replication; these plasmids are unstable and maintain high copy number (7). The *TUB3* gene was inserted into the vector YEp24 (8), which contains the *URA3* gene, and the *TUB1* gene was inserted into the vector YEp21 (8), which contains the *LEU2* gene.

Before using these constructions in dosage experiments, several controls were done. Because of the known lethality associated with 2μm-borne copies of the β-tubulin gene of either *S. cerevisiae* (J. Thomas, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984) or *Schizosaccharomyces pombe* (13), we were concerned that the constructions that placed the α-tubulin genes on 2μm plasmids might kill the cells. Plasmids that have high-copy-number lethality generally transform yeast cells at low frequency. The few transformants isolated are generally due to gene conversion events or to integration of the plasmid into a chromosome instead of replicating as a free, high-copy-number plasmid. The *TUB1* and *TUB3* plasmids, however, transformed with the same efficiency as control vectors without inserts (data not shown). They also showed the low stability of transmission generally found with freely replicating 2μm plasmids (see below). This result is similar to that found previously by Toda et al. (50), who showed that both of the *Schizosaccharomyces pombe* α-tubulin genes can exist on high-copy-number plasmids without killing the cells.

We show below that extra copies of *TUB3* can suppress the lethality associated with the *TUB1* null mutation. As a control, a similar set of experiments, which gave analogous results, was carried out in parallel, to show that the *TUB1* gene on plasmids could complement the chromosomal *TUB1* disruption (data not shown). This result confirms that the cloned fragments used contain all of the sequences necessary for *TUB1* gene function.

Suppression of the *TUB1* null allele by extra copies of *TUB3*. All three *TUB3* constructions mentioned above were used in attempts to suppress the *TUB1* gene disruptions. Because the *TUB1* null mutation had a recessive lethal phenotype, the simple experiment of transforming a *TUB1* null strain with extra copies of *TUB3* could not be done. Such experiments must be done by transforming diploid strains with the appropriate constructions and allowing the meiotic machinery to segregate plasmids and mutations into spores. Therefore, each construction was transformed into a diploid strain (DBY2254) in which one copy of the *TUB1* gene had already been replaced with the *tub1::HIS3* construction mentioned above. The resulting diploids were sporulated, and tetrads were dissected. Candidate spores for cross-complementation were chosen on the basis of the presence of the *HIS3⁺* marker, the presence of the marker associated with the extra tubulin gene, and the absence of the slow growth previously correlated with the extra chromosomal copy of *TUB1*. No viable spores were recovered that carried the *HIS3* disruption without evidence (slow growth) of extra copies of *TUB3* or *TUB1*. Gel transfer hybridization experiments were then performed to confirm the presence of the extra gene(s) and to rule out the presence of extra, unmarked copies of the *TUB1* gene.

Experiments with all three of the *TUB3* constructions gave candidate spores for cross-complementation. Figure 3 shows gel transfer hybridization analysis of DNA from several spore colonies digested with *Bgl*II and probed with the *TUB1* *Bgl*II fragment. Several complete tetrads were recovered from the heterozygous *TUB1* null strain transformed with

TUB3 on the 2μm plasmid (pRB316) (Fig. 3, lanes 1 through 4). In this tetrad, all spores grew equally well, chromosomal markers segregated 2:2, and Ura3$^+$ segregated 4:0. The plasmid did not contain a BglII site and so was expected to give several high-molecular-weight bands representing open and closed circular forms of the plasmid and also larger bands due to recombination with endogenous 2μm plasmid. All four spores showed plasmid bands; the His3$^-$ spores had only intact TUB1, and the His3$^+$ spores had only disrupted TUB1. The intensity of the plasmid bands strongly suggested that the TUB3 plasmid could suppress the TUB1 null allele, because it was retained in all viable TUB1$^-$ cells (see below). Lanes 6 through 10 show similar results from spores from the TUB1 heterozygous null strain transformed with TUB3 on the centromere plasmid (pRB325). All grew well and showed the expected plasmid band at 11.3 kb. The His3$^-$ spores had only intact TUB1, while the His3$^+$ spores had only disrupted TUB1. Lane 11 shows a wild-type strain transformed with pRB325. Finally, lanes 12 and 13 show two candidate spores from the TUB1 heterozygous null transformed with TUB3 on the integrating plasmid directed to the URA3 locus. Both grew well, implying that they did not have an extra copy of chromosome 13; both showed the 11.2-kb band expected from a single integration event of the TUB3 plasmid at the URA3 locus; both lacked intact TUB1. In complete tetrads from this dissection (6 of 16), the extra copy of TUB3 segregated 2:2, as expected for a single gene. From these experiments we conclude that as little as one extra copy of TUB3 can suppress, to some degree, the lethality produced by the TUB1 null allele.

To confirm that an extra copy of chromosome 13 was not required for the suppression, one of the candidate spores, whose DNA is shown in Fig. 3, lane 3 (genotype tub1::HIS3, TUB3 on 2μm plasmid, ADE4$^+$ his3), was crossed to a strain carrying ade4 and his3 mutations. Both Ade4$^+$ and His3$^+$ segregated 2:2, confirming the presence of only a single copy of chromosome 13 in the ADE4$^+$ parent.

We next wanted to confirm that the centromeric and 2μm plasmids existed in the cells as free replicons and that they were essential for the suppression observed. To do this, the stability of the plasmid-borne marker was examined in strains in which the plasmid was thought to supply an essential function, and also in their meiotic sisters, in which the plasmid was presumably dispensable. As mentioned above, we were concerned that the construction that placed the TUB3 gene on a 2μm plasmid might have integrated a single copy into a chromosome instead of replicating as a free, high-copy-number plasmid. Cells were grown to saturation in rich medium, plated on rich plates, and replica plated to test for the presence of the plasmid marker. The 2μm plasmid was present in all cells with no functional copy of TUB1, while it was present in only 2% of the cells with an intact TUB1 gene. This result is illustrated by the intensity of the plasmid bands in Fig. 3, lanes 1 through 4. The plasmid bands in the His3$^+$ spores were much more intense because the same cultures that were used to check plasmid stability were used to prepare DNA (i.e., they were grown in rich medium without selection for the URA3 plasmid marker). For comparison, lane 5 shows a wild-type strain transformed with pRB316 and grown with selection for Ura3$^+$. The chromosomal bands were much less intense because less DNA was loaded, but the plasmid bands were of approximately equal intensity to those in the His3$^-$ lanes. The centromeric plasmid gave analogous results. It was present in 100% of TUB1$^-$ cells but was present in only 65% of TUB1$^+$ cells after overnight growth in rich medium. From

these results, we conclude that the plasmids exist as unstable autonomous replicons. The loss of the plasmid from a TUB1$^-$ cell leads to the death of the cell, giving the appearance of plasmid stability.

To extend the suppression analysis to other known microtubule functions, we examined the mating and sporulation behavior of TUB1 null strains. Two strains carrying the chromosomal TUB1 null allele and TUB3 on a centromere or 2μm plasmid could be mated. The resulting diploids sporulated efficiently and segregated chromosomal markers 2:2, demonstrating that extra TUB3 genes can suppress the TUB1 null allele for all of the known functions of microtubules in yeast. Such strains also grew at a wide variety of temperatures, from 11 to 37°C.

Complementation of the TUB3 null. We next wanted to see whether an extra copy of the TUB1 gene could suppress the benomyl hypersensitivity and low spore viability associated with the TUB3 disruption. A strain (DBY2287) carrying the tub3::TRP1 disruption was crossed to a strain (DBY2282) with a duplication of the TUB1 locus containing two full copies of TUB1 marked with the LEU2 gene. Tetrads were dissected, and spore colonies were patched onto several different concentrations of benomyl in rich plates and onto supplemented minimal plates so the duplication marker and the disruption marker could be followed. The surprising result was that the TUB1 duplication with or without the TUB3 disruption grew better than the wild type on concentrations of benomyl from 15 to 55 μg/ml (Table 5, compare lines 6, 7, and 9). Thus, an extra copy of TUB1 more than compensated for the loss of TUB3. In the presence of the TUB1 duplication, the poor spore viability associated with the TUB3 disruption did not occur (0 of 43 spores of this genotype died, from diploids heterozygous for the TUB1 duplication and the TUB3 disruption).

Since extra copies of either gene can cure all of the known defects produced by a null mutation in the other, we conclude that there is no detectable qualitative functional difference between the proteins produced by the two genes.

Effect of α-tubulin gene dosage on benomyl resistance. To complete the analysis of the effect of α-tubulin gene dosage on resistance to benomyl, several crosses were done between strains carrying various tubulin mutations. First, a cross was done between two strains carrying extra integrated copies of TUB1 and TUB3 (DBY2282 and DBY2283). Crosses were also done between extra-copy strains (DBY2282 and DBY2284) and a strain carrying the TUB3 null mutation (DBY2287). Finally, crosses were done between these strains and strains (DBY1508 and DBY1520) carrying the β-tubulin benomyl resistance allele tub2-104 (49). The resulting tetrads were tested for resistance to several concentrations of benomyl. Table 5 shows the resulting spore genotypes grouped by easily distinguishable phenotypic classes. The most resistant class was tub2-104 in combination with at least one copy of both TUB1 and TUB3. These strains grew quite well at concentrations near the solubility limit of benomyl in the plates. The next class was tub2-104 with the TUB3 null. These strains papillated heavily at the higher benomyl concentrations, probably due to nondisjunction events which led to higher TUB1 gene dosage. The third class consisted of strains that had a higher α-tubulin gene dosage than the wild type, including those that had two copies of TUB1 and none of TUB3, in addition to those with normal TUB1 and two copies of TUB3. These strains had a definite growth advantage over the wild type, but within the class no consistent distinctions were found. Finally, the strains with one copy of TUB1 and no copies of

TUB3 were the most sensitive to benomyl. Thus, a higher dosage of α-tubulin improves the ability of cells to grow in the presence of benomyl, while lower than normal doses impair this ability, even if the cells carry a resistance allele in the β-tubulin gene.

DISCUSSION

In the preceding paper (35), we reported the isolation of two α-tubulin genes from the budding yeast *S. cerevisiae* by cross-species homology to genes from the distantly related fission yeast *Schizosaccharomyces pombe*. In this paper, we have analyzed the functions of these two genes by varying their functional copy number from zero to many copies. The data presented in these papers demonstrate conclusively that both genes encode functional proteins, but that there are significant differences between the two genes. In single copy, the genes differ markedly in their ability to make, alone, sufficient α-tubulin for cell growth. When their copy number is increased, however, either gene alone is sufficient to provide all of the functions normally ascribed to microtubules in yeast.

TUB1 is essential for growth of normal haploid strains. As anticipated from the fact that the *TUB1* gene apparently makes more assembled protein product than *TUB3* (35), mutations in this gene showed more drastic phenotypes. Even in diploid strains heterozygous for a *TUB1* null allele, several dominant phenotypes were clearly evident, including slow growth and poor sporulation. The *TUB1* gene is essential for the growth of normal haploid strains, as evidenced by our failure to recover any viable normal haploids with the null mutation by either of two methods, sporulation of heterozygous diploids or direct transformation of haploids. Strains were recovered that contained the *TUB1* null mutation, but most of these also contained an extra copy of chromosome 13 carrying an intact copy of both *TUB1* and *TUB3*. Strains were also recovered that contained no intact *TUB1* gene. All of these, however, contained extra copies of chromosome 13 and thus extra copies of *TUB3*. The high rate of recovery of aberrant spores from *TUB1* null heterozygotes can be explained by several models. *TUB1* null heterozygotes grew more slowly than wild-type diploids so that variants with an increased copy number of *TUB1* may take over populations of growing cells. We have preliminary evidence that these strains show an increased rate of chromosome nondisjunction (unpublished data), which would raise the frequency of variants that had higher *TUB1* and *TUB3* dosage through the gain of an additional copy of chromosome 13. Another possible factor in the high rate of abnormal spore recovery may be due to the fact that *TUB1* null heterozygotes sporulate badly. Variants with increased α-tubulin gene dosage might be preferentially picked for tetrad dissection because of better sporulation efficiency. Alternatively, aneuploid spores could have arisen because of aberrant meiosis in the presence of insufficient amounts of α-tubulin (13). These models are not mutually exclusive.

TUB3 is not essential. While null mutations in the *TUB3* gene did not prevent cell growth, mating, or sporulation, the null mutant strains did show phenotypes one might reasonably anticipate from tubulin mutants. One phenotype was hypersensitivity to the antimicrotubule drug benomyl, as has been observed in α-tubulin mutants from other species (25, 50, 53). Another interesting phenotype was the higher death rate of spores that carried the *TUB3* null allele. Preliminary evidence indicates that this phenomenon may be specific for spore germination; stationary-phase mutant cells showed

significantly greater viability than the spores and about the same viability as wild-type cells (data not shown). Observations of the dead spores have shown that most begin to grow and form buds but then arrest growth with a wide variety of aberrant morphologies (data not shown).

TUB3 mutant cells appeared to grow at about the same rate as wild-type cells during colony formation on plates. In liquid culture, however, the mutant had a slightly longer generation time (2.16 versus 1.83 h at 26°C in rich medium). Finally, diploid strains homozygous for the *TUB3* null grew slowly, and we have preliminary evidence that they show an increased rate of chromosome loss compared with heterozygous and wild-type diploids (unpublished data), as might be expected from mutants with a partially defective mitotic spindle.

Either protein alone can perform all functions. The major conclusion of this paper is that either of these two α-tubulin genes can perform all of the functions that have been attributed to microtubules in the yeast life cycle, namely mitosis, meiosis, and nuclear fusion during mating (12, 13, 27, 28, 34, 49–51, 53, 55, 56). Experiments with the *TUB3* null strains proved that a single copy of *TUB1* per haploid genome was sufficient for all of these processes. Experiments with the *TUB1* null strains showed that extra copies of *TUB3* on the chromosome or on plasmids were necessary to carry on these three processes in the absence of *TUB1* function. In all of the experiments, no evidence was found that the *TUB1* and *TUB3* gene products had mutually exclusive functions. In spite of a 10% divergence in the primary structure of their products, the differences between the genes can be explained most simply by differences in the regulation of their level of expression.

Our results join a growing body of evidence that in some species multiple tubulin genes do not encode proteins with restricted functions. In *Drosophila melanogaster* spermatogenesis, a single sperm-specific β-tubulin gene has a role in the meiotic spindles, in nuclear shaping by cytoplasmic microtubules, and in the sperm flagellar axonemes (15, 29). Two recent reports (20, 54) demonstrate that in *Aspergillus nidulans*, the β-tubulin gene normally used for conidiation can be replaced by the vegetative gene. In the unicellular flagellate *Chlamydomonas reinhardi*, the two β-tubulin genes code for identical proteins and the two α-tubulin genes code for proteins that differ at only two positions (41, 59). The fission yeast *Schizosaccharomyces pombe* has only one β-tubulin gene (13), and one of its two α-tubulin genes (50) is not essential (1). Extra copies of the nonessential gene can suppress conditional lethal mutations in the essential gene (50). Finally, a recent report demonstrates that the divergent sequence of a chimeric chicken-yeast β-tubulin gene apparently places no restrictions on its ability to function in cultured mouse cells (6).

There have been demonstrations of electrophoretically distinct tubulins from different structures in the same cell (for an example, see reference 44). There is also ample evidence that multiple tubulin variants coexist in species and in individual cells. Divergent tubulin genes are expressed in tissue-specific development programs in higher organisms (for reviews, see references 10 and 29). Compared to these species, yeast have fewer types of functionally distinct microtubules. Our results therefore may represent an oversimplification of possible functions of divergent tubulin molecules in higher organisms.

Mechanism of benzimidazole drugs. These experiments also have implications for the mechanism of action of benzimidazole antimicrotubule drugs (11, 25, 38). We have

shown that an increased dosage of α-tubulin genes can slightly increase the resistance of wild-type cells to the drug benomyl. On the other hand, decreased gene dosage leads to increased sensitivity even in the presence of a β-tubulin resistance mutation. The hypersensitivity result is consistent with previous evidence from other organisms. Null mutations of the nonessential *Schizosaccharomyces pombe* α2 tubulin gene cause hypersensitivity to benzimidazole drugs (1). Mutations that lead to thiabendazol hypersensitivity in *S. pombe* are found mostly in α- and β-tubulin genes, but mutations to resistance are found mostly in the β-tubulin gene (13, 34, 50, 51, 53, 57). In *S. cerevisiae*, mutations to benomyl resistance are found almost exclusively in the *TUB2* β-tubulin gene (49). In *Aspergillus nidulans*, mutations to benomyl resistance are found in a β-tubulin gene (38), and mutations to hypersensitivity are found in an α-tubulin gene (25). These regularities in mutation effects almost certainly reflect the way in which the tubulin heterodimer or microtubules interact with the drugs. The binding site(s) for the benzimidazole drugs is as yet unidentified; the mutant evidence, however, indicates that the broad outlines of binding are conserved in the several divergent species examined. Sequence analysis of mutations and biochemical binding studies will be required to understand the interaction between tubulin and these drugs.

Similarities to fission yeast. In many respects our results show striking similarity to the results first found in the fission yeast *Schizosaccharomyces pombe*, which also has two α genes and one β gene (13, 50). One of the α genes is not essential (1). As mentioned above, the pattern of mutations to resistance and sensitivity to benzimidazole drugs is similar in the two yeasts. An interesting parallel is found by comparing the behavior of α- and β-tubulin genes in increased copy number. Both organisms are able to tolerate a high copy number of either of their two α-tubulin genes without major defects (50). In contrast, β genes from both species are lethal in high copy number (13; Thomas, Ph.D. thesis). In *S. cerevisiae*, even doubling the copy number of the β-tubulin locus leads to slow growth (J. Thomas and T. Huffaker, personal communication). These results probably have implications for the mechanism by which cells regulate the level of tubulin dimer. One component of a possible model is that the α subunit can be synthesized in excess and only those subunits that dimerize with β are stable. It seems likely that extra β subunit is unhealthy for the cell either because of the accumulation of excess heterodimer or because of some aberrant side reaction.

LITERATURE CITED

1. Adachi, Y., T. Toda, O. Niwa, and M. Yanagida. 1986. Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol. Cell. Biol. 6:2168–2178.
1a. Andreadis, A., Y.-P. Hsu, M. Hermodson, G. Kohlhaw, and P. Schimmel. 1984. Yeast LEU2: repression of mRNA levels by leucine and primary structure of the gene product. J. Biol. Chem. 259:8059–8062.
2. Andreadis, A., Y.-P. Hsu, G. Kohlhaw, and P. Schimmel. 1982. Nucleotide sequence of yeast LEU2 shows 5'-noncoding region has sequences cognate to leucine. Cell 31:319–325.
3. Bach, M. L., F. Lacroute, and D. Botstein. 1979. Evidence for transcriptional regulation of orotidine 5' phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in *E. coli*. Proc. Natl. Acad. Sci. USA 76:386–390.
4. Blackburn, E. H., and J. W. Szostak. 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. 53:163–194.
5. Bolivar, F., R. Rodriguez, P. Green, M. Betlach, H. Heynecker, H. Boyer, J. Crossa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
6. Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules *in vivo*. Cell 44:500–509.
7. Botstein, D., and R. W. Davis. 1982. Principles and practice of recombinant DNA research with yeast, p. 607–638. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. Botstein, D., S. C. Falco, S. Stewart, M. Brennan, S. Sherer, D. Stinchcomb, K. Struhl, and R. Davis. 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8:17–24.
9. Carle, G. F., and M. V. Olson. 1985. An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci. USA 82:3756–3760.
10. Cleveland, D. W., and K. F. Sullivan. 1985. Molecular biology and genetics of tubulin. Annu. Rev. Biochem. 54:331–365.
11. Davidse, L., and W. Flach. 1977. Differential binding of methyl-benzimidazol-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in strains of *Aspergillus nidulans*. J. Cell. Biol. 72:174–193.
12. Delgado, M. A., and J. Conde. 1984. Benomyl prevents nuclear fusion in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 193:188–189.
13. Hiraoka, Y., T. Toda, and M. Yanagida. 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.
13a. Holm, C., D. W. Meeks-Wagner, W. L. Fangman, and D. Botstein. 1986. A rapid, efficient method for isolating DNA from yeast. Gene 42:169–173.
14. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163–168.
15. Kemphues, K. J., T. C. Kaufman, R. A. Raff, and E. C. Raff. 1982. The testis-specific β-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. Cell 31:655–670.
16. Kingsman, A. J., L. Clarke, R. K. Mortimer, and J. Carbon. 1979. Replication in *Saccharomyces cerevisiae* of plasmid pBR313 carrying DNA from the yeast TRP1 region. Gene 7:141–153.
17. Kunes, S., D. Botstein, and M. S. Fox. 1984. Formation of inverted dimer plasmids after transformation of yeast with linearized plasmid DNA. Cold Spring Harbor Symp. Quant. Biol. 49:617–628.
18. Kunes, S., D. Botstein, and M. S. Fox. 1985. Transformation of yeast with linearized plasmid DNA: formation of inverted dimers and recombinant plasmid products. J. Mol. Biol. 184:375–387.
19. Kuo, C., and J. L. Campbell. 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the cdc8-1 mutation. Mol. Cell. Biol. 3:1730–1737.
20. May, G. S., J. Gambino, J. A. Weatherbee, and N. R. Morris. 1985. Identification and functional analysis of β-tubulin genes by site specific integrative transformation in *Aspergillus nidulans*.

J. Cell Biol. 101:712–719.

21. Mortimer, R. K., and D. Schild. 1981. Genetic mapping in *Saccharomyces cerevisiae*, p. 11–26. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The Molecular Biology of the Yeast *Saccharomyces*, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

22. Mortimer, R. K., and D. Schild. 1985. Genetic map of *Saccharomyces cerevisiae*, edition 9, Microbiol. Rev. 49:181–212.

23. Neff, N., J. H. Thomas, P. Grisafi, and D. Botstein. 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function in vivo. Cell 33:211–219.

24. Nogi, Y., and T. Fukasawa. 1984. Nucleotide sequence of the yeast regulatory gene GAL80. Nucleic Acids Res. 12:9287–9298.

25. Oakley, B., and N. R. Morris. 1980. Nuclear movement is β-tubulin dependent in *Aspergillus nidulans*. Cell 19:255–262.

26. Orr-Weaver, T., J. Szostak, and R. Rothstein. 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA 78:6354–6358.

27. Pringle, J. R., S. H. Lillie, A. E. M. Adams, C. W. Jacobs, B. K. Haarer, K. G. Coleman, J. S. Robinson, L. Bloom, and R. A. Preston. 1986. Cellular morphogenesis in the yeast cell cycle, p. 193–209. *In* J. Hicks (ed.), Yeast cell biology. Alan R. Liss, New York.

28. Quinlan, R. A., C. I. Pogson, and K. Gull. 1980. The influence of the microtubule inhibitor methyl benzimidazol-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. J. Cell Sci. 46:341–352.

29. Raff, E. C. 1984. Genetics of microtubule systems. J. Cell Biol. 99:1–10.

30. Ratzkin, B., and J. Carbon. 1977. Functional expression of cloned yeast DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 74:487–491.

31. Rose, M., and D. Botstein. 1983. Structure and function of the yeast URA3 gene. J. Mol. Biol. 170:883–904.

32. Rose, M., P. Grisafi, and D. Botstein. 1984. Structure and function of the yeast URA3 gene: expression in *Escherichia coli*. Gene 29:113–124.

33. Rothstein, R. J. 1983. One-step gene disruption in yeast. Methods Enzymol. 101:202–211.

34. Roy, D., and P. A. Fantes. 1983. Benomyl-resistant mutants of *Schizosaccharomyces pombe* cold-sensitive for mitosis. Curr. Genet. 6:195–202.

35. Schatz, P. J., L. Pillus, P. Grisafi, F. Solomon, and D. Botstein. 1986. Two functional α-tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. Mol. Cell. Biol. 6:3711–3721.

36. Seed, B. 1983. Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*. Nucleic Acids Res. 11:2427–2445.

37. Shaffer, B., I. Brearley, R. Littlewood, and G. R. Fink. 1971. A stable aneuploid of *Saccharomyces cerevisiae*. Genetics 67:483–495.

38. Sheirr-Neiss, G., M. Lai, and N. Morris. 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell 15:639–647.

39. Sherman, F., G. Fink, and C. Lawrence. 1974. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

40. Shortle, D., J. Haber, and D. Botstein. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. Science 217:371–373.

41. Silflow, C. D., R. L. Chisholm, T. W. Conner, and L. P. W.

Ranum. 1985. The two α-tubulin genes of *Chlamydomonas reinhardi* code for slightly different proteins. Mol. Cell. Biol. 5:2389–2398.

42. Snyder, J., and J. McIntosh. 1976. Biochemistry and physiology of microtubules. Annu. Rev. Biochem. 45:699–720.

43. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

44. Stephens, R. E. 1978. Primary structural differences among tubulin subunits from flagella, cilia, and the cytoplasm. Biochemistry 17:2882–2891.

45. Struhl, K., and R. W. Davis. 1977. Production of a functional eukaryotic enzyme in *Escherichia coli*: cloning and expression of the yeast structural gene for imidazoleglycerol phosphate dehydratase (his3). Proc. Natl. Acad. Sci. USA 74:5255–5259.

46. Struhl, K., and R. W. Davis. 1980. A physical, genetic and transcriptional map of the cloned his3 gene region of *Saccharomyces cerevisiae*. J. Mol. Biol. 136:309–332.

47. Struhl, K., D. Stinchcomb, S. Sherer, and R. Davis. 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76:1035–1039.

48. Thomas, J. H., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. Cell 44:65–76.

49. Thomas, J. H., N. F. Neff, and D. Botstein. 1985. Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 112:715–734.

50. Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida. 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.

51. Toda, T., K. Umesono, A. Hirata, and M. Yanagida. 1983. Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. J. Mol. Biol. 168:251–270.

52. Tschumper, G., and J. Carbon. 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the TRP1 gene. Gene 10:157–166.

53. Umesono, K., T. Toda, S. Hayashi, and M. Yanagida. 1983. Two cell division cycle genes NDA2 and NDA3 of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. J. Mol. Biol. 168:271–284.

54. Weatherbee, J. A., G. S. May, J. Gambino, and N. R. Morris. 1985. Involvement of a particular species of β-tubulin (β3) in conidial development in *Aspergillus nidulans*. J. Cell Biol. 101:706–711.

55. Wood, J. S. 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae*. Mol. Cell. Biol. 2:1064–1079.

56. Wood, J. S., and L. H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *S. cerevisiae*. J. Cell Biol. 94:718–726.

57. Yamamoto, M. 1981. Genetic analysis of resistant mutants to antimitotic benzimidazole compounds in *Schizosaccharomyces pombe*. Mol. Gen. Genet. 180:231–234.

58. Yocum, R. R., and M. Johnston. 1984. Molecular cloning of the GAL80 gene from *Saccharomyces cerevisiae* and characterization of a gal80 deletion. Gene 32:75–82.

59. Yongblom, J., J. A. Schloss, and C. D. Silflow. 1984. The two β-tubulin genes of *Chlamydomonas reinhardi* code for identical proteins. Mol. Cell. Biol. 4:2686–2696.

Chapter 4:

Insertions of up to 17 Amino Acids into a Region of α-Tubulin

Do Not Disrupt Function *in vivo*

Introduction:

The α, β-tubulin heterodimer is the major structural component of microtubules, which are intimately involved in eukaryotic cell structure and motility (48). The ultrastructure of microtubules is highly conserved in a wide variety of species. The sequences of α- and β-tubulins are also highly conserved (7, 36). These observations lead to the hypothesis that many of the mechanisms that regulate microtubule structure and function will be conserved as well. We are studying microtubules in yeast, using a combination of genetic and biochemical techniques.

Microtubules in yeasts are elements of structures involved in chromosome and nuclear movement (2, 6, 17, 19, 28, 30). Studies with antimicrotubule drugs and with conditional lethal tubulin mutants have confirmed the role of microtubules in the mitotic and meiotic spindles of yeast as well as in nuclear movement during cell division and mating (10, 13, 34, 35, 38, 51, 52, 53, 54, 57, 58). Tubulin has been purified from yeast and shown to have biochemical properties similar to tubulin from higher eukaryotes (16). Two very different yeasts have been used in genetic studies, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Genetic and molecular cloning experiments have shown that both yeasts have one essential β-tubulin gene and two α-tubulin genes (13, 29, 40, 41, 52). Within each species, the two α genes differ substantially from each other in sequence and differ markedly in their importance for normal cell growth. In both organisms, one of the genes is genetically essential and the other is not (1,41). In both species, however, either gene alone can perform all of the functions normally performed by the pair if present in the cell in sufficient copy number (41, M. Yanagida, personal communication). Thus the differences between the genes are apparently due to level of expression and not to functional differences between the encoded proteins.

Several approaches have been used to study the relation between the primary sequence of

isolated tubulin proteins and their tertiary structure and function. Cleavage of both α- and

β-tubulin to remove carboxy-terminal fragments alters their ability to assemble into

microtubules *in vitro* (39,43). The carboxy termini have also been identified as sites of high

affinity calcium binding (44). Using proteolytic fragments of tubulin, several groups have

identified potential sites of interaction between tubulin and microtubule associated proteins

(24,42). Specific amino acid residues have been identified that are important for microtubule

assembly *in vitro* (3). These sorts of analyses can now be pursued *in vivo*. The *in vivo*

consequences of variation in the sequence of β-tubulin has been investigated through the

expression of a chicken-yeast chimeric β-tubulin in mouse cells. The results indicated that the

variant sequence placed no restrictions on the ability of the protein to assemble into all

microtubules in the cells (4). A recent study has demonstrated that naturally occuring tubulin

variants in mammalian cells show no restriction in their ability to assemble into a wide variety

of microtubular structures (23). The functional consequences of several kinds of sequence

variation in the chicken-yeast chimeric β-tubulin have been described by Fridovich-Keil et al.

(11a).

We have used one of the two α-tubulin genes of the budding yeast *Saccharomyces*

cerevisiae, *TUB3* (40, 41), to study structure-function relationships in a region of α-tubulin

that is extremely variable in cross species comparisons by both length and sequence. We have

perturbed the structure of this region of the *TUB3* protein by inserting into it up to 17

additional amino acids. We show that yeast strains containing only these mutant genes show

no detectable defect in growth, mating, or sporulation.

Materials and Methods:

Strains and Media: Escherichia coli HB101 was used for bacterial transformation and plasmid growth. Bacterial media were made as described by Davis et al. (9). Media for yeast growth and sporulation were made as described by Sherman et al. (45), except that adenine, uracil and tryptophan were routinely added to YPD medium after autoclaving. Benomyl, 98.6%, was a gift from O. Zoebisch, E. I. duPont de Nemours and Co., Inc. It was kept as a 10 mg/ml stock in dimethyl sulfoxide at 4°C and was added to warm YPD medium with swirling immediately before the plates were poured. The yeast strains used in this paper were derived from a set of essentially isogenic S288C strains originally provided by G. R. Fink. The strains used are listed in Table 1.

Gel electrophoresis and nucleic acid preparation: Restriction enzymes, DNA polymerase I, DNA polymerase I large fragment, polynucleotide kinase, T4 DNA ligase, and terminal transferase were purchased from New England Biolabs and used in the buffers described by Maniatis et al. (26). Agarose gel electrophoresis and plasmid DNA isolation were performed as described by Davis et al. (9). Small scale preparations of yeast DNA were prepared by the method of Holm et al. (14).

Hybridization methods: DNA fragments were transferred from agarose gels to Zetapor membrane (AMF Inc.) by the method of Southern (49) with 20x SSPE (26). Hybridizations were done at 42°C in the buffer described by Wahl et al. (56) with nick-translated probes (37). After hybridization, the filters were washed in 2x SSPE, 0.5% SDS at 50°C.

Plasmid constructions: An extensive map and the sequence of the *TUB3* gene has been published elsewhere (40). The BamHI linkers were purchased from Collaborative Research and consisted of the sequences CGGATCCG, CGGGATCCCG, and CGCGGATCCGCG. BamHI linker insertions were made in plasmid pRB325 (41), which is shown in Figure 2. pRB325 was digested with MstII and the 5' overhangs were filled with

Table 1: Yeast strains used in this study.

Strain	Genotype of strain
DBY2254	MATa/MATα, ade2/+, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, lys2-801/+, trp1-1/trp1-1, ura3-52/ura3-52, tub1::HIS3/+
DBY2287	MATa, his3-Δ200, leu2-3,112, trp1-1, ura3-52, tub3::TRP1
DBY2288	MATα, his3-Δ200, leu2-3,112, lys2-801, trp1-1, ura3-52, tub3::TRP1
DBY2375	MATa, his3-Δ200, leu2-3,112, lys2-801, ura3-52, tub3::TRP1
DBY2384	MATα, his3-Δ200, leu2-3,112, lys2-801, ura3-52, tub3::TRP1, tub1::HIS3, TUB3-URA3-2μm(pRB316)
DBY2387	MATa, ade2, his3-Δ200, leu2-3,112, lys2-801, trp1-1 ura3-52, tub3::TRP1, tub1::HIS3, tub3-109-URA3-2μm(pRB592)
DBY2389	MATα, his3-Δ200, leu2-3,112, lys2-801, trp1-1 ura3-52, tub3::TRP1, tub1::HIS3, tub3-117-URA3-2μm(pRB593)
DBY2392	MATα, his3-Δ200, leu2-3,112, lys2-801, trp1-1 ura3-52, tub3::TRP1, tub1::HIS3, tub3-103-URA3-2μm(pRB591)

the large fragment of DNA polymerase I. Linkers were added to the blunt ends and the molecules were recircularized with T4 DNA ligase.

The *TUB3* linker insertion mutations were transferred to the yeast plasmid YEp24 (5) by an *in vivo* recombination technique described in detail elsewhere (25). Briefly, the pRB325 derivatives were digested with SalI and PstI. The 4.6 kb fragments, containing the modified *TUB3* genes plus flanking pBR322 sequences, were purified on an agarose gel. These fragments were mixed with BamHI digested YEp24 DNA and used to transform yeast strain DBY2254 with selection for *Ura3^{+}*. The resulting plasmids were recovered from yeast and transformed into E. coli. All had the expected structure shown in Figure 2.

DNA Sequence Analysis: The sequences of the linker insertions were determined by the method of Maxam and Gilbert (27) using the pRB325 derivatives described above (see Figure 2). The plasmids were digested with ClaI and labeled at their 3' ends by filling the 5' overhangs with the large fragment of DNA polymerase I and α-^{32}P dCTP. The labeled plasmids were recut with XhoI before gel purification of the 0.64 kb fragment for sequencing. The complementary strand was sequenced from the opposite side by cutting with NsiI and labeling the 3' end with α-^{32}P dideoxyATP and terminal transferase as described by Yousaf et al. (59). The labeled plasmids were recut with EcoRI before gel purification of the 0.45 kb fragment for sequencing.

Genetic Techniques and Transformation: Methods of yeast mating, sporulation, and tetrad analysis were as described by Sherman et al. (45). Yeast cells were transformed by the lithium acetate method of Ito et al. (15) as modified by Kuo and Campbell (21) with 1 to 4 micrograms of plasmid DNA and 50 micrograms of sonicated chicken blood DNA (Sigma Chemical Co.) as carrier. Transformants were plated on SD medium supplemented with the appropriate nutrients to select cells with the plasmid.

Preparation of antibodies: Four peptides were purchased from Peninsula Labs and coupled to KLH via an amino terminal cysteine. The peptide called TUB1-N consisted of amino acids 115-122 of the TUB1 protein (Ile-Leu-Gly-Asp-Val-Leu-Asp-Arg). Peptide

TUB3-N consisted of amino acids 115-122 of the TUB3 protein

(Ile-Val-Asp-Glu-Val-Glu-Glu-Arg). Peptide TUB1-C consisted of the last 10 amino acids of

the TUB1 protein (Ala-Asp-Ser-Tyr-Ala-Glu-Glu-Glu-Glu-Phe). Peptide TUB3-C consisted

of the last 8 amino acids of the TUB3 protein (Ala-Asp-Ser-Tyr-Ala-Glu-Glu-Phe). Each

rabbit was injected with 0.5 mg of the conjugate homogenized in Freund's complete adjuvant.

Rabbits 6-340 and 6-341 were injected with the TUB1-N conjugate; rabbits 6-342 and 6-343

were injected with the TUB3-N conjugate; rabbits 6-344 and 6-345 were injected with the

TUB1-C conjugate; rabbits 6-346 and 6-347 were injected with the TUB3-C conjugate. The

rabbits were boosted twice with 0.5 mg of conjugate in Freund's incomplete adjuvant at two

week intervals. The rabbits were bled at two week intervals and the sera were stored in frozen

aliquots. Sera from all of the rabbits injected with the carboxy-terminal peptides reacted very

strongly with the corresponding protein on western blots and in immunofluorescence. The

TUB1-C sera reacted weakly with TUB3 protein; the TUB3-C sera reacted weakly with

TUB1 protein. Neither of the TUB1-N sera reacted with either protein. The TUB3-N sera

reacted very weakly with TUB3 protein.

Protein Preparation and Western Blotting: Yeast cells were grown to a density of

about 1×10^7 cells/ml, harvested by centrifugation and washed in 20 mM Tris-HCl, pH 7.5.

Cells were lysed by boiling for 3 minutes in ESB buffer (2% Sodium dodecylsulfate, 10%

glycerol, 80 mM Tris pH 6.8, 1mM Phenylmethylsulfonylfluoride, 0.1 M dithiothreitol,

0.001% bromophenol blue), vortexed vigorously for one min. with 0.5 mm glass beads, and

boiled for one more min. The proteins were resolved on an 8.5% SDS-polyacrylamide gel

and transferred to nitrocellulose paper (Schleicher and Schuell) by electroblotting at 30 V

overnight. The nitrocellulose paper was blocked with 5% dry milk (Carnation) in 0.01 M Tris

pH 7.4, 0.15 M NaCl for one hour and then probed with the α–tubulin monoclonal antibody

B-5-1-2 (31) for one hour in the same solution. The excess antibody was removed by

washing four times in the Tris-NaCl solution (without milk) over the course of one hour. The

primary antibody was then labeled with ^{125}I labeled rabbit anti mouse IgG (generously

provided by Tom Briner and Malcolm Gefter) in Tris-NaCl for one hour. Finally, the filter

was washed four times during an hour with Tris-NaCl and exposed to Kodak XAR film.

Blots with the anti-peptide antibody were performed using a similar procedure. The primary

antibody, from rabbit 6-346, was used at 1/1000 dilution and detected with ^{125}I protein A at

0.2 mCi/ml.

Results:

Comparison of sequences of α-tubulins from several species reveals a region near the amino terminus which is exceptionally variable (Figure 1). To begin to examine the role of this region in the function of yeast α-tubulin, we have perturbed its structure by inserting extra amino acids into the region. The alterations consisted of 8, 10, or 12 bp BamHI linkers inserted into a blunted MstII site in the *TUB3* α-tubulin gene of the yeast *Saccharomyces cerevisiae* (40). The mutations were initially constructed and examined in the plasmid pRB325 (Figure 2), which carries a yeast centromere (CEN4), replication origin (ARS1), and selectable marker (URA3). Four of each type of linker insertion were examined in detail.

To determine the structure of the mutations, we sequenced all twelve of the plasmids in the region of the inserts. The sequences (Figure 3) revealed that some of the mutations resulted from the insertion of more than one linker. Variation of a single base pair was also observed in the sequences of the ligation sites, perhaps due to incomplete filling of the MstII overlapping ends or to exonuclease digestion of the ends before ligation. Three of the mutations shown in Figure 3 preserved the reading frame of *TUB3* while the rest shifted the frame. The in-frame insertions added 3, 9 or 17 amino acids after the normal amino acid number 43 (see Figure 1). These mutations were designated *tub3-103*, *tub3-109*, and *tub3-117*, respectively.

Tests of Function:

We first assayed the altered genes for their ability to complement one of the phenotypes of a null mutation in *TUB3*, hypersensitivity to the antimicrotubule drug benomyl (41). We transformed strain DBY2375 (*tub3⁻*) with centromere plasmids carrying the linker insertions (see Figure 2). The transformed derivatives were then tested for growth on several concentrations of benomyl. A plasmid carrying intact *TUB3* served as a positive control and

Figure 1. α-tubulin sequences from a variety of species in the variable amino terminal region. α-tubulin sequences are shown from a variety of species starting with amino acid 20. Dashes indicate insertions to bring the sequences into register. Positions of identity with the human sequence are left blank. The large arrowhead indicates the position of the insertion mutations in *TUB3*. The α-tubulin sequences shown are from human keratinocyte (8), porcine brain (32), rat (22), chinese hamster ovary cells (11), mouse α1-4 (55), chicken testis specific cα2 (33), *Drosophila melanogaster* α1-4 (50), *Trypanosome brucei* (18), *Stylonychia lemnae* (12), *Physarum polycephalum* (20), *Chlamydomonas reinhardi* (46), *Schizosaccharomyces pombe* (52), and *Saccharomyces cerevisiae TUB1* and *TUB3* (40).

```
                     20
Human           WELYCLEHGIQPDG--QMPSDKTIGGGDDSFN--------TFFSETGAGKHVPRAV
Porcine                          --            ---------
Rat                              --            ---------
Hamster                          --            ---------
Mouse 1,2,3                      --            ---------
Mouse 4                          --          T---------   C
Chicken testis     F      S      ---TF PP--SS A---------   R  SMS Y    I
Drosophila 1,3                   --      V     ---------
Drosophila 2                     --H     V     S---------
Drosophila 4       L      NL     SLKTKEELTASGSSASVGHDTSANDAR  T    N   Q   SI
Trypanosome        F             --A       VE A ---------
Stylonychia        F             --           A ---------        E       C
Physarum                  N      --      SV   A ---------        SS      I
Chlamydomonas                    --           A ---------                CI
S. pombe 1                G      FPTEN EVHKNNSYLNDGFG-------        Q  F   SI
S. pombe 2                     N --Y NP TASQNS GG S---------       Q  Y   SI
TUB1               S      K      -HLEDGLSKPK  EEG S---------  H    Y  F   I
TUB3               S      KE     -HLEDGLSKPK  EEG S---------  H    Y  F   I
                                         ▲
```

Figure 2. Restriction maps of plasmids. The construction of plasmid pRB325 has been described (41). The *TUB3* coding and intron sequence are shown darkly shaded while the *TUB3* flanking sequences are shown with medium shading. Unshaded regions are derived from pBR322 and the other sequences on the plasmid are labeled. BamHI linker insertions were made into the single MstII site. The *tub3-103*, *tub3-109*, and *tub3-117* alleles were used to construct plasmids pRB591, pRB592, and pRB593 respectively, by the method described in the text. They are analogous to pRB316 (41), which carries the wild-type *TUB3* gene.

pRB325
(11.3 kb)

PstI
EcoRI ClaI
BamHI/
BglII
NsiI

EcoRI

EcoRI

ClaI

MstII

NsiI

XhoI

BamHI/BglII

SalI

PstI

NsiI

amp^r

ARS1

CEN4

URA3

TUB3

ATG

PstI

BglII

XhoI
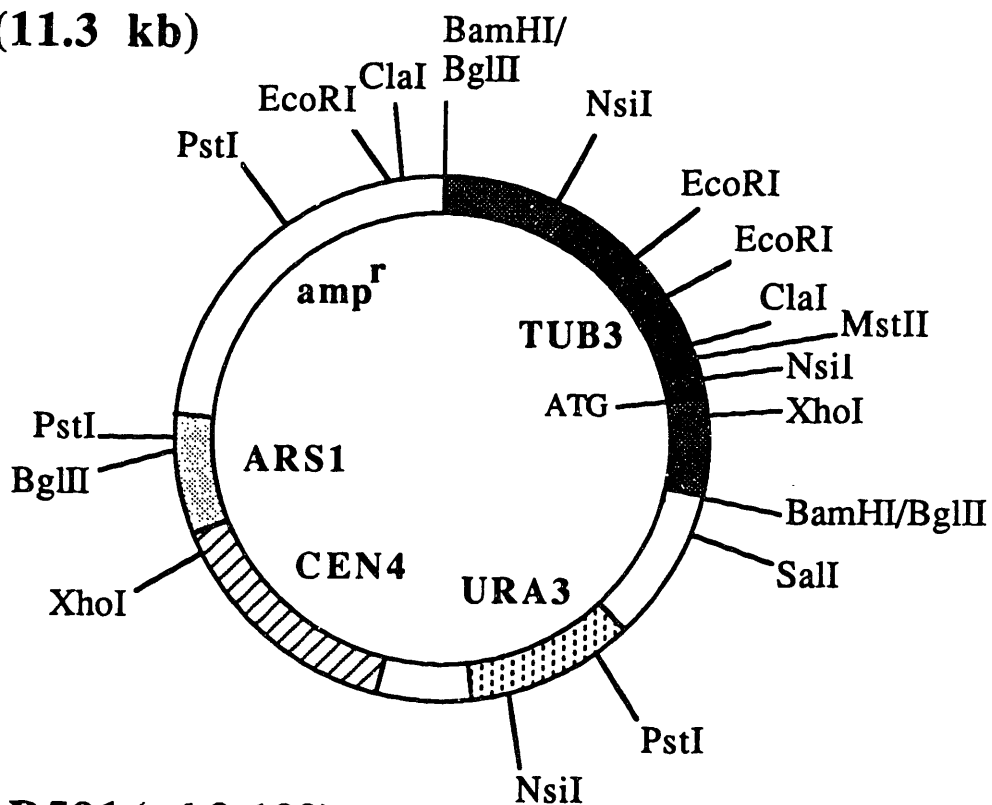
pRB591 (tub3-103)
pRB592 (tub3-109)
pRB593 (tub3-117)
(11.0 kb)

BamHI/BglII

PstI

PstI

PvuI

URA3

TUB3

ATG

MstII::BamHI

BamHI/BglII

SalI

2μ

amp^r

PvuI PstI
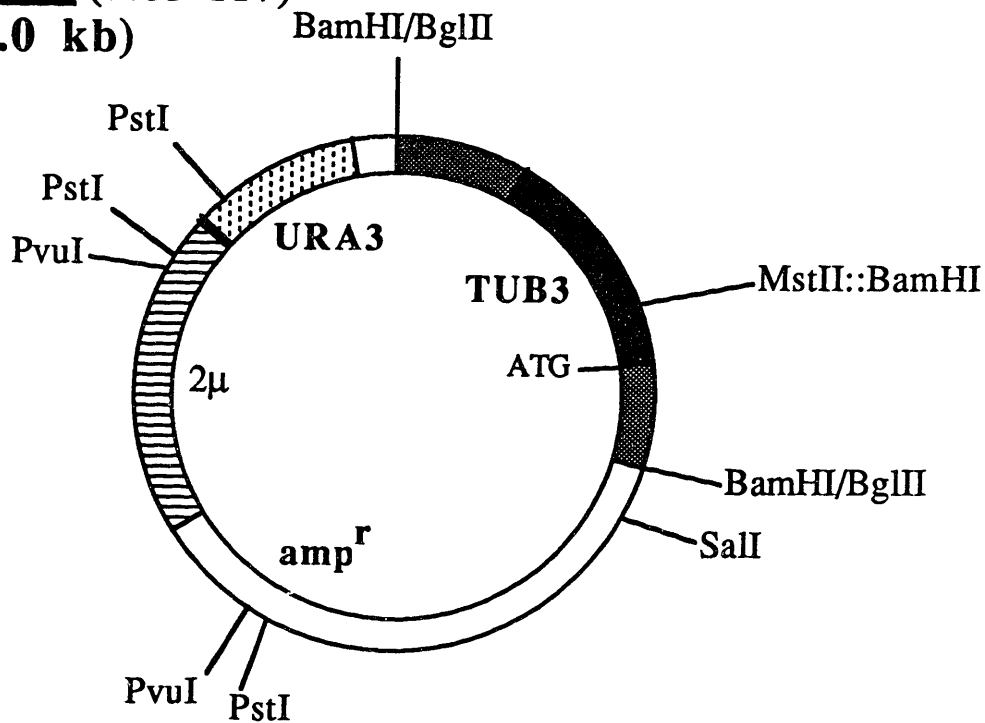
Figure 3. Sequences of *TUB3* BamHI linker insertion mutations. The sequences of

wild-type *TUB3* and the linker insertion mutations are shown with translations below each

line. The numbers in parentheses below each allele number indicate the number of

independent isolates of each mutation. Arrows above the lines indicate sites where single base

pairs were lost during the linker insertion procedure. The added nucleotides are shown in

lower case. The *tub3-103* and *tub3-104* were products of insertion of a 8 bp BamHI linker;

tub3-105, *tub3-106*, and *tub3-107* were from insertion of a 10 bp linker; *tub3-108*, *tub3-109*,

tub3-110, and *tub3-117* were from insertion of a 12 bp linker.

```
TUB3        TTGTCAAAACCT---------------------------------------------AAGGGAGGTGAA
            LeuSerLysPro------------------------------------------------LysGlyGlyGlu


tub3-103    TTGTCAAAACCTacggatccg----------------------------------AAGGGAGGTGAA
  (3)       LeuSerLysProThrAspPro-------------------------------------LysGlyGlyGlu


tub3-104    TTGTCAAAACCTaacggatccg---------------------------------AAGGGAGGTGAA
  (1)       LeuSerLysProAsnGlySer-------------------------------------out of frame


tub3-105    TTGTCAAAACCTacgggatcccg-------------------------------AAGGGAGGTGAA
  (2)       LeuSerLysProThrGlySer-------------------------------------out of frame


tub3-106    TTGTCAAAACCTacgggatcccgcgggatcccgt----------------------AAGGGAGGTGAA
  (1)       LeuSerLysProThrGlySerArgGlyIlePro------------------------out of frame


tub3-107    TTGTCAAAACCTacgggatcccgcgggatcccgcgggatcccgt------------------AAGGGAGGTGAA
  (1)       LeuSerLysProThrGlySerArgGlyIleProArgAspPro-------------------out of frame


tub3-108    TTGTCAAAACCTacgcggatccgcg---------------------------------AAGGGAGGTGAA
  (1)       LeuSerLysProThrArgIleArg----------------------------------out of frame


tub3-109    TTGTCAAAACCTaacgcggatccgcgcgcggatccgcgt-----------------------AAGGGAGGTGAA
  (1)       LeuSerLysProAsnAlaAspProArgAlaAspProArg----------------------LysGlyGlyGlu


tub3-110    TTGTCAAAACCTacgcggatccgcgcgcggatccgcgcgcggatccgcgt-----------AAGGGAGGTGAA
  (1)       LeuSerLysProThrArgIleArgAlaArgIleArgAlaArgIleArg--------------out of frame


tub3-117    TTGTCAAAACCTaacgcggatccgcgcgcggatccgcgcgcggatccgcgcgcggatccgcgtAAGGGAGGTGAA
  (1)       LeuSerLysProAsnAlaAspProArgAlaAspProArgAlaAspProArgAlaAspProArgLysGlyGlyGlu
```

the centromere plasmid with no insert served as a negative control. The experiments revealed that all of the insertion mutant alleles that had perserved the reading frame of *TUB3* had retained the ability to complement the benomyl sensitivity of the *TUB3* null mutant as effectively as the positive control. None of the insertions that had changed the reading frame of *TUB3* retained the ability to complement.

Complementation of benomyl hypersensitivity is only a limited test of the ability of the altered gene products to function as α-tubulins. We therefore decided to test their ability to support growth as the only α-tubulin gene in the cell. Our previous results indicate that the *TUB3* gene in high enough copy number can support mitotic growth, mating and sporulation of yeast in the absence of the *TUB1* α-tubulin gene (41). We performed the test in two steps: first we assayed the mutant genes for their ability to complement a *TUB1* null mutation and then we crossed them to construct strains without wild-type α-tubulin genes.

To insure sufficient copy number of the altered *TUB3* genes, we transferred the mutant alleles to high copy number vectors containing the 2-micron plasmid origin of replication. The transfer was performed using *in vivo* recombination between a DNA fragment carrying the altered genes and the 2-micron plasmid YEp24 (Figure 2, see Materials and Methods). The *in vivo* recombination occurred during transformation of strain DBY2254 (Table1), a diploid strain heterozygous for a disruption of the *TUB1* gene (marked with the yeast *HIS3* gene). The resulting diploids were sporulated and tetrads were dissected.
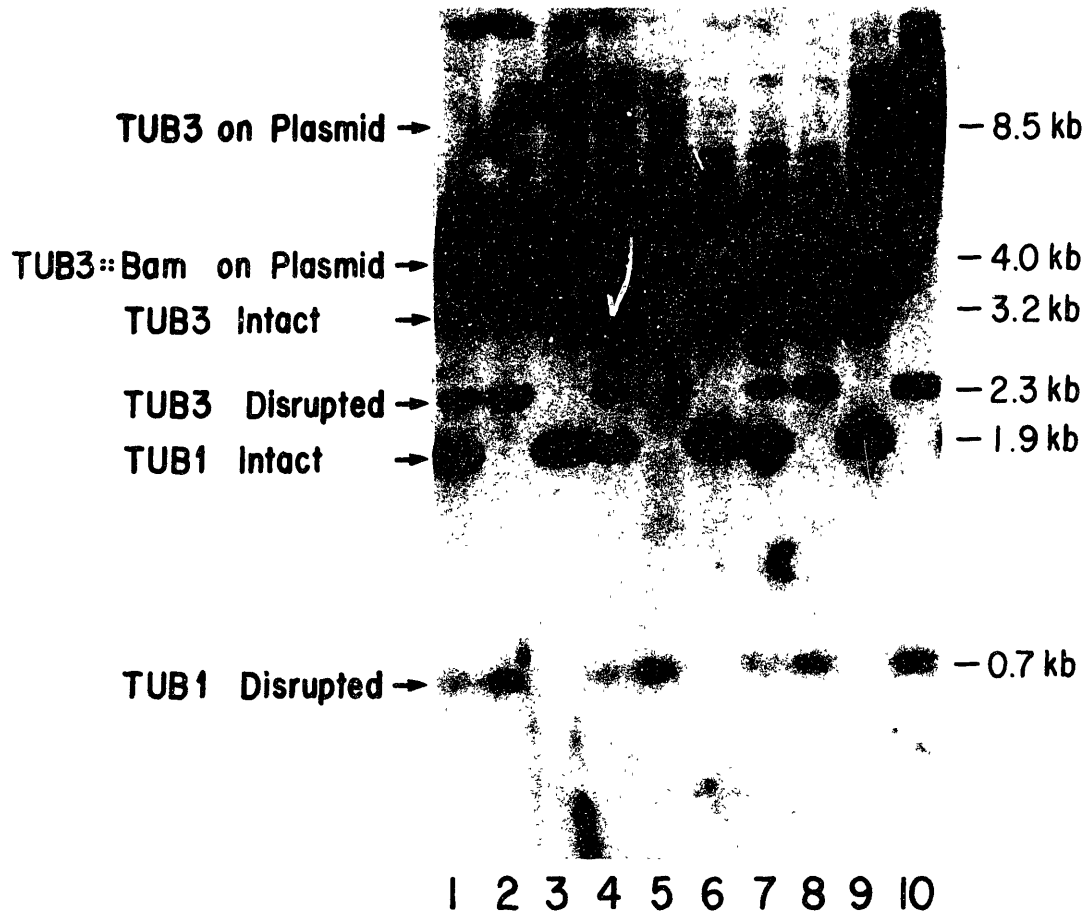
The *TUB1* disruption is normally a recessive lethal mutation. In the presence of extra copies of functional α-tubulin genes, however, the lethality is suppressed (41). To determine whether the mutant alleles could suppress the lethality, the progeny spores were examined for the presence of spores containing the *tub1* disruption, a single copy of wild-type chromosomal *TUB3* and one of the plasmids. Such spores would have two obvious phenotypes; they would be His3$^+$ because the *HIS3* gene is the marker of the *TUB1* disruption; they would be Ura3$^+$ because the *URA3* gene is the marker on the plasmid. In

addition, they would show an absence of slow growth, previously correlated with

abnormalities of chromosome 13, where both α genes map (41). Experiments were done with

all three in-frame insertion mutations along with an out-of-frame mutation as a negative

control. Experiments with all of the in-frame insertions yielded spores of the expected

phenotype while the negative control yielded none. These results indicate that the insertion

mutations will complement the lethality normally associated with the *TUB1::HIS3* gene

disruption in normal haploid cells (41).

The strains from the above experiment contained wild-type *TUB3* on the chromosome and

mutant alleles of *TUB3* on the plasmid. To show that the growth of yeast could be supported

by the mutant *TUB3* alleles alone, these strains were crossed to strains DBY2287 or

DBY2288, which contain a disruption of the *TUB3* gene (marked with the yeast *TRP1* gene).

In these crosses, both the *HIS3* marker and the *TRP1* marker segregated 2:2 and the plasmid

marker segregated 4:0 in most cases. Strains were recovered that contained each of the

plasmids and both the *TUB1* and *TUB3* disruptions. In these strains, the mutated *TUB3*

genes were presumably the only α–tubulin genes in the cells.

To confirm the absence of wild-type α–tubulin genes, we carried out gel transfer

hybridization experiments (Figure 4). DNA was prepared from parental diploids, from His⁺,

Trp⁺, Ura⁺ spores, which should contain only the altered tubulins, and also from their His⁻,

Trp⁻, Ura⁺ sisters, which should contain wild-type chromosomal genes. The DNA was

digested with BamHI, PvuI and BglII, run on an agarose gel, transferred to filter paper, and

analyzed with a *TUB1* probe. Under these digestion conditions, the intact *TUB1* gene yields

a hybridizing fragment of 1.9 kb while the disruption allele yields a fragment of 0.7 kb.

Because of extensive homology between *TUB1* and *TUB3*, the probe will also hybridize to a

band of 3.2 kb from intact *TUB3* and a band of 2.3 kb from the *TUB3* disruption allele (41).

Plasmid borne *TUB3* was expected to yield a band of 4.0 kb if a BamHI linker were present

or a band of 8.5 kb if no BamHI linker were present. As expected, the diploids (lanes 1, 4,

Figure 4. Gel transfer hybridization of total yeast DNA from strains transformed with plasmids bearing the *TUB3* linker insertion mutations. DNA was purified from diploid strains heterozygous for the *tub3::TRP1* and *tub1::HIS3* disruption alleles (41) containing pRB591, pRB592, and pRB593 (lanes 1, 4, and 7 respectively). DNA was also purified from progeny spores (from these diploids) carrying the plasmids plus both disruption alleles (lanes 2, 5, and 8) or from their meiotic sisters carrying the plasmids plus wild-type *TUB3* and *TUB1* (lanes 3,6, and 9). The DNA in lane 10 was from strain DBY2384, which carries wild-type *TUB3* on plasmid pRB316 and the two disruption alleles. In all cases the strains were grown under conditions that selected for maintenance of the plasmids. The DNA was digested with BamHI, BglII, and PvuI, and the fragments were separated on an agarose gel. The DNA was transferred to Zetapore membrane and analyzed with a probe made from a 1.9 kb BglII fragment containing most of the *TUB1* coding sequence. The size and origin of the hybridizing fragments are indicated in the margins.

TUB3 on Plasmid → ——8.5 kb

TUB3::Bam on Plasmid → ——4.0 kb

TUB3 Intact → ——3.2 kb

TUB3 Disrupted → ——2.3 kb

TUB1 Intact → ——1.9 kb

TUB1 Disrupted → ——0.7 kb

1 2 3 4 5 6 7 8 9 10

and 7) showed both sets of chromosomal bands, the His$^+$, Trp$^+$ spores (lanes 2, 5, and 8) showed only the disruption specific bands and the His$^-$, Trp$^-$ spores (lanes 3, 6, and 9) showed the bands from the intact genes. All of the strains presumed to be carrying the plasmid borne *TUB3* insertion mutations showed the expected 4.0 kb band. A control strain, with wild-type *TUB3* on a similar plasmid, showed the expected 8.5 kb band (lane 10).
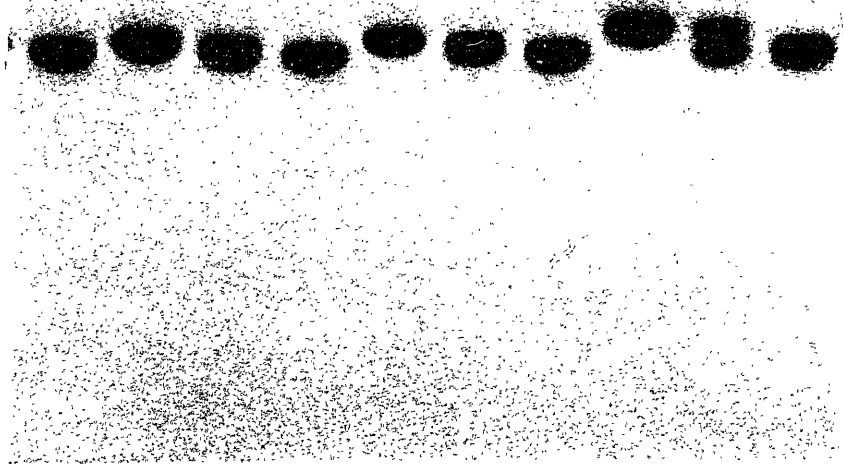
We conclude that the *TUB3* insertion mutations specify α-tubulins that are sufficient for germination and mitotic growth of yeast in the absence of other α-tubulins.

To confirm that these strains contained only the altered α-tubulin proteins, we examined the mobility of the α-tubulins in SDS-polyacrylamide gels by western blotting. Because the mutant alleles encoded from 3 to 17 extra amino acids, their products might be expected to have a slightly lower mobility than wild-type *TUB3* protein. Two antibodies were used. The first was a widely cross-reactive monoclonal anti α-tubulin made against sea urchin tubulin (31, kindly provided by G. Piperno). The second was a rabbit polyclonal antibody made against the peptide encoded by the carboxy terminus of *TUB3* (see Materials and Methods). Both antibodies gave similar results. Figure 5 shows the results obtained by probing whole cell protein extracts with the anti *TUB3* peptide antibody. Lanes 1, 4, 7, and 10 show the results from an extract made from a strain (DBY2384) that contains only wild-type *TUB3*. Lanes 2, 5, and 8 contained extracts from strains carrying only the *tub3-103*, *tub3-109*, and *tub3-117* alleles, respectively. Lanes 3, 6, and 9 show the results obtained when extracts for the three mutants were mixed with wild-type extracts. As expected, the altered proteins migrated more slowly in the gel than the wild-type protein.

We tested the strains carrying only *tub3-103*, *tub3-109*, or *tub3-117* for any obvious defects. All of them grew well at temperatures from 14°C to 37°C. Based on observations of colony size after 2 days of growth at 30°C, mutant strains grew at about the same rate as the wild type. To extend the analysis to other known microtubule functions in yeast, we tested the *TUB3* alleles for their ability to support mating and sporulation of yeast. All

Figure 5. Western blot analysis of proteins from the *TUB3* mutant alleles. Total cell protein was prepared from strain DBY2384, containing only wild-type *TUB3* (lanes 1, 4, 7, and 10) and from strains DBY2392 (*tub3-103*), DBY2387 (*tub3-109*), and DBY2389 (*tub3-117*) in lanes 2, 5, and 8 respectively. Lanes 3, 6, and 9 contain equal mixes of the three mutant extracts, respectively, with wild-type (DBY2384) extract. The proteins were separated on an 8.5% acrylamide gel, transferred to nitrocellulose paper and probed with an antibody made to the C-terminus of TUB3.

three mutations allowed strains to mate and sporulate with about the same efficiency as the wild-type controls (data not shown). We conclude that the insertions show no detectable phenotype in any of the known microtubule dependent processes in yeast.

Discussion:

We have shown that insertion of 3, 9, or 17 amino acids into a variable region near the amino terminus of yeast α-tubulin has no detectable effect on the function of the protein. Strains carrying only the altered α-tubulins were indistinguishable from the wild type in mitotic growth, mating and sporulation, the three processes in yeast known to depend on microtubules (10, 13, 34, 35, 38, 51, 52, 53, 54, 57, 58).

Comparisons in this area between α-tubulins from a wide variety of species are shown in Figure 1. Among eukaryotes from humans to chlamydomonas the region is quite well conserved, with several notable exceptions. The chick testis sequence shows considerable divergence by length and by sequence (33). The *Drosophila* α-4 and all four yeast α-tubulin sequences of this region have no appreciable homology to the other sequences (40, 50, 52). The divergence of *S. pombe* α1 and *Drosophila* α-4 is especially noticeable because they contain 4 and 11 additional amino acids, respectively, relative to most of the other sequences. The sequence comparisons plus our mutational analysis strongly suggest that this region can tolerate considerable variation without losing any of the highly conserved functions of α-tubulin. These observations suggest that variabiltiy in this region occurs because it can be tolerated, not because it specifies an important function for the protein. Deletion analysis will be required to prove that this region no specific function in yeast, and to determine whether it has any minimum length. It is possible that the other eukaryotes retain a subset of microtubule functions, dependent on this region, that yeasts do not have. Definitive tests of such a model will only be possible through mutational analysis of α-tubulin in a eukaryote, such as *Drosophila*, that combines the ability to test altered genes *in vivo* with a wide variety of microtubular structures and functions. Functional analysis of other regions of tubulin, however, will continue to be easily accomplished using the sophisticated genetics available in yeasts.

The identification of regions of proteins that can tolerate sequence variation has been useful in a number of instances. Notable examples include the phage T4 rIIB gene product and *E. coli* β-galactosidase (47). One possible use for such a region in α-tubulin would be the construction of functional mutant alleles whose products could be detected by probes specific to the inserted region. Such proteins could be used for structural studies of tubulin and microtubules. They also could be used as a starting point to construct variant tubulins that could be identified in a background of normal tubulin proteins (for example see 11a).

It is informative to compare our results to those of Fridovich-Keil et al. (11a). This paper examines the functional consequences of a number of sequence variations in a chicken-yeast chimeric β-tubulin expressed in mouse cells. The sequence alterations examined included a deletion, several additional chimeric constructions, and a number of small insertions of 2 or 4 amino acids made through linker insertions. Most of the insertion alleles examined encoded tubulins with major functional defects, including an inability to assemble into microtubules. The only mutant alleles whose gene products were able to assemble included an insertion and a deletion at the carboxy terminus and 3 of the 4 chimeric constructions. Most regions of tubulin thus appear to be very sensitive to variations in length. The regions so far identified that are not sensitive to insertions include the variable amino terminal region of α-tubulin and the carboxy terminus of β-tubulin (also a region of considerable variation in sequence comparisons).

Literature Cited:

1. Adachi, Y., T. Toda, O. Niwa, and M. Yanagida. 1986. Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol. Cell. Biol. 6: 2168-2178.

2. Adams, A.E.M., and J.R. Pringle. 1984. Localization of actin and tubulin in wild-type and morphogenetic mutants of *Saccharomyces cerevisiae*. J. Cell Biol. 98: 934-945.

3. Blank, G.S., M.B. Yaffe, J. Szasz, E. George, T.L. Rosenberry, and H. Sternlicht. 1986. The role of Lys 394 in microtubule assembly. Ann. N. Y. Acad. Sci 466: 467-481.

4. Bond, J.F., J.L. Fridovich-Keil, L. Pillus, R.C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. Cell 44: 461-468.

5. Botstein, D., S.C. Falco, S. Stewart, M. Brennan, S. Sherer, D. Stinchcomb, K. Struhl, and R. Davis. 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8: 17-24.

6. Byers, B. 1981. Cytology of the yeast life cycle, p. 59-96. In J.N. Strathern, E.W. Jones, and J.R. Broach (ed.), The Molecular Biology of the Yeast *Saccharomyces*, vol. 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

7. Cleveland, D.W., and K.F. Sullivan. 1985. Molecular biology and genetics of tubulin. Annu. Rev. Biochem. 54: 331-365.

8. Cowan, N.J., P.R. Dobner, E.V. Fuchs, and D.W. Cleveland. 1983. Expression of human α-tubulin genes: interspecies conservation of 3' untranslated regions. Mol. Cell. Biol. 3: 1738-1745.

9. Davis, R., D. Botstein and J. Roth. 1980. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

10. Delgado, M.A., and J. Conde. 1984. Benomyl prevents nuclear fusion in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 193: 188-89.

11. Elliot, E.M., G. Henderson, F. Sarangi, and V. Ling. 1986. Complete sequence of three α-tubulin cDNAs in Chinese hamster ovary cells: each encodes a distinct α-tubulin isoprotein. Mol. Cell. Biol. 6: 906-913.

11a. Fridovich-Keil, J.L., J.F. Bond, and F. Solomon. 1987. Domains of β-tubulin essential for conserved functions *in vivo*. Mol. Cell. Biol. 7: 3792-3798.

12. Helftenbein, E. 1985. Nucleotide sequence of a macronuclear DNA molecule coding for α-tubulin from the ciliate *Stylonychia lemnae*. Special codon usage: TAA is not a translation termination codon. Nucl. Acid. Res. 13: 415-433.

13. Hiraoka, Y., T. Toda, and M. Yanagida. 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.

14. Holm, C., D.W. Meeks-Wagner, W.L. Fangman, and D. Botstein. 1986. A rapid,

efficient method for isolating DNA from yeast. Gene 42: 169-173.

15. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163-168.

16. Kilmartin, J. 1981. Purification of yeast tubulin by self-assembly in vitro. Biochemistry 20: 3629-3633.

17. Kilmartin, J., and A. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. J. Cell Biol. 98: 922-933.

18. Kimmel, B.E., S. Samson, J. Wu, R. Hirschberg, and L.R. Yarbrough. 1985. Tubulin genes of the African trypanosome *Trypanosoma brucei rhodesiense* : nucleotide sequence of a 3.7 kb fragment containing genes for α- and β-tubulins. Gene 35: 237-248.

19. King, S.M., and J.S. Hyams. 1982. The mitotic spindle of *Saccharomyces cerevisiae* : assembly, structure, and function. Micron 13: 93-117.

20. Kraemmer, G., M. Singhofer-Wowra, K. Seedorf, M. Little, and T. Schedl. 1985. A plasmodial α-tubulin cDNA from *Physarum polycephalum* : nucleotide sequence and comparative analysis. J. Mol. Biol. 183: 633-638.

21. Kuo, C., and J.L. Campbell. 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the CDC8 gene and two genes that compensate for the cdc8-1 mutation. Mol. Cell. Biol. 3: 1730-1737.

22. Lemischka, I.R., S. Farmer, V.R. Racaniello, and P.A. Sharp. 1981. Nucleotide

sequence and evolution of a mammalian α–tubulin messenger RNA. J. Mol. Biol. 151: 101-120.

23. Lewis, S.A., W. Gu, and N.J. Cowan. 1987. Free intermingli᠎ of mammalian β–tubulin isotypes among functionally distinct microtubules. Cell 49: 539-548.

24. Littauer, U.Z., D. Giveon, M. Thierauf, I. Ginzburg, and H. Ponstingl. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. Proc. Natl. Acad. Sci. USA 83: 7162-7166.

25. Ma, H., S. Kunes, P.J. Schatz, and D. Botstein. 1987. Introduction of cloned genes and functional elements to plasmids by recombination in yeast. Gene 58: 201-216.

26. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

27. Maxam, A.M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. 65: 499-560.

28. Moens, P.B., and E. Rapport. 1971. Spindles, spindle plaques, and meiosis, in the yeast Saccharomyces cerevisiae (Hansen). J. Cell. Biol. 50: 344-361.

29. Neff, N., J.H. Thomas, P. Grisafi and D. Botstein. 1983. Isolation of the β–tubulin gene from yeast and demonstration of its essential function in vivo. Cell 33: 211-219.

30. Peterson, J.B., and H. Ris. 1976. Electron-microscopic study of the spindle and

chromosome movement in the yeast *Saccharomyces cerevisiae.* J. Cell Sci. 22: 219-242.

31. Piperno, G., M. LeDizet, X.-j. Chang. 1987. Microtubules containing acetylated α-tubulin in mammalian cells in culture. J. Cell Biol. 104: 289-302.

32. Ponstingl, H., E. Krauhs, M. Little, and T. Kempf. 1981. Complete amino acid sequence of α-tubulin from porcine brain. Proc. Nat. Acad. Sci. USA 78: 2757-2761.

33. Pratt, L.F., S. Okamura, D.W. Cleveland. 1987. A divergent testis-specific α-tubulin isotype that does not contain a coded C-terminal tyrosine. Mol. Cell. Biol. 7: 552-555.

34. Pringle, J.R., S.H. Lillie, A.E.M. Adams, C.W. Jacobs, B.K. Haarer, K.G. Coleman, J.S. Robinson, L. Bloom, and R.A. Preston. 1986. Cellular morphogenesis in the yeast cell cycle, p. 193-209. In J. Hicks, ed., Yeast Cell Biology, Alan R. Liss, New York.

35. Quinlan, R.A., C.I. Pogson and K. Gull. 1980. The influence of the microtubule inhibitor, methyl benzimidazol-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae.* J. Cell Sci. 46: 341-352.

36. Raff, E.C. 1984. Genetics of microtubule systems. J. Cell Biol. 99: 1-10.

37. Rigby, P., M. Dieckman, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251.

38. Roy, D., and P.A. Fantes. 1983. Benomyl-resistant mutants of *Schizosaccharomyces*

pombe cold-sensitive for mitosis. Curr. Genet. 6: 195-202.

39. Sackett, D.L., B. Bhattacharyya, and J. Wolff. 1985. Tubulin subunit carboxyl termini determine polymerization efficiency. J. Biol. Chem. 260: 43-45.

40. Schatz, P.J., L. Pillus, P. Grisafi, F. Solomon, and D. Botstein. 1986. Two functional α-tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. Mol. Cell. Biol. 6: 3711-3721.

41. Schatz, P.J., F. Solomon, and D. Botstein. 1986. Genetically essential and nonessential α-tubulin genes specify functionally interchangeable proteins. Mol. Cell. Biol. 6: 3722-3733.

42. Serrano, L., J. Avila, and R.B. Maccioni. 1984. Controlled proteolysis of tubulin by subtilisin: localization of the site for MAP2 interaction. Biochemistry 23: 4675-4681.

43. Serrano, L., J. de la Torre, R.B. Maccioni, and J. Avila. 1984. Involvement of the carboxyl-terminal domain of tubulin in the regulation of its assembly. Proc. Natl. Acad. Sci. USA 81: 5989-5993.

44. Serrano, L., A. Valencia, R. Caballero, and J. Avila. 1986. Localization of the high affinity calcium binding site on tubulin molecule. J. Biol. Chem. 261: 7076-7081.

45. Sherman, F., G. Fink, and C. Lawrence. 1974. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

46. Silflow, C.D., R.L. Chisholm, T.W. Conner, and L.P.W. Ranum. 1985. The two

α–tubulin genes of *Chlamydomonas reinhardi* code for slightly different proteins. Mol. Cell Biol. 5: 2389-2398.

47. Silhavy, T.J., and J.R. Beckwith. 1985. Uses of *lac* fusions for the study of biological problems. Microbiol. Rev. 49: 398-418.

48. Snyder, J., and J. McIntosh. 1976. Biochemistry and physiology of microtubules. Annu. Rev. Biochem. 45: 699-720.

49. Southern, E., 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

50. Theurkauf, W.E., H. Baum, J. Bo, and P.C. Wensink. 1986. Tissue-specific and constitutive α–tubulin genes of *Drosophila melanogaster* code for structurally distinct proteins. Proc. Natl. Acad. Sci. USA 83: 8477-8481.

51. Thomas, J.H., N.F. Neff and D. Botstein. 1985. Isolation and characterization of mutations in the β–tubulin gene of *Saccharomyces cerevisiae*. Genetics 112: 715-734.

52. Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida. 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.

53. Toda, T., K. Umesono, A. Hirata, and M. Yanagida. 1983. Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe*. J. Mol. Biol. 168: 251-270.

54. Umesono, K., T. Toda, S. Hayashi, and M. Yanagida. 1983. Two cell division cycle genes NDA2 and NDA3 of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. J. Mol. Biol. 168: 271-284.

55. Villasante, A., D. Wang, P. Dobner, P. Dolph, S.A. Lewis, and N.J. Cowan. 1986. Six mouse α-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. Mol. Cell. Biol. 6: 2409-2419.

56. Wahl, G.M., M. Stern, and G.R. Stark 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. USA 76: 3683-3687.

57. Wood, J.S. 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae*. Mol. Cell. Biol. 2: 1064-1079.

58. Wood, J.S. and L.H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *S. cerevisiae*. J. Cell Biol. 94: 718-726.

59. Yousaf, S.I., A.R. Carroll, B.E. Clarke. 1984. A new and improved method for 3'-end labelling DNA using [α-^{32}P]ddATP. Gene 27: 309-313.

Chapter 5:

Isolation and Characterization of Mutations in the *TUB1* α–Tubulin

Gene of the Yeast *Saccharomyces cerevisiae*

Introduction:

The α-,β-tubulin heterodimer polymerizes into microtubules, structures that are involved in many aspects of eukaryotic cell structure and motility (Dustin, 1984). By electron and light microscopy, microtubules in yeast appear in intranuclear mitotic and meiotic spindles and in extranuclear arrays (Adams and Pringle, 1984; Byers, 1981; Byers and Goetsch, 1975; Kilmartin and Adams, 1984; King and Hyams, 1982; Matile et al., 1969; Moens and Rapport, 1971; Peterson and Ris, 1976). Evidence obtained through the use of antimicrotubule drugs (Davidse and Flach, 1977; Oakley and Morris, 1980; Sheirr-Neiss et al., 1978) has demonstrated the role of microtubules in chromosome separation on spindles and in nuclear movement during mitotic growth and mating (Delgado and Conde, 1984; Pringle et al., 1986; Quinlan et al., 1980; Wood, 1982; Wood and Hartwell, 1982). Conditional-lethal mutations in tubulin genes cause cell cycle arrests that are consistent with the role of microtubules in spindle function and nuclear movement (Hiraoka et al., 1984; Huffaker et al., 1988b; Roy and Fantes, 1983; Stearns and Botstein, 1988; Thomas et al., 1985; Toda et al., 1984; Toda et al., 1983). These mutants also display defects in meiotic spindles and in nuclear fusion during mating (Hiraoka et al., 1984; Huffaker et al., 1988; Toda et al., 1984; Thomas, 1984). Tubulin has been purified from yeast and shown to have biochemical properties similar to tubulin from higher eukaryotes (Kilmartin, 1981). We are using a combination of genetic, biochemical, and structural information to dissect molecular mechanisms responsible for microtubule function in yeast.

The single β-tubulin gene (TUB2) of the yeast Saccharomyces cerevisiae has been isolated, sequenced, and shown to be essential for growth (Neff et al., 1983). Two α-tubulin genes, TUB1 and TUB3, have been isolated, sequenced, and shown to encode proteins that are components of yeast microtubules (Chapter 2). The functional differences between these two genes have been examined through the construction of null mutations and by increasing their copy number on chromosomes and on plasmids (Chapter 3). Experiments with null alleles of

TUB3 demonstrated that *TUB3* was not essential for mitosis, meiosis or mating, although several minor phenotypes were observed. On the other hand, the *TUB1* gene was essential for growth of normal haploid strains. The difference between these two genes was not due to functional differences between the proteins, because extra copies of either gene could suppress the defects caused by a null mutation in the other. The number and functions of tubulin genes in *S. cerevisiae* are strikingly similar to those of the distantly related fission yeast *Schizosaccharomyces pombe* (Hiraoka et al., 1984, Toda et al., 1984; Adachi et al., 1986; M. Yanagida, personal communication).

Several different techniques have been used to isolate tubulin mutants of *S. cerevisiae* and *Schizosaccharomyces pombe*. The earliest mutants were isolated on the basis of altered sensitivity to benzimidazole antimicrotubule drugs (Roy and Fantes, 1983; Thomas et al., 1985; Toda et al., 1983; Umesono et al., 1983; Yamamoto et al., 1980). In addition to altered drug sensitivity, some of these mutants also exhibited conditional-lethal cell growth. Tubulin alleles have also been identified in screens for conditional-lethal mutants unable to undergo nuclear division (Toda et al., 1983). The most direct way to obtain tubulin mutants is through mutagenesis of the cloned gene, a technique made possible by the development of sophisticated techniques for manipulating yeast genes in vivo (Botstein and Davis, 1982). Using the integrating plasmid method of Shortle et al. (1984), Huffaker et al. (1988b) have isolated several mutations in the *TUB2* gene of *S. cerevisiae*. Additionally, using noncomplementation analysis, Stearns and Botstein (1988) have isolated new alleles of *TUB2* and also the first conditional-lethal alleles of the α-tubulin gene *TUB1*. This method, which was used previously with the tubulin genes of *Drosophila* (Raff and Fuller, 1984), relies on the failure of complementation between strains carrying recessive mutations in different genes that encode components of some functional complex.

To date, the tubulin mutants discussed above have been used mostly to determine the role of microtubules in the life cycle of yeast. A complete understanding of microtubule function in yeast, however, will require the identification of many other genes whose products

participate in microtubular structures. One major approach to identifying such genes starts with the isolation of tubulin mutants, followed by suppression analysis, noncomplementation analysis, or other methods that allow the identification of interacting genes (for review see Huffaker et al, 1988a). A second strategy relies on the *de novo* identification of mutants with phenotypes related to microtubule function (eg. Rose and Fink, 1987; Schild et al., 1981; Thomas and Botstein, 1986; Baum et al., 1986a, b). A third approach begins with biochemical identification of protein components of microtubular structures (Pillus and Solomon, 1986; Snyder and Davis, 1986). These three methods will continue to yield complementary information about yeast microtubule function.

To explore the functions of α-tubulin in yeast and to lay the groundwork for future studies of microtubules, we have isolated 70 conditional-lethal alleles of the *TUB1* gene. Because of the inefficiency of the integrating plasmid (Shortle et al., 1984) method for the isolation of mutations in essential genes, we have used a technique, called the "plasmid shuffle", that relies on replacement of one plasmid by another (Boeke et al., 1988). We report on fine structure mapping of these mutations and on detailed studies of the phenotypes that they display.

Materials and Methods:

Bacterial strains and media, electrophoresis, DNA preparations, and hybridizations were as previously described (Chapter 2, Chapter 3).

Strains and Media:

Media for yeast growth and sporulation were made as described by Sherman et al. (1974), except that adenine, uracil and tryptophan were routinely added to YPD after autoclaving. Benomyl, 98.6%, was a gift from E. I. Dupont de Nemours and Company, Inc. It was kept as a 10mg/ml stock in dimethyl sulfoxide at 4 degrees and was added to warm YPD with swirling immediately before pouring plates. 5-fluoroorotic acid (5-FOA) was purchased from PCR Inc. It was used to select for yeast strains without a functional *URA3* gene (Boeke et al., 1984). The powder was dissolved directly in warm medium with stirring before pouring the plates. Because it was found to be more effective at warmer temperatures, 5-FOA was used at a concentration of 0.5 mg/ml in 37° plates and at 1.0 mg/ml in 14° and 26° plates. The yeast strains used in this paper were derived from a set of essentially isogenic S288C strains originally provided by G. R. Fink. The strains used are listed in Table 1.

Genetic Techniques and Transformation:

Methods of yeast mating, sporulation and tetrad analysis were as described by Sherman et al. (1974). Yeast cells were transformed by the lithium acetate method of Ito et al. (1983) as modified by Kuo and Campbell (1983) using 1 to 4 micrograms of plasmid DNA with 50 micrograms of chick blood DNA (Sigma) as carrier. Transformants were plated on SD medium supplemented with the appropriate nutrients in order to select cells with the plasmid.

Plasmid Constructions:

The plasmid pRB306, a pBR322 derivative carrying the *TUB1* gene, has been described

previously (Chapter 2, Fig. 1). The integrating plasmid pRB337, that was used in attempts to

isolate *TUB1* mutations, consisted of the 1.9 kilobase (kb) BglII fragment from PRB306

inserted into the BamHI site of YIp5 (Botstein et al., 1979). The BglII fragment contained all

of the *TUB1* coding sequences except the first exon of 9 codons and about 1/4 of the single

TUB1 intron. The vector YIp5 is a pBR322 derivative containing the *URA3* gene of yeast as

a selectable marker.

The plasmid pRB539 (Fig. 1) consisted of a 3.1 kb SphI to BglII fragment, containing the

entire *TUB1* gene, inserted between the SphI and BamHI sites of YCp402. YCp402 is a

replication-competent yeast centromere vector containing the yeast genes *CEN4*, *ARS1*, and

LEU2 (Appendix1-Ma et al., 1987). pRB539 was constructed using the method described in

Appendix 1 (Ma et al., 1987) by in vivo recombination between the

TUB1-URA3-CEN4-ARS1 plasmid pRB326 (Chapter 3) and a *LEU2* containing fragment of

pRB327 (Chapter 3).

The plasmid pRB316 (Fig. 1) contained the *TUB3* gene inserted into the *URA3-2μ*

plasmid YEp24 (Botstein et al., 1979). Its construction has been described previously

(Chapter 3).

In Vitro Mutagenesis:

Misincorporation mutagenesis was performed essentially as described by Shortle et al.

(1982). Plasmid DNA was nicked randomly with DNAse I in 50 mM Tris pH 7.5, 5 mM

$MgCl_2$, 0.01% gelatin, 100 μg/ml ethidium bromide until all of the molecules were converted

to open circular form (as determined by mobility on an agarose gel). The nicks were

expanded to single stranded gaps using 0.05 units/μl of *Micrococcus luteus* DNA polymerase

I (PL Biochemicals, Inc.) at 26° for 60 min. in 70 mM Tris pH 8.0, 7 mM $MgCl_2$, 1 mM

ß-mercaptoethanol. The efficiency of gap formation was assayed by attempting to ligate the

gapped plasmids into closed circles in parallel with ligation of control nicked plasmid. The

gaps were filled and reclosed in the presence of 3 of the 4 deoxynucleotide triphosphates

(0.125 mM), *M. luteus* DNA polymerase (0.008 units/µl), and T4 DNA ligase (2.5 units/µl) in 60 mM Tris pH 8.0, 20 mM ß-mercaptoethanol, 1 mM MgAcetate, 2mM $MnCl_2$, 0.5 mM ATP.

The four gap filling reactions were designated -A, -C, -G, and -T according to which nucleotide was missing. The level of mutagenesis was determined by transforming samples of each of the four reaction mixtures into *E. coli* to check for loss of function of the nutritional marker on the plasmid. To check loss of *URA3* function on plasmid pRB337, we used *E. coli* DB6507 *(F⁻, leuB6, pyrF74::Tn5, proA2, recA13, hsdS20 (r⁻, m⁻), ara-14, lacY1, galK2, rpsL20 (Smʳ), xyl-5, mtl-1, supE44).* Since the *URA3* gene complemented the phenotype of the *pyrF* mutation, loss of *URA3* function resulted in uracil auxotrophy among a fraction of the mutagenized plasmids. To check for loss of *LEU2* function on plasmid pRB539, we used strain HB101 *(F⁻, leuB6, proA2, recA13, hsdS20 (r⁻, m⁻), ara-14, lacY1, galK2, rpsL20 (Smʳ), xyl-5, mtl-1, supE44).* Functional *LEU2* complemented the phenotype of the *leuB* mutation, so the level of mutagenesis was estimated by determining the fraction of Leu⁻ transformants from the 4 mutagenesis reaction mixtures.

The four gap filling reaction mixtures were divided into 8 independent pools and were amplified separately in *E. coli*. In all cases, each mutagenized pool of DNA was amplified from about 40,000 independent *E. coli* transformants. The pools were numbered as follows: Pools 1 and 2 were from the -A reaction mixture; pools 3 and 4 were from the -C reaction; pools 5 and 6 were from the -G reaction; pools 7 and 8 were from the -T reaction. Table 2a lists the mutagenized pool from which each of the *TUB1* mutants was isolated.

Hydroxylamine mutagenesis of pRB539 was carried out using the method described by Rose and Fink (1987). 0.35 g of hydroxylamine hydrochloride and 0.09 g of NaOH were dissolved in water to make 5 ml of solution. 0.5 ml of this solution was added to 10 µg of plasmid in each of 12 tubes. The tubes were covered and incubated at 37° for 24 hr. After incubation, 10 µl of 5 M NaCl and 50 µl of 1 mg/ml bovine serum albumin were added to each tube to quench the reaction, followed by 1 ml of ethanol to precipitate the DNA. After

incubation at -20° for 60 min., the tubes were spun in a microcentrifuge for 10 min. to pellet

the DNA. The DNA was washed with cold ethanol, dissolved in 10 mM Tris, 1 mM EDTA

pH 8.0, and used directly for yeast transformation. This DNA was designated pool 9.

The number of mutations isolated from each mutagenized pool (see below) was as follows:

Pool 1 (7), Pool 2 (7), Pool 3 (5), Pool 4 (7), Pool 5 (4), Pool 6 (6), Pool 7 (11), Pool 8 (9),

Pool 9 (14). Thus hydroxylamine was the most efficient mutagen, although it only produces

a limited spectrum of changes.

"Plasmid Shuffle" Mutant Isolation:

The 9 pools of mutagenized plasmid pRB539, 8 from misincorporation mutagenesis and 1

from hydroxylamine mutagenesis, were introduced into yeast strain DBY2384. Before

plating, the cells were incubated in SD minimal medium containing 45 μg/ml leucine and 30

μg/ml lysine for 30 min. 0.33 ml of cells were spread on each plate containing SD plus 30

μg/ml lysine and 20 μg/ml uracil. The preincubation in limiting amounts of leucine was found

to increase the transformation frequency about 4 fold. The master transformation plates were

incubated at 26° for 3 to 4 days.

The master plates were replica-plated to SD + lysine + uracil (control plates) and to SD +

lysine + 5.6 μg/ml uracil + 5-FOA (5-FOA plates, see above for concentrations) at 14° and

37°. In an initial experiment, colonies were selected that would not grow on the 5-FOA plates

at one or both temperatures, but would grow on the control plates at all temperatures. These

strains were picked and retested on the same plates at 14°, 26°, and 37°. 160 of these

strains, carrying presumptive TUB1 loss-of-function mutations (allele numbers 101-260) on

the LEU2 plasmid, were frozen and were not characterized further. Strains carrying

conditional-lethal mutations in TUB1 were identified as those that would: [1] grow at 26° on

5-FOA plates, but not at 14° and/or 37° on 5-FOA, and [2] grow at all temperatures on

control plates. The two mutations that resulted in both temperature-sensitivity (ie.

warm-sensitive, called Ts) and cold-sensitivity (Cs) were isolated in these initial experiments

along with several others that were only Ts or Cs. The two Ts/Cs alleles were designated *tub1-501* and *tub1-502*.

In later experiments, strains that carried conditional-lethal *TUB1* mutations were identified as those that grew on 5-FOA at 14° but not at 37° (or vice versa), but grew at both temperatures on control plates. These candidates were then retested on control and 5-FOA plates at 14°, 26°, and 37°. Strains that exhibited conditional-lethality only at 14° or 37° on 5-FOA plates were struck out on rich (YPD) medium at 26°. To confirm that the mutant phenotype was due to the *TUB1* gene on the *LEU2* plasmid, replicas of these plates were incubated at nonpermissive (14° or 37°) and at permissive temperature (26°) on YPD and on appropriate SD plates to check for auxotrophies. Only Leu$^+$, Ura$^-$ colonies of authentic mutants exhibited conditional-lethality. The one tight Ts allele isolated was designated *tub1-603*. The 67 Cs alleles were named *tub1-701* through *tub1-767*. The *TUB1-LEU2* plasmids were isolated from all of these strains by transformation of DNA into *E. coli*, and their structure was confirmed by restriction analysis (data not shown). The plasmids were then retransformed into DBY2384 to allow confirmation of the conditional-lethal phenotypes caused by the mutations. Leu$^+$, Ura$^-$ strains from these experiments were used for all subsequent characterization of the phenotypes caused by the mutations.

Fine Structure Mapping:

The *TUB1* mutations were localized to regions of the gene using the method of Kunes et al. (1987). Plasmids carrying the *TUB1* mutations were cleaved with SphI, that cut at a single site 1 kilobase (kb) upstream of the start codon, between the yeast sequences and the backbone pBR322 sequences of the plasmid. A series of DNA fragments from pRB539, the wild-type plasmid, were isolated from agarose gels (Fig. 2, Fig. 3). These fragments overlapped the SphI site of the plasmid and extended into the *TUB1* coding region to different extents. Each of the cut mutant plasmids were mixed with each of the wild-type "healer" fragments and introduced into yeast DBY2384 by the LiAc method. Cotransformation of a

cut plasmid with a restriction fragment that overlaps the cut site results in high frequency recombination to regenerate the original plasmid (Kunes et al., 1985). Cells that had received the plasmid were selected on SD + lysine + uracil plates at 26°. To test whether the recombination event regenerated the wild-type coding sequence, the transformant colonies were replica-plated to 5-FOA plates (see above) at the nonpermissive temperature. Colonies containing wild-type TUB1-LEU2 plasmid could grow on 5-FOA plates while colonies containing the mutant TUB1-LEU2 plasmids could not. The interval in which each mutation mapped was defined by the shortest fragment that yielded wild-type colonies at a frequency above background.

Because we used a set of healer fragments that all started from one side of the TUB1 gene, the mapping data only defined the minimum amount of the TUB1 coding sequence sufficient to correct the defect(s) that caused the conditional phenotype. Therefore, this procedure did not rule out the existence of mutations in multiple intervals.

Two of the mutations, tub1-501 and tub1-502 caused both Ts and Cs phenotypes. The mapping procedure for these two mutations involved replica-plating to both nonpermissive temperatures. Since Ts^+ colonies were always Cs^+ and vice versa, we concluded that the same mutation caused both phenotypes.

Temperature Shift Experiments:

Mutants were grown in YPD medium at the permissive temperature and their growth was monitored by absorbance measurements at 600 nm (A_{600}). These measurements were used to determine the generation time at permissive temperature that is shown in Table 2a (see below). When the cultures reached an A_{600} of 0.1 (about 1×10^6 cells/ml), a portion of the culture was shifted to the nonpermissive temperature. At this time, samples of the permissive temperature cultures were fixed by mixing them 1:1 with 10% formaldehyde for 2 to 12 hrs. After fixation, the cells were centrifuged at 2k for 2 min., washed in 0.1 M KPO_4 pH 6.5, and stored in 1.2 M sorbitol, 0.1 M KPO_4 pH 7.5 at 4° until used. These fixed samples were

used for immunofluorescence (see below), cell counts in a Coulter counter, and counts of cell division cycle morphology. Also at this time, portions of the cultures were sonicated briefly to disrupt clumps and plated on YPD plates at permissive temperature for viable cell count determinations.

Samples were taken from the shifted cultures at intervals of about one generation time (as determined by the growth of the wild type). These samples were fixed for use in immunofluorescence or cell counts as above and were also plated at permissive temperature for viable counts.

Determination of Growth Rates:

Data from Coulter or viable cell counts or from A_{600} measurements were plotted on a Macintosh personal computer using CricketGraph software as $\log_2$(cell count or A_{600}) vs. time in hrs. The slope of a least-squares line through the points was determined by the program. The generation time (or half-life of dying cells) was computed by taking the reciprocal of the slope.

Anti-Tubulin Immunofluorescence:

Formaldehyde-fixed cells were prepared for anti-tubulin immunofluorescence by a variation of the method of Kilmartin and Adams (1984). The walls of the fixed cells were digested with 100 µg/ml zymolyase 60,000 (Seikagaku Kogyo) in 1.2 M sorbitol, 25 mM ß-mercaptoethanol, 0.1 M KPO$_4$ pH 7.5 for 30 min. at 30°. The cells were centrifuged at 2k for 2 min. and were resuspended in PBS buffer (50 mM KPO$_4$, 150 mM NaCl pH 7.2). Multiwell slides were treated with 1 mg/ml polylysine 400,000 (Sigma). After the polylysine dried, the slides were washed for 1 min. in water and allowed to dry. The cells were mounted on the slides and then washed three times in PBS and once in PBS + 1 mg/ml bovine serum albumin (BSA). The primary antibody was YOL1/34 anti-yeast-α-tubulin (Kilmartin et al., 1982) diluted 1/250 in PBS/BSA. It was used for 1 hr. at room temperature followed by 4

washes with PBS. The secondary antibody was fluorescein labeled Goat-anti-Rat (Cappel) diluted 1/100 in PBS. Incubation was for 1 hr. at room temperature followed by 4 washes with PBS. The cells were then stained with a 1 µg/ml solution of the DNA stain 4',6'-diamidino-2-phenylindole (DAPI) in PBS for 5 min. followed by 4 washes with PBS. The cells were mounted in 1 mg/ml p-phenylenediamine, 90% glycerol/10% PBS pH 9.0 and observed with a Zeiss microscope equipped for epifluorescence. A Zeiss Neofluor 63x objective was used for both epifluorescence and Nomarski optics. The DAPI-specific and fluorescein-specific filters produced images free of detectable crossover. Photography was with hypersensitized Kodak Technical Pan 2415 film (Lumicon Co.) developed with Kodak D-19 developer.

Table 1. Yeast Strains Used in this Study:

Strain	Genotype of Strain
DBY2375	*MATa, his3-Δ200, leu2-3,112, lys2-801, ura3-52, tub3::TRP1*
DBY2376	*MATα, ade4, his3-Δ200, leu2-3,112, ura3-52, tub3::TRP1*
DBY2379	*MATα, ade4, his3-Δ200, leu2-3,112, ura3-52*
DBY2056	*MATα, ura3-52*
DBY2384	*MATα, his3-Δ200, leu2-3,112, lys2-801, ura3-52, tub1::HIS3, tub3::TRP1, TUB3-URA3-2μm* (pRB316)
DBY2395	*MATα, his3-Δ200, leu2-3,112, lys2-801, ura3-52, tub1::HIS3, tub3::TRP1, tub1-501-LEU2-CEN4-ARS1* (pRB594)
DBY2396	as DBY2395 except *tub1-502* (pRB595)
DBY2397	as DBY2395 except *tub1-603* (pRB598)
DBY2398	as DBY2395 except *tub1-703* (pRB603)
DBY2399	as DBY2395 except *tub1-704* (pRB604)
DBY2400	as DBY2395 except *tub1-705* (pRB605)
DBY2401	as DBY2395 except *tub1-709* (pRB609)
DBY2402	as DBY2395 except *tub1-713* (pRB613)
DBY2403	as DBY2395 except *tub1-714* (pRB614)
DBY2404	as DBY2395 except *tub1-716* (pRB616)
DBY2405	as DBY2395 except *tub1-717* (pRB617)
DBY2406	as DBY2395 except *tub1-719* (pRB619)
DBY2407	as DBY2395 except *tub1-723* (pRB623)
DBY2408	as DBY2395 except *tub1-724* (pRB624)
DBY2409	as DBY2395 except *tub1-727* (pRB627)
DBY2410	as DBY2395 except *tub1-728* (pRB628)
DBY2411	as DBY2395 except *tub1-729* (pRB629)
DBY2412	as DBY2395 except *tub1-730* (pRB630)
DBY2413	as DBY2395 except *tub1-731* (pRB631)
DBY2414	as DBY2395 except *tub1-733* (pRB633)
DBY2415	as DBY2395 except *tub1-735* (pRB635)
DBY2416	as DBY2395 except *tub1-737* (pRB637)
DBY2417	as DBY2395 except *tub1-738* (pRB638)
DBY2418	as DBY2395 except *tub1-741* (pRB641)
DBY2419	as DBY2395 except *tub1-742* (pRB642)
DBY2420	as DBY2395 except *tub1-743* (pRB643)
DBY2421	as DBY2395 except *tub1-744* (pRB644)
DBY2422	as DBY2395 except *tub1-746* (pRB646)
DBY2423	as DBY2395 except *tub1-747* (pRB647)
DBY2424	as DBY2395 except *tub1-749* (pRB649)
DBY2425	as DBY2395 except *tub1-750* (pRB650)
DBY2426	as DBY2395 except *tub1-758* (pRB658)
DBY2427	as DBY2395 except *tub1-759* (pRB659)
DBY2428	as DBY2395 except *tub1-760* (pRB660)
DBY2429	as DBY2395 except *tub1-761* (pRB661)
DBY2430	as DBY2395 except *tub1-763* (pRB663)
DBY2431	as DBY2395 except *tub1-764* (pRB664)
DBY2432	as DBY2395 except *tub1-767* (pRB667)
DBY2433	as DBY2395 except *TUB1* (pRB539)

Results:

Isolation of α-tubulin mutants:

In order to explore the functions of α-tubulin in the yeast *Saccharomyces cerevisiae*, we isolated a large set of α-tubulin mutants. This yeast has two α-tubulin genes, *TUB1* and *TUB3*, that differ in their importance for normal cell growth. *TUB1* is essential for growth of normal haploid strains, while *TUB3* is not. The difference between these two genes is not due to functional differences between the proteins, because extra copies of either gene can suppress the defects caused by a null mutation in the other (Chapter 3). Because of this functional similarity between the two proteins, we isolated mutants of the more strongly expressed *TUB1* gene in a strain with it functional *TUB3*. This strategy prevented interference by *TUB3* with the phenotypes of *TUB1* mutants. We used two strategies with widely different degrees of success.

Attempts to Isolate *TUB1* Mutants Using Integrating Plasmids:

In our initial attempts to isolate *TUB1* mutants, we used the method of Shortle et al. (1984). This method employs an integrating plasmid (with selectable marker but without a yeast replication origin) carrying a version of the gene in question that has been truncated at one end. When such a plasmid is cleaved in the sequences of the gene in question and used in yeast transformation, it will integrate into the chromosome at the locus of the gene by homologous recombination (Orr-Weaver et al, 1981). This integration event produces a partial duplication of the gene containing one functional and one nonfunctional copy, with the plasmid sequences and selectable marker between the two copies. If the plasmid is mutagenized before transformation, some of the mutations will be incorporated into the intact version of the gene. Thus, the method can produce recessive or dominant conditional-lethal mutations in essential genes (Shortle et al., 1984).

The plasmid used, pRB337, contained a fragment of the *TUB1* gene missing the first exon

of 9 codons and the 5' splice site of the single intron. The plasmid contained the *URA3* gene

to permit selection of strains with the plasmid. The strain used, DBY2375, contained a large

insertion/deletion in the *TUB3* gene. To confirm that the plasmid behaved as expected, we

transformed DBY2375 to Ura$^+$ with unmutagenized plasmid that had been cut with XbaI to

direct integration to the *TUB1* locus. When one of the transformants was crossed to

DBY2379, we observed several properties consistent with expected behavior of the plasmid.

First, the *URA3* marker was linked to *TUB3* (PD:NPD:TT = 6:0:9), consistent with

integration at the *TUB1* locus (Chapter 3). Second, the Ura3$^+$ spores showed expression

levels of *TUB1* consistent with the presence of only one functional copy of *TUB1* at the

partially duplicated locus. Expression was tested by measuring resistance of the progeny of

the cross to various concentrations of benomyl, an assay that is sensitive to levels of α-tubulin

gene dosage (Chapter 3).

We mutagenized the plasmid in vitro by base misincorporation (Shortle et al., 1982), that

is reported to cause random base changes at single sites or short stretches uniformly around

the plasmid (see Materials and Methods). The level of mutagenesis was checked by

introducing the DNA into *E. coli* strain DB6507 *(pyrF⁻)*. The *URA3* gene complemented the

phenotype of the *pyrF* mutation to allow growth on medium without uracil, so the fraction of

Ura⁻ transformants was an indication of the amount of mutagenesis. DB6507 transformants

from 4 independent mutagenized pools yielded an average of 1.5% Ura⁻'s (20/1356), while

unmutagenized plasmid yielded none (0/254) . Since 11% of the plasmid consisted of *URA3*

sequences, about 14% (1.5/11) of the plasmids received lesions of sufficient severity to

disrupt *URA3* function, presumably at random sites around the plasmid. The mutagenized

DNA was divided into 8 pools, amplified in *E. coli* (about 60,000 independent plasmids per

pool), and used in large scale transformations.

We screened about 40,000 Ura$^+$ transformant colonies for temperature-sensitive (Ts) or

cold-sensitive (Cs) mutants by replica-plating from 26°C master plates to plates at 37° and

14°. Of 41 temperature-sensitive candidates picked and retested, 8 did not retest as Ts, 7

were Ts for *URA3* function, and 26 were Ts mutants. Of 70 Cs candidates picked, 49 did not retest as Cs, 1 was Cs for *URA3*, and 20 were Cs mutants. As a next step, we needed to test these mutants to distinguish authentic *TUB1* mutants from mutants with defects in some other gene. Because of ambiguous results obtained by others in complementation tests with tubulin mutants (T. Huffaker and T. Stearns, personal communication), we checked the remaining 26 Ts and 20 Cs candidates for linkage between the *URA3* marker on the integrating plasmid and the conditional-lethal allele. All *TUB1* mutations should have been linked to *URA3*, though linkage would not have proven that the mutation was in *TUB1*. The Ts and Cs candidates were crossed to DBY2376 and 8 tetrads from each cross were dissected and analyzed. In no case did the conditional phenotype cosegregate with Ura3$^+$. In 3 of the crosses with Ts candidates, the progeny displayed the high levels of spore lethality expected from crosses with aneuploids (Mortimer and Schild, 1981). In 5 of the crosses with Cs candidates, no obvious Cs phenotype segregated in the cross. In the rest of the crosses (23 with Ts mutants and 15 with Cs mutants), the conditional phenotype segregated 2:2 and Ura3$^+$ segregated 2:2, but the two were unlinked. We concluded that all of these mutants were spurious nuclear mutations that arose during the growth of the strain or during the transformation procedure. We found no authentic *TUB1* mutants by this method.

We were interested to discover the origin of these spurious conditional-lethal mutations. One possibility was that they arose because of the partial tubulin defects in the starting *tub3*$^-$ strain. One known property of *tub3*$^-$ strains was their increased rate of chromosome loss (Chapter 3). During growth of this strain, variants could have arisen with extra copies of any of the yeast chromosomes. Since some chromosome abnormalities cause sickness and conditional-lethality (eg. see Chapter 3), it seemed possible that many of the spurious mutants were the result of disomy. One prediction of this model was that the conditional allele, if caused by disomy, should have been centromere linked. To test this, 3 Ts strains and 3 Cs strains were crossed to strain DBY2056. The resulting spores were examined to check for an overabundance of parental ditype and nonparental ditype tetrads when scored for the

centromere linked marker *leu2* and the conditional-lethality. In no case did the conditional-lethal mutation behave as if it were centromere linked. We concluded that most of the conditional alleles isolated were non-centromere-linked nuclear mutations that arose either during growth of the strain or during transformation, as has been observed previously (Shortle et al., 1984). Because of this high level of nonspecific background, we used the technique described below to isolate *TUB1* mutants.

Isolation of *TUB1* Mutants Using the "Plasmid Shuffle":

The "plasmid shuffle" is a method for isolating mutants with defects in essential genes (Boeke et al., 1988; see Fig. 1). In brief, a strain is constructed with a chromosomal deletion of the gene of interest. The essential function is supplied by a copy of the gene on a plasmid; the plasmid also carries a marker for which a negative selection exists. A second plasmid carrying the gene and a different selectable marker is mutagenized and then introduced into the strain. Transformant colonies, selected for the presence of the second plasmid, are then screened at various temperatures after replica-plating to two different media. One medium selects against the first plasmid. The other is the same as the transformation medium selecting for the second plasmid but allowing the first to be lost. Colonies that contain null mutations of the copy of the gene on the second plasmid will be unable to lose the first plasmid at any temperature because the gene is essential for growth. They will score as negatives at all temperatures on the negative selection plates. Because the second plasmid can replicate, they will grow on the original transformation medium at any temperature. Colonies containing conditional-lethal alleles of the copy of the gene on the second plasmid will grow on the negative selection plates at some temperatures but not others. They will grow on the original transformation medium at all temperatures. One of the advantages of this technique is that colonies carrying spurious conditional-lethal nuclear mutations should not grow on either medium at the nonpermissive temperature and thus can be eliminated early in the screening process. Furthermore, colonies carrying defects in the selectable marker or replication

Figure 1. Plasmid shuffle strategy for isolating *TUB1* mutations. The starting strain,

DBY2384, contained deletions of both *TUB1* and *TUB3* on the chromosome and *TUB3* on

the plasmid pRB316. It was transformed to Leu$^+$ with the *TUB1* plasmid pRB539, that had

been mutagenized in vitro. Colonies were grown at 26° on plates lacking leucine. The

colonies were replica-plated on to 5-FOA plates to select those cells that had lost the

unmutagenized *TUB3* plasmid. Colonies were selected that did not grow at 37° or 14° on

5-FOA.
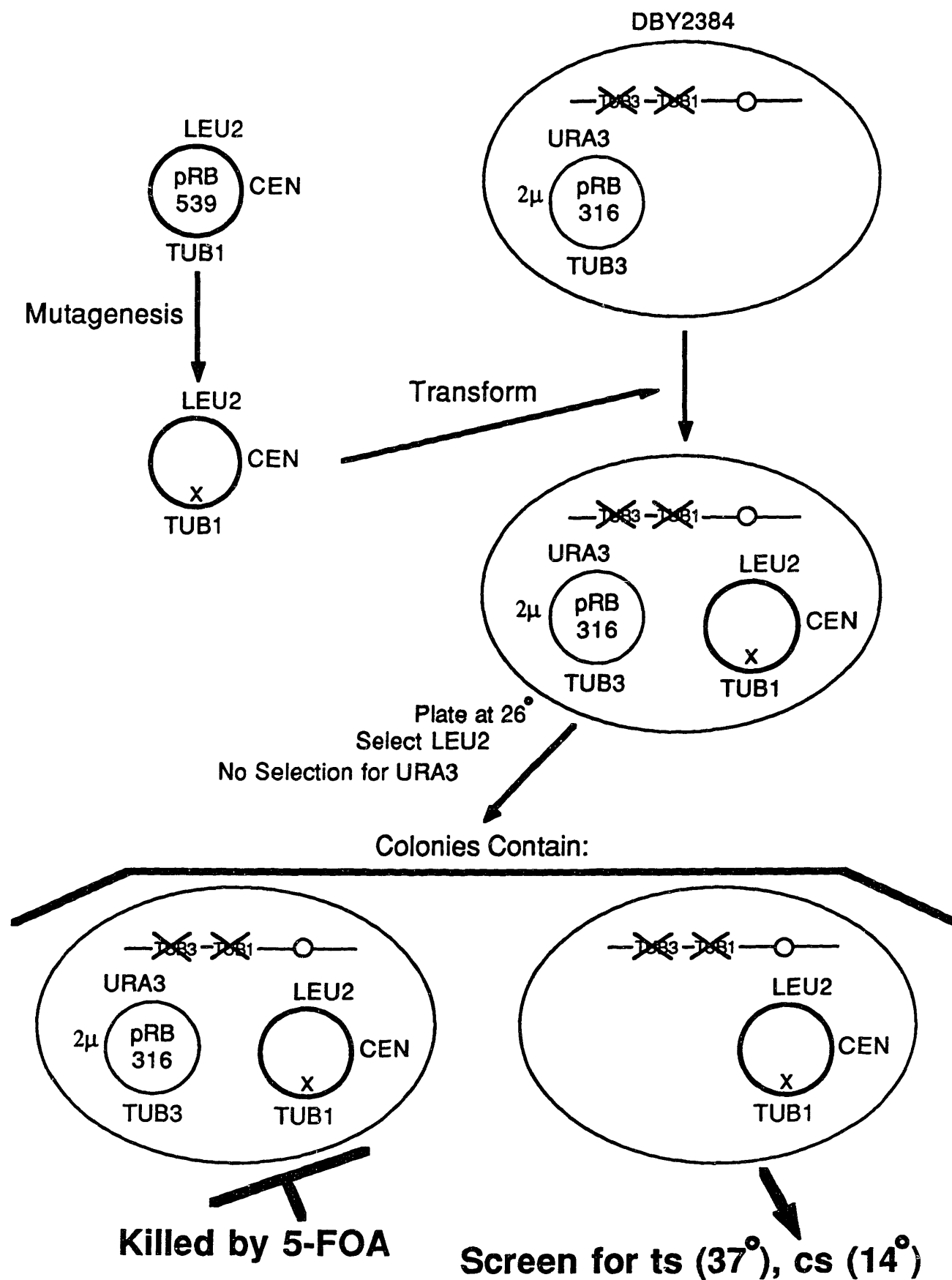
functions of the second plasmid will show growth defects at some or all temperatures on the original transformation medium.

The strategy used for isolating *TUB1* mutants is shown in Figure 1. Because of the functional equivalence of *TUB1* and *TUB3*, we deleted both from chromosome 13 in the starting strain (DBY2384). Functional α-tubulin was supplied from a *TUB3-URA3-2μm* plasmid (pRB316). The *URA3* gene was chosen because the drug 5-fluoro-orotic acid (5-FOA; Boeke et al., 1984) allows selection against Ura3$^+$ cells. A *2μ* origin of replication on the plasmid normally leads to high copy number and low stability (Botstein et al., 1979). The plasmid, however, appeared to be completely stable in DBY2384, presumably because loss of the plasmid was lethal. The tubulin genotype of the strain was confirmed by gel transfer hybridization analysis (see Chapter 4, Fig. 4, lane 10). DBY2384 was transformed to Leu2$^+$ with the *TUB1, LEU2, CEN4, ARS1* containing plasmid, pRB539, that had been mutagenized in vitro. Such centromere (*CEN*) plasmids normally exist as low copy, but fairly stable plasmids (Clarke and Carbon, 1985). We used two mutagenesis methods, base misincorporation (Shortle et al., 1982) and treatment with hydroxylamine (Rose and Fink, 1987), as described in Materials and Methods. Approximately 56,000 transformant colonies were screened for the presence of *TUB1* mutations (see Materials and Methods).

By screening for strains unable to grow without the *TUB3* plasmid at any temperature, we isolated 160 strains that carried loss-of-function mutations in the *TUB1* gene. These *TUB1* alleles were designated *tub1-101* through *tub1-160*. The strains were frozen and were not characterized further.

By screening for strains unable to grow at certain temperatures without the *TUB3* plasmid, we isolated 70 conditional-lethal alleles of *TUB1*. These experiments revealed a definite bias in the ability of *TUB1* to mutate to temperature(warm)- versus cold-sensitivity: 67 of the alleles resulted in cold-sensitivity (Cs), 1 resulted in temperature-sensitivity (Ts), and 2 displayed both Ts and Cs phenotypes (Ts/Cs). The Ts/Cs alleles were designated *tub1-501* and *tub1-502*; the Ts allele was called *tub1-603*; the Cs alleles were numbered *tub1-701*

through *tub1-767*. To confirm that the mutations were in the *TUB1* gene on the plasmid, we

isolated the plasmids and reintroduced them into DBY2384. All of them enabled the

transformant strains to grow at all temperatures on medium without leucine when the covering

TUB3 plasmid was present, but only at certain temperatures when it was not present. The

ability to grow at all temperatures on medium without leucine demonstrated that the mutations

did not substantially affect the replication functions or the *LEU2* gene on the mutagenized

plasmids. This observation also demonstrated that all of the *TUB1* alleles could be

suppressed by *TUB3* in high copy number. When the *TUB3* plasmid was lost, however, all

of the strains showed a clear conditional-lethal phenotype.

Fine Structure Mapping:

To confirm that these mutations were in the *TUB1* gene and to localize them to distinct

regions of the gene, we performed fine structure mapping by the method of Kunes et al.

(1987). We selected 38 of the mutant plasmids for this analysis on the basis of the growth

characteristics of the corresponding strains (see below). The mapping strategy and results are

shown in Figs. 2 and 3. The mutant plasmids were digested with SphI, that cuts 1.0

kilobases (kb) upstream of the *TUB1* start codon. The cut plasmids were introduced into

DBY2384 along with one of a series of fragments of the wild-type plasmid, that overlapped

the cut site and contained progressively more of the *TUB1* sequences. When a cut plasmid

was introduced into yeast along with a homologous "healer" fragment that overlapped the cut

site, homologous recombination took place to regenerate the original plasmid (Kunes et al.,

1985). By using several healer fragments containing progressively increasing amounts of a

gene, mutations in the cut plasmids could be localized to deletion intervals (Kunes et al.,

1987). This was accomplished by determining the shortest of the healer fragments that could

restore wild-type activity to the mutant gene (Fig. 2).

The strain used for these experiments, DBY2384, was the strain used in the original

mutant isolation. It contained chromosomal deletions of both *TUB1* and *TUB3* along with a

Figure 2. Strategy for fine structure mapping of *TUB1* mutations. The plasmids containing the *TUB1* mutations, represented by the circle, were cleaved with SphI at a site 1 kb upstream of the *TUB1* start codon. They were introduced into yeast along with "healer" fragments of a similar plasmid that contained the wild-type *TUB1* gene (represented by arcs). The TUB1 coding sequence is represented by the widened portion of the circle. The X represents a hypothetical mutation in deletion interval F. Recombination between this mutant plasmid and healer E (or shorter healers) would result in regeneration of the original mutant plasmid. Recombination between the mutant plasmid and healer F or G would produce some wild-type recombinants. The production of wild-type recombinants was detected by replica-plating to an appropriate medium at the nonpermissive temperature (see Materials and Methods).
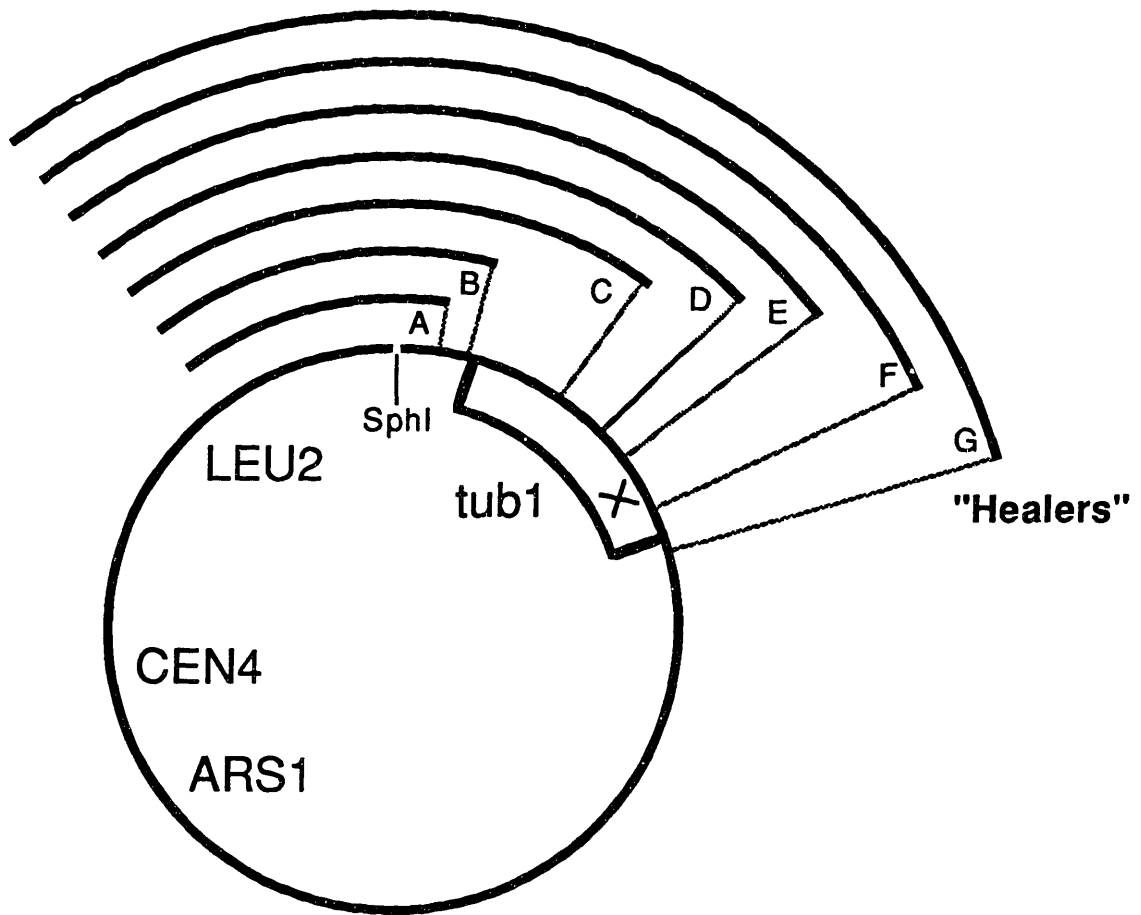
Healer	WT Recombinants
A	-
B	-
C	-
D	-
E	-
F	+
G	+

Figure 3. Results for fine structure mapping of *TUB1* mutations. The central line represents

the *TUB1* gene, with several restriction sites indicated to the right. The wide shaded region

represents the coding sequence of *TUB1*. The left side of the Figure shows the *TUB1*

mutations, tabulated according to the mutagenesis method and the interval in which they

mapped. Mutations that originated from independent pools are shown in separate columns.

The DNA fragments from pRB539 used in mapping are shown on the right as dark lines.

Healer A was a 1.95 kb XhoI to KpnI fragment. Healer B was a 2.05 kb BglII to KpnI

fragment. Healer C was a 3.1 kb BstXI fragment. Healer D was a 2.85 kb HindIII to KpnI

fragment. Healer E was a 2.9 kb BstEII fragment. Healer F was a 3.25 kb ClaI fragment.

Healer G was a 3.65 kb EcoRV to KpnI partial digestion fragment.

	NH₂OH	-A	-C	-G	-T	
	758 759 760	703		724	501, 730 603	735 738 741
	763 764	705	719		727, 731	
		714				
		704, 709			733	744,750
	761 767				728, 729	502,747 743,749

NH_2OH

Mutagenesis

XhoI — A

BglII — B

AUG
BglII

BstXI — C

EcoRV — D
HindIII

BstEII — E

ClaI — F

TAA

EcoRV — G

HindIII

EcoRV

BglII

"Healers"
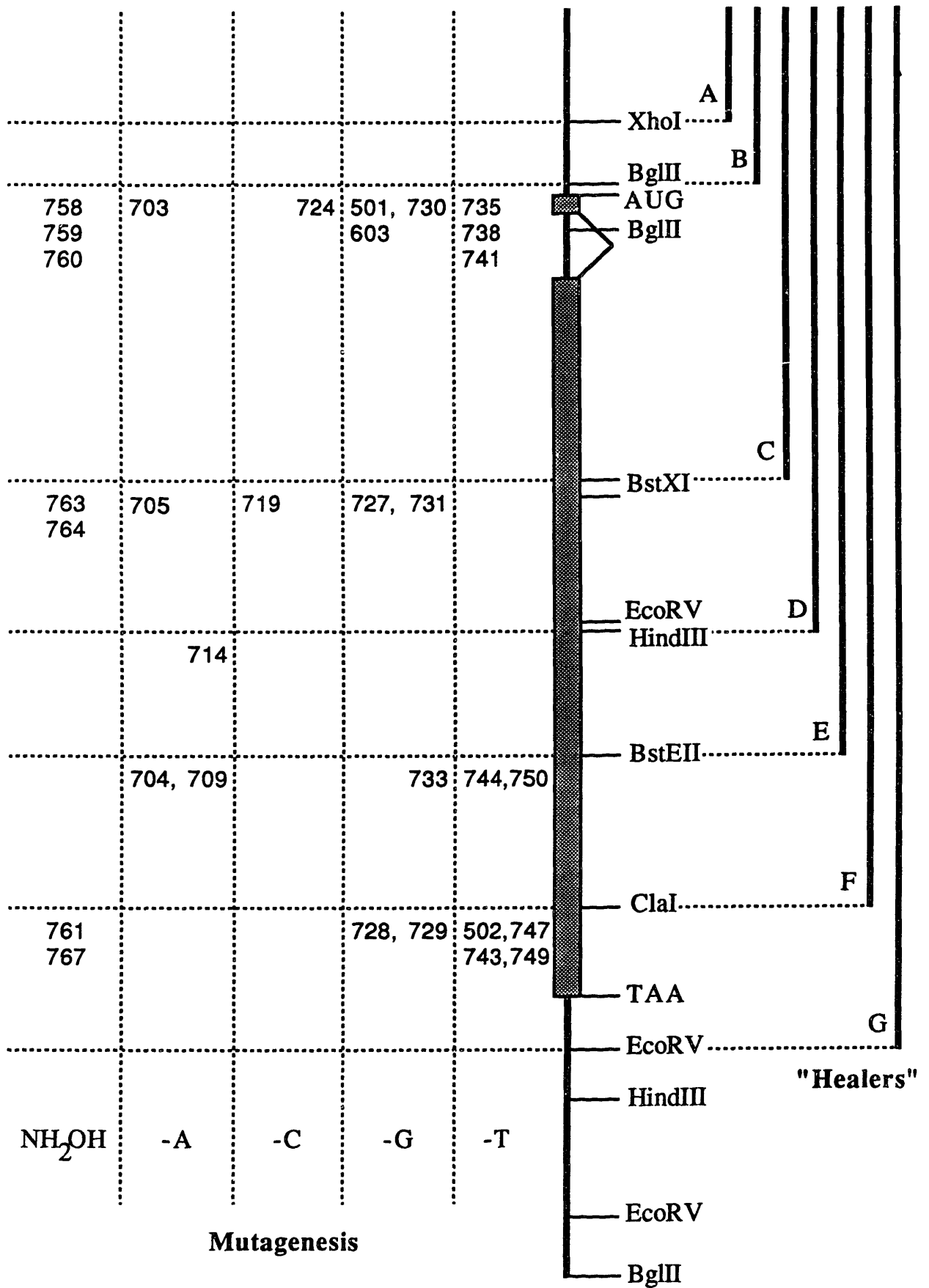
URA3-2μ plasmid containing *TUB3*. To score for return of the wild-type phenotype, transformant colonies were replica-plated to medium containing 5-FOA at nonpermissive temperature. Since this medium kills Ura3$^+$ cells, growth is dependent on the *TUB1* gene on the *LEU2 CEN* plasmid. Colonies that could not grow on this medium at nonpermissive temperature still contained the *TUB1* mutation. Those that could grow presumably contained wild-type *TUB1*, produced by recombination of the mutant plasmid with the wild-type healer fragment. As is shown in Fig. 3, most (31/38) of the *TUB1* mutations mapped in the *TUB1* coding sequences. They occurred in all deletion intervals and were distributed fairly evenly across the gene. The rest of the mutations (7/38) produced sufficiently leaky phenotypes that they proved impossible to map.

Characterization of *TUB1* mutants:

S. cerevisiae tubulin mutants generally arrest growth at the nonpermissive temperature as large budded cells with an undivided nucleus, thus demonstrating that the primary role of microtubules in the cell cycle is in mitosis (Thomas et al., 1985, Stearns and Botstein, 1988, Huffaker et al., 1988). The results of Huffaker et al. (1988) also suggested that cytoplasmic microtubules are involved in nuclear movement to the mother-bud neck. We characterized this large set of *TUB1* mutants to determine the range of phenotypes that could be produced by defects in α-tubulin and to determine what correlations exist between various defects.

We performed all further characterization of these mutations using strains in which the mutant *TUB1* allele on the *CEN* plasmid was the only intact α-tubulin gene in the cells. We constructed these strains by transforming DBY2384 to Leu$^+$ with the mutant plasmids and then selecting for loss of the *TUB3-URA3-2μ* plasmid on 5-FOA medium.

We first determined the optimum permissive growth temperature of the strains by comparing their growth at 20°C, 26°, and 30° to that of a similar strain containing wild-type *TUB1* on the *CEN* plasmid. As expected, all of the Cs strains grew most quickly relative to the wild type at the warmest temperature (30°) and the Ts strain grew best at the coolest

temperature (20°). We used 11° as the nonpermissive temperature for all Cs mutants and 37° as the nonpermissive temperature for all Ts mutants. We chose a set of 38 mutants for detailed study, based on fairly normal growth at the permissive temperature. These mutants included both of the Ts/Cs mutants (501 and 502), the Ts mutant (603), and 36 of the Cs mutants (7xx). As shown in Table 2a, most of these mutants grew at about the same rate as the wild type in liquid medium at permissive temperature.

Sensitivity to Benomyl:

Benomyl is a member of a set of compounds called benzimidazoles, that generally cause instability of microtubules. Hypersensitivity to benzimidazole antimicrotubule compounds is a common phenotype of both α- and β-tubulin mutants in a variety of species (Chapter 3, Adachi et al., 1986; Huffaker et al., 1988; Oakley and Morris, 1980; Stearns and Botstein, 1988; Toda et al., 1984; Umesono et al., 1983). Many previous tubulin mutants have been isolated in screens designed to detect altered sensitivity to these compounds (Sheirr-Neiss et al., 1978; Thomas et al., 1985; Umesono et al., 1983). Because our *TUB1* mutants were isolated only on the basis of temperature-conditional-lethality, we were interested in determining the generality of the drug sensitivity phenotype. As shown in Table 2b, 35 of the 38 mutants exibited hypersensitivity to the benzimidazole benomyl when grown on a series of different concentrations of the drug. The other 3 mutants showed the same resistance to the drug as the wild-type control. None of the mutants were more resistant than the control. Thus, benomyl hypersensitivity is a nearly universal phenotype of conditional-lethal α-tubulin mutants.

Analysis of Mutants at Nonpermissive Temperature:

To examine further the phenotypes associated with α-tubulin defects, we analyzed mutants after a shift from permissive to nonpermissive temperature. We grew the mutants to a density of 10^6 cells/ml at permissive temperature in liquid medium, shifted them to nonpermissive

TUB1 allele	Mutagenized Pool	Mutagenesis Method	Plasmid Number	Map Interval	Generation Time at Permissive Temp. (hrs)
WT	–	–	539	–	1.7 (30°)
Class 1:					
704	1	-A	604	F	2.2
709	2	-A	609	F	1.7
724	4	-C	624	C	1.6
728	5	-G	628	H	1.6
729	6	-G	629	H	1.7
738	7	-T	638	C	1.6
744	7	-T	644	F	1.7
750	8	-T	650	F	1.6
759	9	HA	659	C	1.7
760	9	HA	660	C	1.7
767	9	HA	667	H	1.6
Class 2:					
714	2	-A	614	E	1.8
730	6	-G	630	C	1.7
733	6	-G	633	F	1.7
741	7	-T	641	C	1.9
758	9	HA	658	C	1.8
Class 3:					
703	1	-A	603	C	1.7
705	1	-A	605	D	2.2
713	2	-A	613	nd	1.7
716	3	-C	616	nd	1.7
717	3	-C	617	nd	1.7
719	3	-C	619	D	2.1
723	4	-C	623	nd	1.8
727	5	-G	627	D	1.6
731	6	-G	631	D	1.6
735	7	-T	635	C	1.6
737	7	-T	637	nd	1.6
742	7	-T	642	nd	1.8
743	7	-T	643	H	1.7
746	8	-T	646	nd	1.6
747	8	-T	647	H	1.9
749	8	-T	649	H	1.7
761	9	HA	661	H	nd
763	9	HA	663	D	1.6
764	9	HA	664	D	1.7
Ts and Ts/Cs:					
WT	–	–	539	–	3.8 (20°)
603	5	-G	598	C	4.3 (20°)
WT	–	–	539	–	1.9 (26°)
501	5	-G	594	C	1.8 (26°)
502	7	-T	595	H	1.9 (26°)

Table 2a. Properties of the TUB1 mutants and wild-type (WT) controls. The mutagenized pools from which the mutant genes were isolated are numbered as described in Materials and Methods. The mutagenesis methods -A, -C, -G, and -T refer to pools of plasmid pRB539 mutagenized by misincorporation. HA refers to pool 9, which was mutagenized with hydroxylamine. The plasmid numbers refer to pRB numbers of the plasmids containing the TUB1 mutations. The map interval column shows the interval to which each mutation mapped (see Fig. 3). The last column shows the generation time of each mutant determined at the permissive temperature as described in Materials and Methods (permissive temperature for unmarked mutants was 30°). The mutants are grouped according to their microtubule phenotype (Table 2b).

TUB1 allele	Growth on Benomyl	Microtubule and Nuclear Phenotype
WT (11°)	++++	normal
Class 1:		
704	+	
709	+	
724	+	
728	+	
729	+	very few or no MT's, enormous cells with large buds, single nucleus in
738	+	random location with respect to the neck, nuclei sometimes disorganized.
744	+	
750	+	
759	+	
760	+	
767	+	
Class 2:		
714	+	
730	+	many extra MT's, especially cytoplasmic ones, enormous cells with large
733	++	buds, single nucleus usually in or near neck, nuclei sometimes disorganized.
741	+	
758	+	
Class 3:		
703	++	disorganized MT's, predominantly short forms, large cells, disorganized nuclei.
705	+	disorganized MT's, large cells, disorganized nuclei.
713	+	fewer disorganized MT's and dots, large cells, disorganized nuclei.
716	+	fairly normal MT's, some MT's and nuclei disorganized, normal sized cells.
717	+	fawer disorganized MT's, predominantly short forms, large cells, disorganized nuclei.
719	++	more MT's, many short spindles, large cells, disorganized nuclei.
723	+	fairly normal.
727	+	fairly normal but slightly fewer MT's.
731	+	fewer disorganized MT's, large cells, disorganized nuclei.
735	++	very disorganized MT's, large cells, disorganized nuclei.
737	++++	large budded cells with bright, long intranuclear spindle, normal cytoplasmic MT's.
742	+++	more very disorganized MT's, large cells, many multinucleate cells, disorganized nuclei.
743	+	fewer disorganized MT's, large cells, disorganized nuclei.
746	++	large budded cells with bright, long intranuclear spindle, normal cytoplasmic MT's.
747	+	fairly normal but lots of short spindles, large cells.
749	+	fewer disorganized MT's, large cells, disorganized nuclei.
761	++++	nd
763	+	very disorganized MT's, many cells with none, large cells, disorganized nuclei.
764	++++	very disorganized MT's, no apparent breaks at SPB, large cells, disorganized nuclei.
Ts and Ts/Cs:		
WT (37°)	++++	normal
603 (37°)	+	very disorganized MT's, large cells, disorganized nuclei.
WT (11°)	++++	normal
501 (11°)	+	very few MT's, large cells with single nucleus in random location, disorganized nuclei.
501 (37°)	+	very disorganized MT's, large cells, disorganized nuclei.
502 (11°)	+	fairly normal, some cells with less MT's.
502 (37°)	+	fewer disorganized MT's, large cells, disorganized nuclei.

Table 2b. Properties of the TUB1 mutants and wild-type (WT) controls. Growth of the mutants at permissive temperature was scored at various concentrations of benomyl on a scale of + to ++++. The microtubule and nuclear phenotypes of mutant cells (and wild-type controls) were observed after shifts to nonpermissive temperature for 2 generations. Temperatures in parentheses next to the allele numbers indicate the nonpermissive temperature used. Unmarked mutants were scored at 11°.

TUB1 allele	Cell Count Increase at Nonpermissive	Half Life (hrs) at Nonpermissive	Cell Division Cycle			
			Unbudded	Small Bud	Large Bud	Multi-Bud
WT (11°)	32	13 (Gen)	34	26	33	7
Class 1:						
704	2.4	nd	13	11	70	6
709	2.6	nd	14	9	71	6
724	1.7	8.0	5	1	93	1
728	1.7	8.5	8	1	88	3
729	1.6	8.3	8	0	90	2
738	2.9	23	16	8	75	1
744	1.6	8.7	11	2	80	7
750	1.8	8.3	11	4	77	8
759	2.4	13	7	1	87	5
760	2.5	12	7	1	89	3
767	1.6	8.3	10	1	87	2
Class 2:						
714	2.1	nd	10	4	82	4
730	2.1	20	10	7	75	8
733	1.8	14	15	5	78	2
741	2.5	20	23	18	52	7
758	2.8	21	13	7	65	15
Class 3:						
703	2.7	nd	16	8	50	26
705	3.4	nd	30	14	49	7
713	2.5	nd	21	9	51	19
716	8.0	nd	22	12	55	11
717	3.0	nd	26	7	49	18
719	5.1	nd	31	16	45	8
723	4.5	13	30	17	45	8
727	3.5	15	23	16	52	9
731	2.1	8.0	19	10	53	18
735	1.5	12	16	5	64	15
737	3.9	15	11	9	76	4
742	7.0	poor growth	34	17	39	10
743	3.0	16	18	12	60	10
746	3.0	13	13	11	71	5
747	4.4	39	29	17	49	5
749	4.5	24	14	9	70	7
761	nd	nd	nd	nd	nd	nd
763	3.0	24	24	8	56	12
764	2.5	14	21	6	70	3
Ts and Ts/Cs:						
WT (37°)	8.0	2.1 (Gen)	30	26	41	3
603 (37°)	2.3	1.5	28	11	54	7
WT (11°)	32	13 (Gen)	34	26	33	7
501 (11°)	2.3	7.9	18	7	68	7
501 (37°)	3.1	2.3	32	11	51	6
502 (11°)	6.1	very poor growth	18	26	53	3
502 (37°)	3.8	6.0	26	10	61	3

Table 2c. Properties of the TUB1 mutants and wild-type (WT) controls. Cultures of mutants and wild-type controls were shifted to nonpermissive temperature and several phenotypes were observed. Cell numbers were determined in a Coulter counter and compared to the number of cells just before the shift. The Cell Count Increase column shows the relative number of cells after 5 generations (11° cells) or after 3 generations (37° cells). Viable counts were determined by plating cells at intervals after the shift. These counts were used to determine the half-life of the cells after the shift. The wild type showed an increase in the number of viable cells, so the generation time is shown marked (Gen). The percentages of the cells in each of the morphological classes were determined 2 generations after the shift. At least 200 cells were counted. Buds were considered to be small if their diameter was less than half of that of the mother cell body.

temperature, and observed the following phenotypes over a time course: [1] To determine the rate at which the mutant alleles caused loss of cell viability, samples of the cultures were plated at permissive temperature to obtain viable counts at a series of time points. Table 2c shows the results of this analysis, expressed as a half-life at the nonpermissive temperature. [2] To determine the number of new cells produced by the mutants after the shift, cells were fixed at several time points and counted in a Coulter counter. Table 2c shows the number of cells after about 5 generations, relative to the starting number at the shift. [3] The morphology of cells fixed about 2 generations after the shift was examined by phase-contrast microscopy. The relative fractions of unbudded, small budded, large budded and multi-budded cells are shown in Table 2c, based on counts of at least 200 cells each. Counts of the mutant cells growing at permissive temperature just before the shift demonstrated that normal distributions of cell morphologies existed (data not shown). [4] Cells just before the shift and cells 2 generations after the shift were examined for microtubular structures by indirect immunofluorescence (Kilmartin and Adams, 1984). In most cases, mutants examined at the permissive temperature were indistinguishable from the wild type, that had similar microtubular structures at all temperatures. Table 2b contains descriptions of the microtubule phenotypes and of the nuclear morphologies of the cells after 2 generations at nonpermissive temperature. Photographs of representative mutants are shown in Figures 4, 5, and 6.

The mutants shown in Tables 2a, b, and c are grouped into three classes according to the morphologies of their microtubules after 2 generations at nonpermissive temperature. Class 1, represented by the *tub1-724* mutant, had very few or no microtubular structures (Fig. 4). The vast majority of the cells were large-budded with the nucleus randomly located in the cell. Class 2, represented by the *tub1-741* and *tub1-758* mutants, contained excess microtubular structures, especially in the cytoplasm (Fig. 5). They also tended to arrest at the large-budded stage, but the nucleus was located in the neck of the bud in a majority of the cells. Class 3 was a very heterogeneous group containing the rest of the mutants, most of which displayed a wide variety of aberrant microtubule structures and disorganized nuclei (Fig. 5).

Figure 4. Microscopic analysis of the *tub1-724* mutant. The left panel shows anti-tubulin immunofluorescence analysis of a field of *tub1-724* mutant cells after two generations (24 hrs) at the nonpermissive temperature (11°). The right panel shows the same cells viewed with Nomarski optics in addition to DAPI epifluorescence (to view DNA). In this panel, the white areas correspond to the DAPI staining regions. The bar represents 10 μm.

anti-tubulin
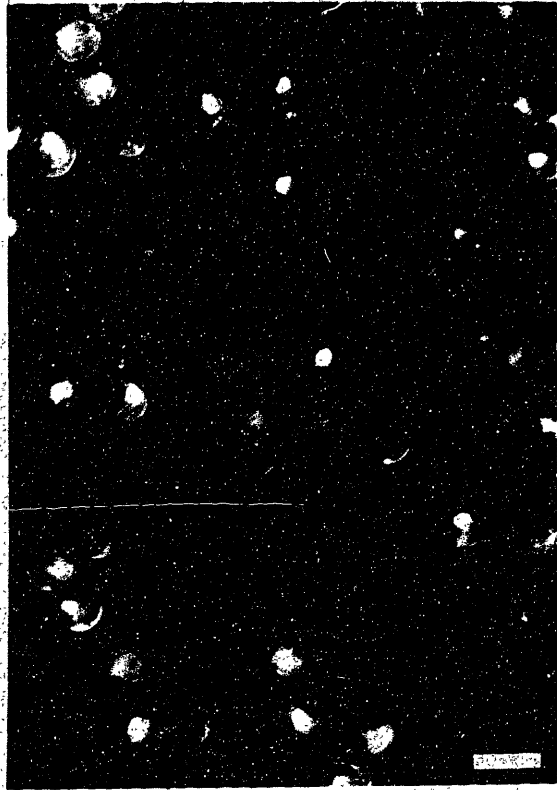
DAPI
+Nomarski

724 - 11°

Figure 5. Microscopic analysis of *TUB1* mutants. The left panel of each pair shows anti-tubulin immunofluorescence and the right panel shows a combination of Nomarski optics and DAPI epifluorescence. The left side of the Figure contains images of the *tub1-741* and *tub1-758* mutants after 2 generations (24 hrs) at the nonpermissive temperature (11°). The right side contains similar images of the *tub1-705* mutant. The bar represents 10 µm.

anti-tubulin	DAPI +Nomarski	anti-tubulin	DAPI +Nomarski

741, 758 - 11°   705 - 11°
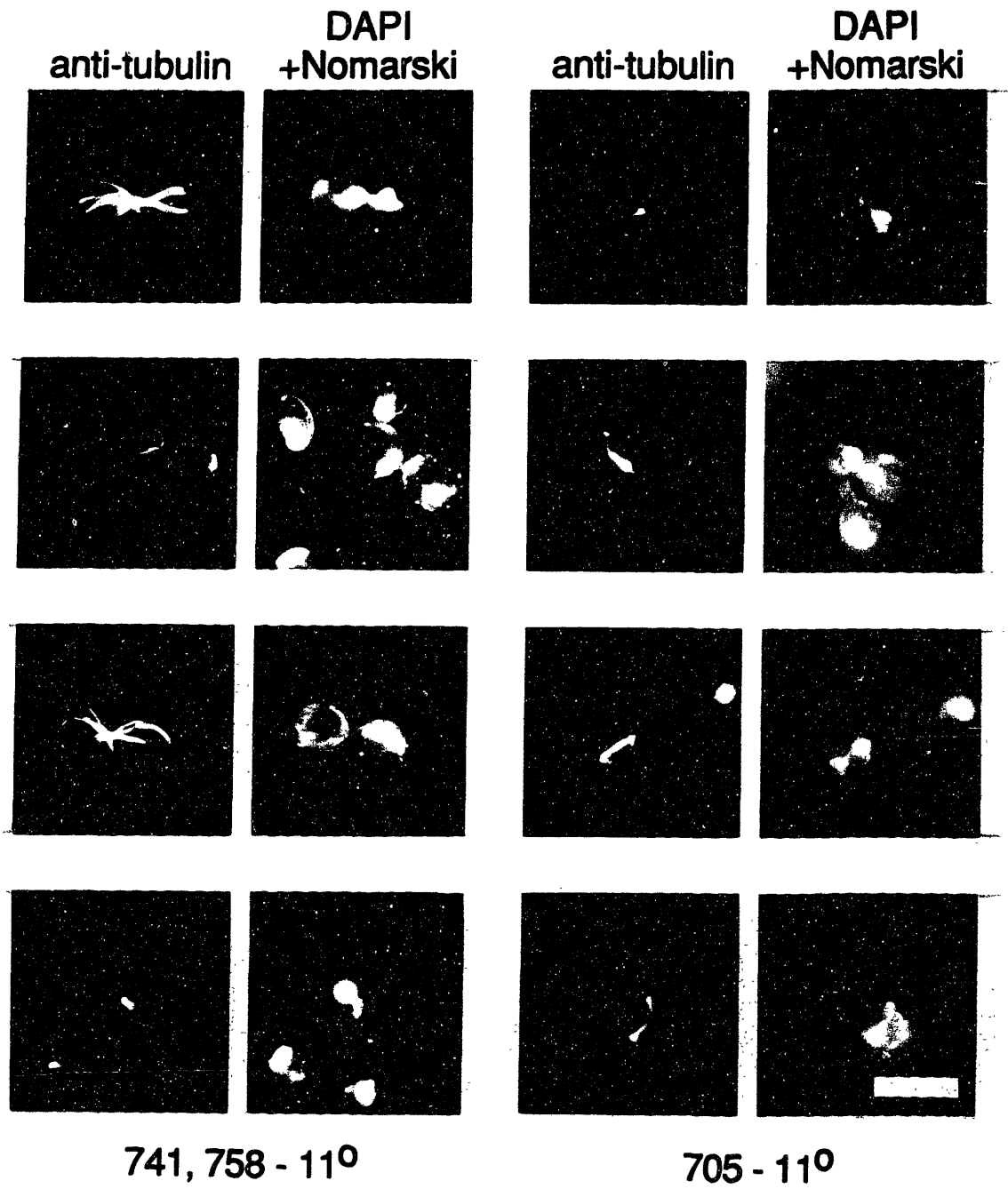
Figure 6. Microscopic analysis of *TUB1* mutants. The left panel of each pair shows anti-tubulin immunofluorescence and the right panel shows a combination of Nomarski optics and DAPI epifluorescence. The left side of the Figure contains images of the *tub1-737* and *tub1-746* mutants after 2 generations (24 hrs) at the nonpermissive temperature (11°). The right side contains similar images of the wild-type control for comparison. The bar represents 10 μm.

anti-tubulin   DAPI +Nomarski   anti-tubulin   DAPI +Nomarski

737, 746 - 11°   Wild Type
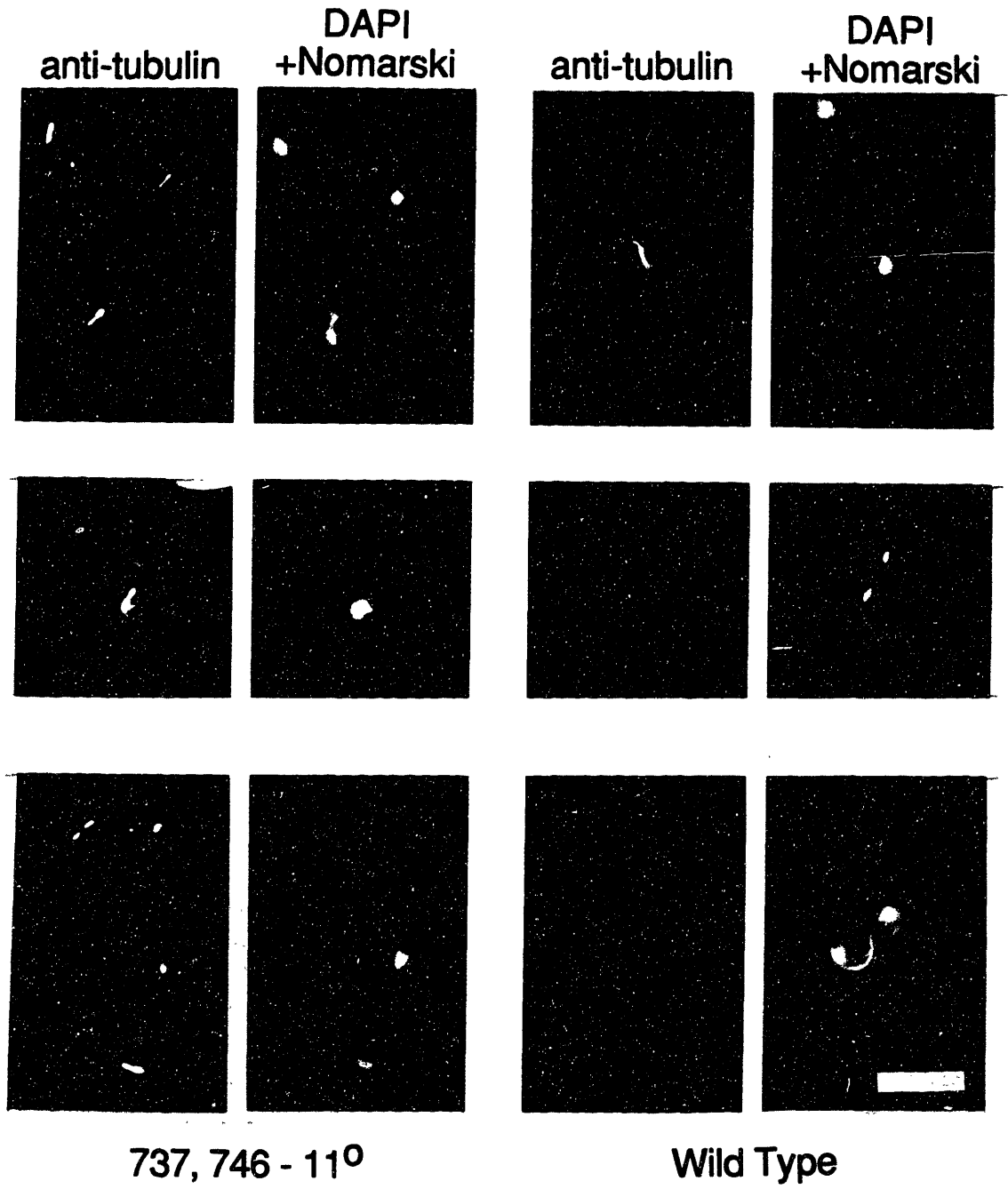
Examination of these classes permits us to make several generalizations. All of the mutants accumulated an excess of large-budded cells at the nonpermissive temperature compared to the wild type under the same conditions (Table 2c). Several of the mutants accumulated more than 85% large budded cells. Thus, *TUB1* qualifies as a CDC gene as defined by Hartwell et al. (1970). Most of the mutants, however, do not fit this criterion. But the fact that all of the mutants showed an excess of large budded cells suggests that they all were defective in part of the cell cycle necessary for progression to cytokinesis. The observation that several of the mutant cultures contained an excess of multi-budded cells supports this hypothesis (Table 2c).

The mutants with gross defects in microtubule assembly (class 1 with no microtubules and class 2 with many) showed better cell cycle arrests, on average, than the class 3 mutants (79 % large budded vs 56 % for class 3). Class 1 and class 2 mutants also generally exhibited a smaller increase in the number of cells after the shift (2.1 fold vs 3.8 fold). Thus mutants with the strongest microtubule assembly defects had the tightest phenotypes. The class 1 mutants lost viability more quickly than the other groups (11 hrs. half-life vs 18 hrs.), implying that the loss of microtubules did not simply stop the cell cycle, but resulted in aberrant events that led to relatively rapid cell death.

Most of the class 3 mutants did not have a uniform cell or microtubule morphology after 2 generations at nonpermissive temperature. We observed many abnormal arrangements of microtubules and nuclei. A significant fraction of cells had multiple nuclei or no nuclei. An example of this disorganized phenotype is shown in Figure 5, that contains several photographs of the *tub1-705* mutant. The top set of pictures shows a cell with misoriented microtubules that radiate from one pole. The second set shows a cell with a fairly normal spindle. In the third set of pictures, the cell has a normal spindle and separated chromosomes that are restricted to one of the daughter cells. The fourth cell appears to have completed spindle elongation and breakdown in a single cell body. The lack of uniformity of morphology in the shifted *tub1-705* mutant and the other mutants in class 3 implies that the tubulin defect did not prevent the execution of a single single step necessary for progression

of the cell cycle.

Of all of the mutants, only two, that contained the *tub1-737* and *tub1-746* alleles, displayed a fairly uniform morphology that resembled a normal stage of the cell cycle. Both arrested with medium-length intranuclear spindles and a normal complement of cytoplasmic microtubules (Figure 6). The intranuclear spindles appeared to be less bright in the center. Both alleles allowed the production of a relatively large number of new cells after the shift (3.9 fold and 3.0 fold, respectively). These mutants may represent rare examples of defects in tubulin that affect a specific stage in spindle elongation.

Discussion:

We have isolated 70 conditional-lethal mutants in the *TUB1* α-tubulin gene of the yeast *Saccharomyces cerevisiae*. Because of the functional similarity between *TUB1* and the minor α-tubulin gene *TUB3*, we characterized the mutants in a strain lacking *TUB3*. The *TUB1* gene had a definite bias in its ability to mutate to cold-sensitivity (Cs) versus temperature (warm) sensitivity (Ts). Of the 70 mutants isolated, 67 were Cs, 1 was Ts, and 2 were both (Ts/Cs).

We have studied a subset of these mutants, characterizing several phenotypes after shifts to nonpermissive temperature. Although only a minority of the mutants fit the definition of CDC mutants (greater than 85% arrest at uniform morphology, Hartwell et al., 1970), all of them accumulated excess large budded cells after the shift. This result is consistent with the previously determined role of microtubules in yeast mitosis (Hiraoka et al., 1984; Huffaker et al., 1988b; Pringle et al., 1986; Quinlan et al., 1980; Roy and Fantes, 1983; Stearns and Botstein, 1988; Thomas et al., 1985; Toda et al., 1984; Toda et al., 1983; Wood and Hartwell, 1982). The fact that most of the mutants did not arrest with a clear CDC phenotype probably indicates that they died from causes other than simple arrest of the cell cycle. One likely reason for death is progression through an abnormal mitosis, that would lead to lethal chromosome imbalances.

Our results are also consistent with the other hypothesized role of microtubules in the *S. cerevisiae* mitotic cell cycle, specifically nuclear migration before mitosis (Pringle et al., 1986; Huffaker et al., 1988). Nuclear movement to the bud neck in tubulin mutants has been correlated with the presence of cytoplasmic microtubules (Huffaker et al., 1988). Our results are consistent with the hypothesis that cytoplasmic microtubules are necessary for nuclear migration. The nuclei of mutants that lost almost all of their microtubules were located randomly with respect to the neck. Mutants that accumulated excess cytoplasmic microtubules, however, generally displayed a nucleus in the neck.

The mutants with gross microtubule assembly defects generally had the most uniform cell cycle arrest and the strongest block to the production of additional cells after a shift to the nonpermissive temperature. These included mutants with very few microtubules and also mutants with excess microtubules. The mutants that lost microtubules after the shift tended to die more quickly than the others. Perhaps the loss of microtubules resulted in aberrant events during mitosis that led to lethality rather than simple cell cycle arrest. Of all tubulin mutants in *S. cerevisiae*, the one that retains viability for the longest time while still showing a strong cell cycle block is *tub2-104* (Thomas et al., 1985; T. Huffaker, personal communication; Our unpublished results). This mutant retains an intranuclear spindle at nonpermissive temperature while losing almost all cytoplasmic microtubules (Huffaker et al., 1988). The persistence of an intranuclear spindle may be important in preserving the viability of yeast cells blocked in spindle elongation.

Very few of our mutants displayed arrested growth phenotypes with a uniform morphology that resembled any normal stage of the cell cycle. Two exceptions to this rule were the *tub1-737* and *tub1-746* mutants. Both of these mutants accumulated as large budded cells (76% and 71%, respectively) with medium length, intranuclear spindles that resembled those of wild-type cells. Their cytoplasmic microtubules were also normal in appearance (Figure 6). The intranuclear spindles were less bright in the center, as if the chromosome-to-pole microtubules did not extend all of the way to the middle of the spindle. It is possible that these mutants had fairly specific defects in some stage of anaphase movement.

Comparison of the map positions of the mutations with their corresponding microtubule phenotypes revealed no obvious correlations between the two. Mutations that caused the loss of almost all microtubules after shift occurred in most deletion intervals. Similarly, mutations that caused accumulation of excess microtubules and mutations that led to disorganized microtubules were spread fairly uniformly across the coding sequences.

The Significance of Benomyl Hypersensitivity:

Benomyl is a member of a set of compounds called benzimidazoles, that generally cause fairly specific defects in microtubule assembly (Davidse and Flach, 1977; Oakley and Morris, 1980; Sheirr-Neiss et al., 1978). The specificity of these drugs for tubulin was most clearly demonstrated by the observation that mutations that caused resistance to very high levels of benomyl occurred exclusively in the *TUB2* β-tubulin gene of *S. cerevisiae* (Thomas et al., 1985). Of 38 *TUB1* α-tubulin mutants that we tested, 35 were hypersensitive to drug benomyl and none was resistant. Such hypersensitivity is a common phenotype of both α and β-tubulin mutants in a variety of species (Chapter 3, Adachi et al., 1986; Huffaker et al., 1988; Oakley and Morris, 1980; Stearns and Botstein, 1988; Toda et al., 1984; Umesono et al., 1983). On the other hand, resistance to benzimidazoles is usually found in β-tubulin but not in α-tubulin genes (Sheirr-Neiss et al., 1978; Thomas et al., 1985; Umesono et al., 1983). Mutations in the *TUB2* β-tubulin gene isolated on the basis of cold-sensitivity had a different pattern of benomyl sensitivity than our *TUB1* mutants. Of 5 that were examined by Huffaker et al.(1988), one was resistant, two were hypersensitive, and two had about the same sensitivity as the wild type. Comparison of these results suggests that α- and β-tubulin have different roles in the interaction between tubulin and these drugs.

The observation that 35 of 38 conditional-lethal α-tubulin mutants were hypersensitive suggests that benomyl hypersensitivity may be a sensitive probe for a variety of defects in microtubule function. This hypothesis is supported by the observation that overlapping subsets of nontubulin genes were identified in screens for benomyl hypersensitive mutants and screens for mutants that lost chromosomes at an elevated rate (A. Hoyt and T. Stearns, personal communication). Thus, benomyl hypersensitivity may be a very useful criterion for identifying genes involved in many aspects of microtubule function in yeast.

Contrasts between mutagenesis strategies:

We used two strategies in attempts to isolate conditional-lethal mutations in the *TUB1*

gene. Initial attempts used a mutagenized integrating plasmid that produced a partial

duplication of the gene upon homologous recombination with the chromosomal copy (Shortle

et al., 1984). After screening 40,000 transformants, we picked 46 conditonal lethal

candidates for examination. We found that most of these contained nuclear mutations of

unknown origin that were unlinked to the *TUB1* gene. None of them were *TUB1* mutant.

They probably arose during growth of the strain or during the transformation procedure. In

contrast, a plasmid replacement method, commonly called the plasmid shuffle (Boeke et al.,

1988), yielded 70 authentic *TUB1* conditional-lethal mutants from 56,000 transformants

screened.

There are several possible explanations for the different success rates with the two

methods. The level of mutagenesis, as assayed by loss of *URA3* or *LEU2* function in *E. coli*,

was about 4 times higher for the plasmid used for the plasmid shuffle. There are also several

theoretical problems with the integrating plasmid strategy. Because the method relies on

recombination between plasmid-borne and chromosomal copies of the gene, the functional

copy produced will usually contain unmutagenized sequences. The recombination event also

produces a bias towards mutagenesis of one end of the gene. If there is any way for the yeast

to distinguish between endogenous and newly introduced DNA sequences, it may favor the

endogenous sequences during recombination. Reconstruction experiments with known

mutants on integrating plasmids have shown a surprisingly low frequency of incorporation of

the mutation into the functional copy of the gene (M. Rose, personal communication; Teresa

Dunn, personal communication). The other major problem with this technique is that it is

laborious to screen among candidates for authentic mutants. We screened candidates by

crossing them to a wild type strain and checking for linkage between the conditional-lethal

mutation and the plasmid integration marker. Assuming that the mutations were cleanly

recessive, such a screen also could be performed using a complementation test. Such a test,

however, would require either transformation of each strain with a plasmid carrying the gene or the existence of previously isolated alleles of the gene.

The plasmid shuffle method does not have several of these problems. Since the method does not rely on recombination between mutagenized and unmutagenized copies of the gene, there is no bias towards one end of the gene. Almost all of the mutagenized copies of the gene will be unchanged by the transformation process. As used here, the method also had a built-in suppression test to distinguish *TUB1* mutants from random chromosomal mutants produced by either growth or transformation of the starting strain. We demanded that the mutants show conditional-lethal growth only on plates that selected for the absence of a *TUB3* plasmid (that carried the only other α-tubulin gene in the cells). In the case of a single essential gene with no duplicated function, this test would be a complementation test using the unmutagenized copy of the gene.

As used by us, the plasmid shuffle method did have the drawback of identifying only recessive alleles. A simple modification of the procedure, however, would allow the isolation of dominant mutations also. Loss of the plasmid carrying the unmutagenized copy of the gene could be selected in an initial replica-plating step at the permissive temperature. During a second round of replica-plating, colonies then could be screened for a conditional-lethal mutation, in the absence of interference from the unmutagenized gene. To distinguish between authentic alleles of the gene and other random nuclear mutations, the growth properties of the original transformant colonies could be examined at the nonpermissive temperature on medium that selected for retention of the plasmid carrying the unmutagenized copy of the gene, but not the plasmid with the mutagenized copy.

Thus, the plasmid shuffle method can be generalized to allow isolation of both recessive and dominant alleles of essential genes in yeast. In our hands, it has been a far more efficient technique than the integrating plasmid/partial duplication method.

Future Prospects:

The yeast mitotic spindle is still poorly understood. Although the genes encoding the tubulins have been isolated and characterized, very few other proteins of the spindle have been identified. A major reason for choosing yeast as an experimental organism is the ability to use genetic techniques to identify new components of complicated systems (for review see Huffaker et al., 1988a). One common approach starts with the isolation of mutations in one component of a system, which then are used to identify new genes involved in the system, using suppression analysis. Suppression analysis relies on the correction of a defect in some part of a system by a compensating change in some other part. When used with genes whose products form complexes, this analysis often yields mutations in genes whose products physically interact with the product of the original gene (Jarvik and Botstein, 1975). We hope to use the *TUB1* mutations described herein to identify new components of the yeast spindle apparatus through suppression analysis.

One reason for isolating and characterizing such a large set of mutations in *TUB1* is that we hope to identify alleles likely to yield suppressor mutations in new genes involved in spindle function. An important aspect of this identification is the effect of the mutations on microtubule assembly. Our analysis of *TUB1* mutations has revealed three classes of microtubule assembly phenotypes: [1] the loss of microtubules, [2] the accumulation of excess microtubules, and [3] the appearance of disorganized or nonfunctional microtubular structures. Mutations that caused phenotypes in the first two categories had a gross effect on microtubule assembly, which was probably the primary reason for the defect in microtubular function. Therefore, any compensating suppressor mutation would have to correct that assembly defect. Since α– and β–tubulin associate very tightly to form a dimer, and since that dimer is able to form microtubules without the presence of other proteins, most of the suppressor mutations might occur in the *TUB2* β–tubulin gene or as intragenic revertants of the original α–tubulin mutation. These mutations would not identify new spindle genes. It is possible that revertants of the third class of mutations, which did not cause gross microtubule

assembly defects, might yield a wider variety of suppressor mutations. Mutations such as *tub1-737* and *tub1-746*, which appeared to cause a specific defect in spindle movement, might be the best candidates for suppressor analysis. Such analysis is currently in progress.

References:

Adachi, Y., T. Toda, O. Niwa, and M. Yanagida. 1986. Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol. Cell. Biol. 6: 2168-2178.

Adams, A.E.M., and J.R. Pringle. 1984. Localization of actin and tubulin in wild-type and morphogenetic mutants of *Saccharomyces cerevisiae*. J. Cell Biol. 98: 934-945.

Baum, P., F. Clement, and B. Byers. 1986a. Yeast gene required for spindle pole body duplication: homology of its product with Ca^{2+}-binding proteins. Proc. Natl. Acad. Sci. USA 83: 5512-5516.

Baum, P., L. Goetsch, and B. Byers. 1986b. Genetics of spindle pole body regulation. p. 155-158., in Yeast Cell Biology, J. Hicks, ed., Alan R. Liss, Inc., NY.

Boeke, J.D., F. LaCroute, G.R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197: 345-347.

Boeke, J.D., J. Trueheart, G. Natsoulis, and G.R. Fink. 1987. 5-fluoro-orotic acid as a selective agent in yeast molecular genetics. In Methods in Enzymology, vol. 154, Recombinant DNA Part E, R. Wu and D. Grossman eds., pp. 164-175.

Botstein, D., S.C. Falco, S. Stewart, M. Brennan, S. Sherer, D. Stinchcomb, K. Struhl, and R. Davis. 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8: 17-24.

Botstein, D., and R.W. Davis. 1982. Principles and practice of recombinant DNA research with yeast. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 607-638.

Byers, B. 1981. Cytology of the yeast life cycle. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 59-96.

Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J. Bacteriol. 124: 511-523.

Clarke, L., and J. Carbon. 1985. The structure and function of yeast centromeres. Ann. Rev. Genet. 19: 29-55.

Cleveland, D.W., and K.F. Sullivan. 1985. Molecular biology and genetics of tubulin. Ann. Rev. Biochem. 54: 331-365.

Davidse, L., and W. Flach. 1977. Differential binding of methyl-benzimidazol-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in strains of Aspergillus nidulans. J. Cell. Biol. 72: 174-193.

Delgado, M.A., and J. Conde. 1984. Benomyl prevents nuclear fusion in Saccharomyces cerevisiae. Mol. Gen. Genet. 193: 188-89.

Dustin, P. 1984. Microtubules. Springer-Verlag, New York.

Hartwell, L.H., J. Culotti and S. Reid. 1970. Genetic control of the cell division cycle in yeast I: Detection of mutants. Proc. Natl. Acad. Sci. USA 66: 352-359.

Hiraoka, Y., T. Toda, and M. Yanagida. 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.

Huffaker, T.C., M.A. Hoyt, and D. Botstein. 1988a. Genetic analysis of the cytoskeleton of yeast. Ann. Rev. Genet. (in press).

Huffaker, T.C., J.H. Thomas, and D. Botstein. 1988b. Diverse effects of β-tubulin mutations on microtubule assembly and function J. Cell Biol. (submitted).

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163-168.

Jarvik, J., and D. Botstein. 1975. Conditional lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. Proc. Natl. Acad. Sci. USA 72: 2738-2742.

Kilmartin, J. 1981. Purification of yeast tubulin by self-assembly in vitro. Biochemistry 20: 3629-3633.

Kilmartin, J., and A. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. J. Cell Biol. 98: 922-933.

King, S.M., and J.S. Hyams. 1982. The mitotic spindle of *Saccharomyces cerevisiae:* assembly, structure, and function. Micron 13: 93-117.

Kunes, S., D. Botstein, and M.S. Fox. 1985. Transformation of yeast with linearized plasmid DNA: Formation of inverted dimers and recombinant plasmid products. J. Mol. Biol. 184: 375-387.

Kunes, S., H. Ma, K. Overbye, M.S. Fox, and D. Botstein. 1987. Fine structure recombinational analysis of cloned genes using yeast transformation. Genetics 115: 73-81.

Kuo, C., and J.L. Campbell. 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the CDC8 gene and two genes that compensate for the cdc8-1 mutation. Mol. Cell. Biol. 3: 1730-1737.

Ma, H., S. Kunes, P.J. Schatz, and D. Botstein. 1987. Construction of plasmids by recombination in yeast. Gene 58: 201-216.

Matile, P., H. Moor, and C.F. Robinow. 1969. Yeast Cytology. p. 219-302. in The Yeasts, vol. I, A.H. Rose and J.S. Harrison., Academic Press, NY.

Moens, P.B., and E. Rapport. 1971. Spindles, spindle plaques, and meiosis, in the yeast *Saccharomyces cerevisiae* (Hansen). J. Cell. Biol. 50: 344-361.

Morris, N.R., M.H. Lai, and C.E. Oakley. 1979. Identification of a gene for α–tubulin in *Aspergillus nidulans.* Cell 16: 437-442.

Mortimer, R.K., and D. Schild. 1981. Genetic mapping in *Saccharomyces cerevisiae.* In:

The Molecular Biology of the Yeast Saccharomyces, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, vol. 1, pp. 11-26.

Neff, N., J.H. Thomas, P. Grisafi and D. Botstein. 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function in vivo. Cell 33: 211-219.

Oakley, B., and N.R. Morris. 1980. Nuclear movement is β-tubulin dependent in *Aspergillus nidulans*. Cell 19: 255-262.

Olmsted, J.B. 1986. Microtubule-associated proteins. Ann. Rev. Cell Biol. 2: 421-457.

Orr-Weaver, T., J. Szostak, and R. Rothstein. 1981. Yeast transformation: a model system for the study of recombination Proc. Nat. Acad. Sci. USA 78: 6354-6358.

Peterson, J.B., and H. Ris. 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. J. Cell Sci. 22: 219-242.

Pillus, L., and F. Solomon. 1986. Components of microtubular structures in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 83: 2468-2472.

Pringle, J. R., and L. H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In: The Molecular Biology of the Yeast Saccharomyces, vol. 1, edited by Strathern, J.N., E.W. Jones, and J.R. Broach, Cold Spring Harbor Monograph Series, Cold Spring Harbor, NY, pp. 97-142.

Pringle, J.R., S.H. Lillie, A.E.M. Adams, C.W. Jacobs, B.K. Haarer, K.G. Coleman, J.S.

Robinson, L. Bloom, and R.A. Preston. 1986. Cellular morphogenesis in the yeast cell cycle. In Yeast Cell Biology, J. Hicks, ed., Alan R. Liss, New York.

Quinlan, R.A., C.I. Pogson and K. Gull. 1980. The influence of the microtubule inhibitor, methyl benzimidazol-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. J. Cell Sci. 46: 341-352.

Raff, E.C. 1984. Genetics of microtubule systems. J. Cell Biol. 99: 1-10.

Raff, E.C., and M.T. Fuller. 1984. Genetic analysis of microtubule function in *Drosophila*. In Molecular Biology of the Cytoskeleton, Cold Spring Harbor Press, Cold Spring Harbor, NY., p. 293-304.

Rose, M.D., and G.R. Fink. 1987. *KARI*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell 48: 1047-1060.

Roy, D., and P.A. Fantes. 1983. Benomyl-resistant mutants of *Schizosaccharomyces pombe* cold-sensitive for mitosis. Curr. Genet. 6: 195-202.

Schild, D., H.N. Ananthaswamy, and R.K. Mortimer. 1981. An endomitotic effect of a cell cycle mutation of *Saccharomyces cerevisiae*. Genetics 97: 551-562.

Sheirr-Neiss, G., M. Lai, and N. Morris. 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell 15: 639-647.

Sherman, F., G. Fink, and C. Lawrence. 1974. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shortle, D., P. Grisafi, S.J. Benkovic, and D. Botstein. 1982. Gap misrepair mutagenesis: efficient site-directed induction of transition, transversion, and frameshift mutations in vitro. Proc. Natl. Acad. Sci. USA 79: 1588-1592.

Shortle, D., P. Novick and D. Botstein. 1984. Construction and genetic characterization of temperature-sensitive alleles of the yeast actin gene. Proc. Nat. Acad. Sci. USA 81: 4889-4893.

Snyder, M., and R.W. Davis. 1986. Molecular analysis of chromosome segregation in yeast. Yeast 2: s363.

Stearns, T., and D. Botstein. 1988. Unlinked noncomplementation: Isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. Genetics (submitted).

Thomas, J.H. 1984. Genes controlling the mitotic spindle and chromosome segregation in yeast. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.

Thomas, J.H., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. Cell 44: 65-76.

Thomas, J.H., N.F. Neff and D. Botstein. 1985. Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 112: 715-734.

Toda, T., Y. Adachi, Y. Hiraoka, and M. Yanagida. 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.

Toda, T., K. Umesono, A. Hirata, and M. Yanagida. 1983. Cold-sensitive nuclear division arrest mutants of the fission yeast *Schizosaccharomyces pombe.* J. Mol. Biol. 168: 251-270.

Umesono, K., T. Toda, S. Hayashi, and M. Yanagida. 1983b. Two cell division cycle genes NDA2 and NDA3 of the fission yeast *Schizosaccharomyces pombe* control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. J. Mol. Biol. 168: 271-284.

Wood, J.S. 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae.* Mol. Cell Biol. 2: 1064-1079.

Wood, J.S. and L.H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *S. cerevisiae.* J. Cell Biol. 94: 718-726.

Yamamoto, M. 1981. Genetic analysis of resistant mutants to antimitotic benzimidazole compounds in *Schizosaccharomyces pombe.* Mol. Gen. Genet. 180: 231-234.

Appendix 1:

Plasmid Construction by Recombination in Yeast

Plasmid construction by homologous recombination in yeast

(*Saccharomyces cerevisiae*; transformation; plasmid recombination; YCp50 derivatives; YEp420 [previously called *β72*] derivatives)

Hong Ma, Sam Kunes, Peter J. Schatz and David Botstein

Department of Biology. Massachusetts Institute of Technology, Cambridge. MA 02139 (U.S.A.) Tel. (617)253-3623 (laboratory)

SUMMARY

We describe a convenient method for constructing new plasmids that relies on interchanging parts of plasmids by homologous recombination in *Saccharomyces cerevisiae*. A circular recombinant plasmid of a desired structure is regenerated after transformation of yeast with a linearized plasmid and a DNA restriction fragment containing appropriate homology to serve as a substrate for recombinational repair. The free ends of the input DNA molecules need not be homologous in order for efficient recombination between internal homologous regions to occur. The method is particularly useful for incorporating into or removing from plasmids selectable markers, centromere or replication elements, or particular alleles of a gene of interest. Plasmids constructed in yeast can subsequently be recovered in an *Escherichia coli* host. Using this method, we have constructed an extended series of new yeast centromere, episomal and replicating (YCp, YEp, and YRp) plasmids containing, in various combinations, the selectable yeast markers *LEU2, HIS3, LYS2, URA3* and *TRP1*.

INTRODUCTION

The molecular analysis of gene function in the yeast *S. cerevisiae* has been aided tremendously by the development of methods for the manipulation of

Correspondence to: Dr. D. Botstein, Department of Biology, Massachusetts Institute of Technology, Rm. 56-721, Cambridge, MA 02139 (U.S.A.) Tel. (617)253-6725.

Abbreviations: Ac, acetate; Amp or Ap, ampicillin; bp, base pair(s); kb, kilobases or 1000 bp; $P_{gall-10}$, promoter region between the yeast *GAL1* and *GAL10* genes; ^{R} or ^{r}, resistance; SD, yeast synthetic minimal medium with dextrose; SDS, sodium dodecyl sulfate; Tc or Tet, tetracycline; ::, novel joint.

genes using recombinant DNA technology (Botstein and Davis, 1982). During the process of analysing a particular cloned gene, one often finds it necessary to change the plasmid's selectable marker, or to move the cloned gene to a different plasmid; for example, from a low-copy-number centromere plasmid to a multicopy 2-μm plasmid or the reverse. Also, genetic analyses often require introducing many new alleles of a cloned gene onto a particular plasmid for subsequent studies of function. These objectives are normally attained through the use of in vitro techniques, which can be complex and tedious.

Methods that rely on homologous recombination

in yeast following transformation (Hinnen et al., 1978) have been employed in manipulating the chromosomal loci of cloned genes (see, for example, Winston et al., 1983). The power and applicability of these methods has been greatly expanded by the use in transformation of plasmid DNA containing double strand breaks, which are highly reactive for homologous recombination in yeast (Orr-Weaver et al., 1981; 1983). Here we extend these methods to the manipulation of plasmid-borne genes. We describe a fast and reliable method for plasmid construction that is based on the efficient repair of a linearized plasmid by recombination with a homologous DNA restriction fragment during yeast transformation (Kunes et al., 1985). The method is demonstrated by constructing a number of new yeast vectors, using the common pBR322 (Bolivar et al., 1977) backbone of the existing array of yeast vectors (Botstein et al., 1979; Broach, 1983) to provide homologous regions for recombination.

MATERIALS AND METHODS

(a) Strains, plasmids and media

The yeast strains used in this study are all derived from S288C (*MATα SUC2 gal2*). Strains bearing the following markers were used in transformation with a plasmid containing the corresponding wild-type clone: *ura3*-52 (Carlson et al., 1984), *his3-Δ200* (Struhl, 1985a), *lys2-Δ120* (Simchen et al., 1985), *leu2-3,2*-112 (Botstein et al., 1979) and *trp1-Δ901* (Hieter et al., 1985). The plasmids YRp7, YEp6 and YEp21 (Botstein et al., 1979), and pSI4 (Broach, 1983) have been described. The yeast centromere plasmid YCp50 (C. Mann, personal communication) *RI* contains the 1.75-kb *Pvu*II-*Eco*ʏ̌ fragment of *CEN4* (Mann and Davis, 1986) and the 0.84-kb *Eco*RI-*Hin*dIII fragment of *ARS1* (Tschumper and Carbon, 1980) blunt-end ligated into the *Pvu*II site of YIp5 (Botstein et al., 1979), with all junction sites destroyed. The yeast episomal plasmid YEp420, previously called β72, was constructed by blunt-end *ng* ligation into the *Pvu*II site of YIp5 the 1.58-kb *Hpa*I-*Hin*dIII fragment of the yeast 2-μm circle (form B) (Broach, 1983; Hartley and Donelson, 1980) containing the putative replication origin. The

plasmid pGM65 (provided by G. Maine, Biotechnica International Inc., Cambridge, MA) contains a 0.85-kb *Eco*RI-*Bam*HI fragment of the *GAL10-GAL1* promoter region (Yocum et al., 1984) inserted between the *Eco*RI and *Bam*HI sites of YEp420, with the *GAL1* promoter at the *Bam*HI end and the *GAL10* promoter at the *Eco*RI end. The *Bam*HI site is from a *Bam*HI-linker that was ligated to the *GAL* promoter sequence just upstream from the *GAL1* start codon. Plasmid pPL7 (J. Mullins, personal communication) is a derivative of pBR322 with a 90-bp polylinker fragment inserted between the *Eco*RI and *Bam*HI sites. Plasmids pRB315 and pRB328 are derivatives of pPL7 containing the 0.82-kb *Eco*RI-*Pst*I fragment bearing the *TRP1* gene (lacking *ARS1*; Tschumper and Carbon, 1980) and the 1.77-kb *Bam*HI fragment with the *HIS3* gene (Struhl, 1985b), respectively, as described in Schatz et al. (1986). Plasmid pRB506 is a derivative of pPL7 with the 5.3-kb *Cla*I-*Xba*I fragment containing the *LYS2* gene (see map in Barnes and Thorner, 1986) cloned between the *Cla*I and *Xba*I sites. The maps of plasmids YCp50, YEp420, YRp7, pGM65, and pSI4 are shown in Fig. 1, and those of YEp21, YEp6, pPL7, pRB315, pRB328, pRB506 are shown in Fig. 2.

Yeast was grown in SD medium (Sherman et al., 1979) supplemented with 0.01% of all amino acids, uracil, and adenine, except that the appropriate amino acid or base was absent when selection was applied. *E. coli* was grown in LB medium in the presence of 50 μg Ap/ml or 15 μg Tc/ml, when appropriate.

(b) DNA manipulations

were

Yeast DNA was isolated by a modified version of the procedure described by Winston et al. (1983). Yeast was harvested from 10 ml stationary phase cultures and resuspended in 400 μl 0.1 M EDTA pH 8.0 in 1.5-ml microfuge tubes. To each tube was added 100 μl of zymolyase (zymolyase-100; 2 mg/ml; Seikagaku Kogyo Co., Ltd., 2-9 Nihonbashi-honcho, Chuo-ku, Tokyo, 103 Japan), followed by incubation at 37°C for 1-2 h; 90 μl of 6% SDS 0.7 M Tris base was added to each tube with mixing by inversion. The tubes were then incubated at 65°C for 30 min. After the addition of 80 μl 5 M KAc and gentle mixing, the tubes were incubated on ice for
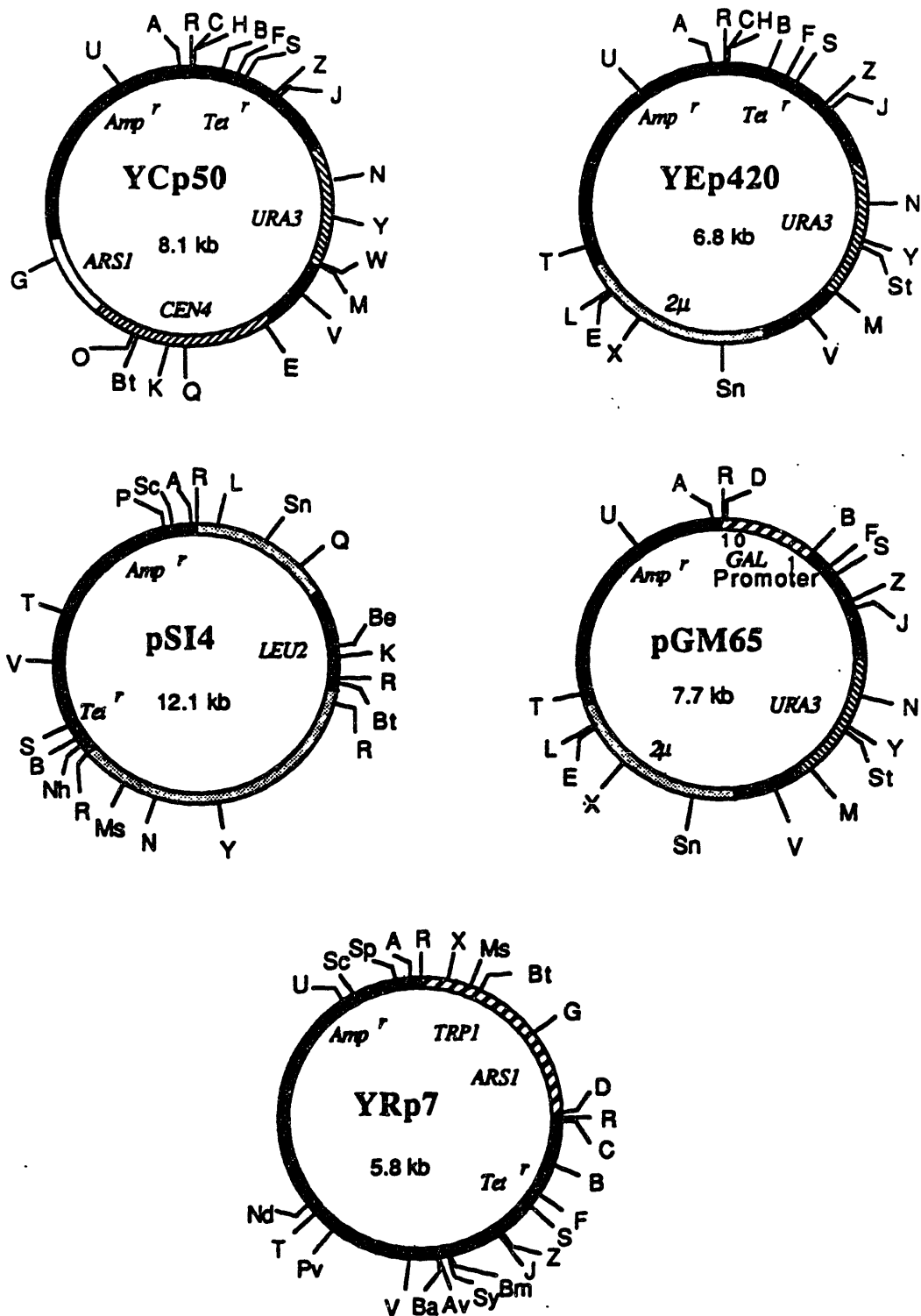
Fig. 1. Restriction maps of the plasmids used to generate linearized plasmids for vector construction by recombination. The restriction site positions are derived from the published sequences of the various components (see MATERIALS AND METHODS, section a). For each plasmid depicted, the lengths of differently shaded regions are proportional to their actual lengths. Only the *Eco*RI and known unique sites are shown. Restriction enzymes: A, *Aat*I; Av, *Ava*I; B, *Bam*HI; Ba, *Bal*I; Be, *Bst*EI; Bh, *Bss*HII; Bm, *Bsm*I; Bs, *Bsp*MI; Bt, *Bst*XI; C, *Cla*I; D, *Dra*III; E, *Spe*I; F, *Sph*I; G, *Bgl*II; H, *Hind*III; J, *Nru*I; K, *Kpn*I; L, *Bcl*I; M, *Sma*I; Ms, *Mst*II; N, *Nco*I; Nd, *Nde*I; Nh, *Nhe*I; O, *Xho*I; P, *Pst*I; Pv, *Pvu*II; Q, *Hpa*I; R, *Eco*RI; S, *Sal*I; Sc, *Sca*I; Sn, *Sna*BI; Sp, *Ssp*I; St, *Stu*I; Sy, *Sty*I; T, *Tth*111I; U, *Pvu*I; V, *Bsp*MII; W, *Ava*III(*Nsi*I); X, *Xba*I; Y, *Apa*I; Z, *Xma*III(*Eag*I). The solid black regions are pBR322 sequences, the stippled ones are yeast 2-μm sequences, and other regions are indicated explicitly on the maps.
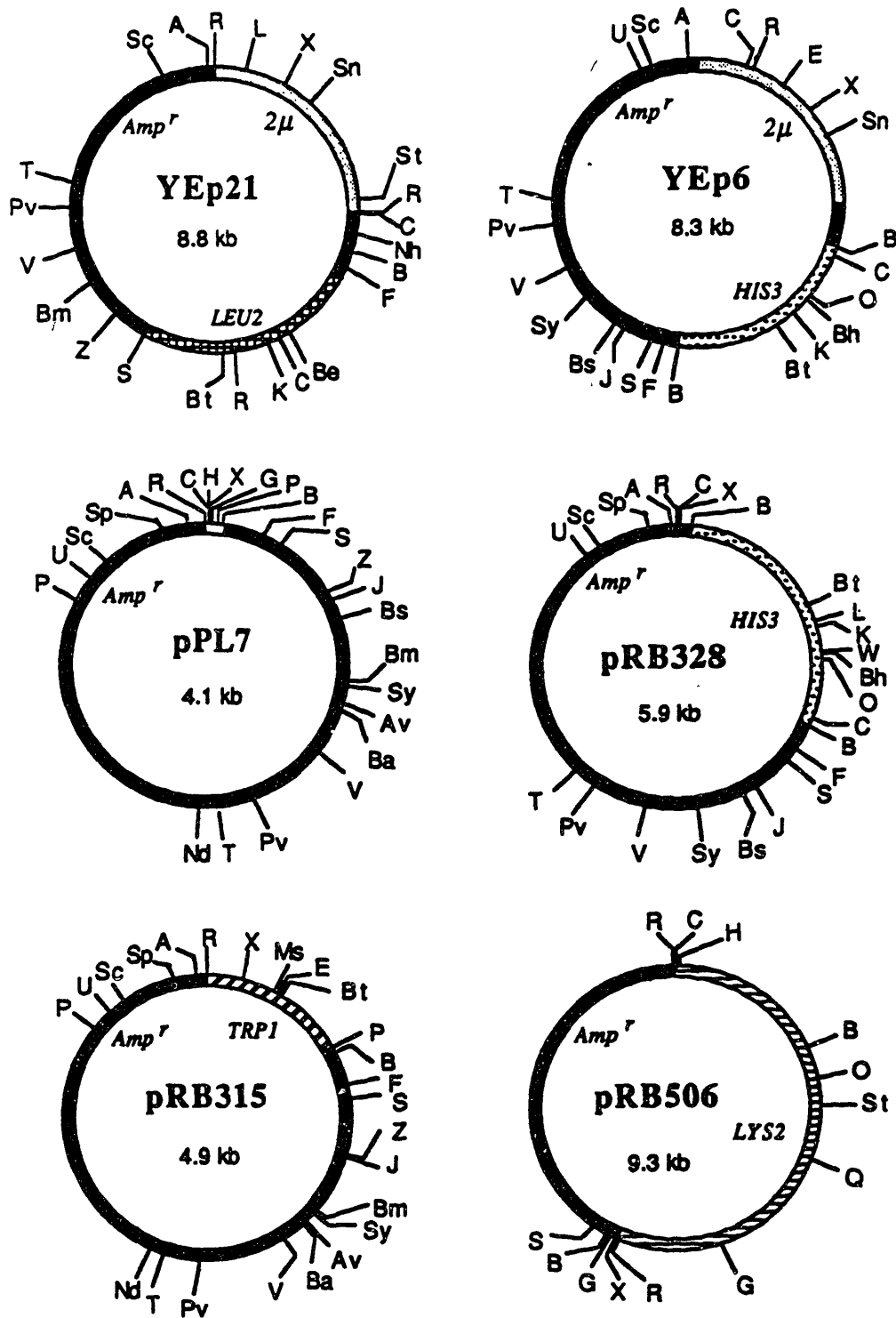
Fig. 2. Restriction maps of the plasmids providing homologous DNA fragments for the recombinational repair of linearized plasmids. Restriction sites are deduced from known sequences when available (see MATERIALS AND METHODS, section a). Within each plasmid, the lengths of differently shaded regions are proportional to their actual lengths. See legend of Fig. 1 for restriction site codes. Only the BamHI, ClaI, EcoRI and known unique sites are shown. During the construction of YEp6, a portion of pBR322 was deleted, so that the precise position of the joints between 2-μm and pBR322 DNA are not known (Struhl et al., 1979). Restriction analysis indicates that the ClaI and HindIII sites of pBR322 were lost, but the AatI and EcoRV sites are still present. The information on pRB506 is not complete because the sequence of the LYS2 fragment is not available. The solid black regions are pBR322 sequences.

1–2 h. The samples were centrifuged in a microfuge for 15 min (12 000 × g). The resulting supernatants were transferred to new tubes, filled with ethanol and mixed by gentle inversion. The precipitate was recovered by centrifugation in a microfuge for 15 s. The pellets were rinsed with cold ethanol, air-dried, and dissolved in 400 μl 10 mM Tris 1.0 mM EDTA pH 8.0 by incubation at room temperature for 1–2 h with gentle agitation. The samples were then centrifuged in the microfuge for 15 min and the supernatants were transferred to new tubes. To recover plasmids, 1% of the DNA solution was used to transform E. coli (Davis et al., 1982).

(c) Transformation of yeast

Yeast transformation was performed by a modified version (Kuo and Campbell, 1983) of the LiAc method (Ito et al., 1983). Typically, to obtain a few thousand transformants, a DNA mixture containing 0.05–0.2 μg of a linearized plasmid, 0.5–1.0 μg of a DNA restriction fragment, and 50 μg of sonicated

chicken erythrocyte DNA (Calbiochem-Behring, La Jolla, CA) was added to about 2×10^8 competent yeast cells.

RESULTS AND DISCUSSION

(a) Introduction of new selectable markers to plasmids

The characterization of a cloned yeast gene often calls for the introduction of new selectable markers to the gene-carrying plasmid. For example, many gene clones are isolated from plasmid libraries that bear a particular selectable marker, such as the yeast URA3 gene, that may not suffice in subsequent studies. The introduction of a new selectable marker can be accomplished by using a DNA fragment containing the new marker to repair by recombination a linearized plasmid bearing the cloned gene. Three basic ways in which this reaction can be
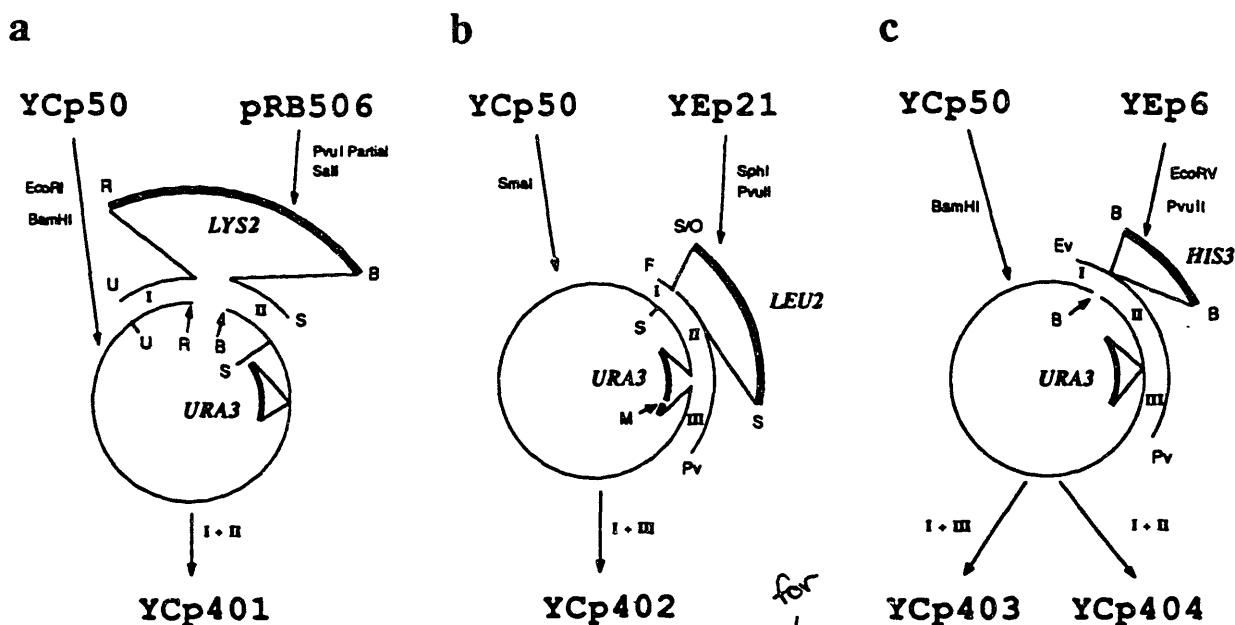

Fig. 3. Introduction of new selectable markers to a plasmid by recombination in yeast. (a) A lys2 strain was transformed with YCp50 cut with EcoRI + BamHI and pRB506 with PvuI (partial) + SalI, applying selection of Lys⁺ transformants. Recombination events in intervals I and II generate YCp401. (b) A leu2 strain was transformed with YCp50 cut with SmaI and YEp21 with SphI + PvuII, applying selection for Leu⁺ transformants. The formation of a LEU2 carrying plasmid requires recombination events in intervals I and III. (c) A his3 strain was transformed with a mixture of YCp50 cut with BamHI and YEp6 cut with EcoRV + PvuII, applying selection for His⁺ transformants. Recombination events in homologous intervals I and III produce YCp401, while recombination events in intervals I and II produce YCp402. Restriction enzyme codes: B, BamHI; Ev, EcoRV; F, SphI; M, SmaI; O, XhoI; Pv, PvuII; R, EcoRI; S, SalI; U, PvuI.
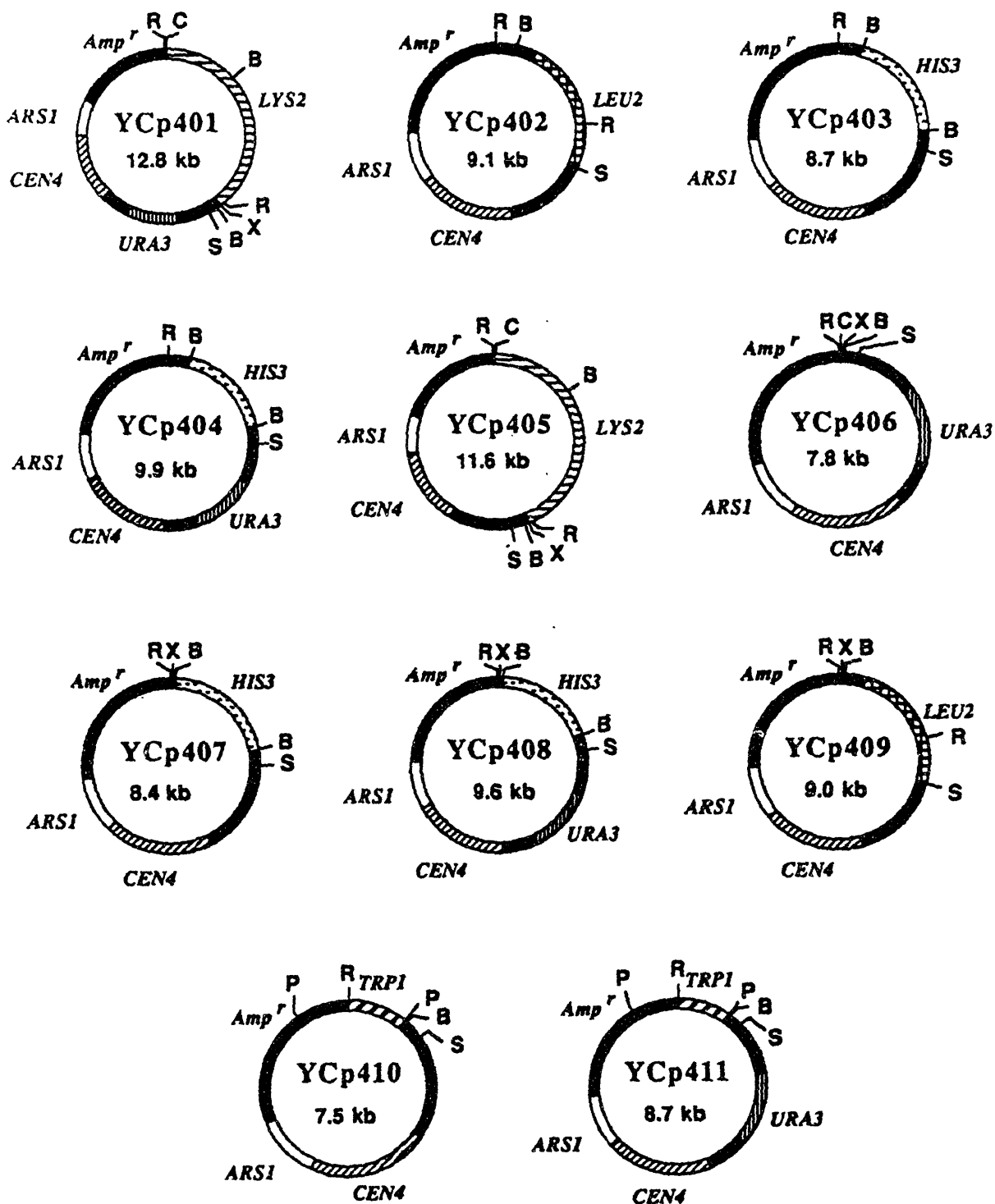
Fig. 4. Restriction maps of YCp50 derivatives constructed by recombination in yeast. For each plasmid depicted, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B, BamHI; C, ClaI; P, PstI; R, EcoRI; S, SalI; X, XbaI. For the positions of other sites, see the map of YCp50 in Fig. 1 and those of plasmids providing the various DNA fragments in Fig. 2. The yeast sequences are indicated and the solid black regions are from pBR322.

(1) Simple insertion

A DNA fragment that can serve as a substrate for the recombinational repair of a linearized plasmid must span the break of the plasmid, and contain two regions of overlapping homology where recombination can occur to generate a circular product. As shown in Fig. 3a, a marker located on a DNA fragment between these two regions of overlapping homology would necessarily be incorporated into this repair product. In the example shown in Fig. 3a, the yeast *URA3 CEN4 ARS1* vector YCp50 was linearized by *Eco*RI + *Bam*HI cleavage and introduced into yeast along with the *LYS2*-containing fragment of a *Sal*I + partial *Pvu*I digest of pRB506. The *LYS2* insert lies between the two regions of overlapping homology labeled I and II. Lys⁺ selection resulted in a high frequency of transformants. Ten of these transformants were tested for growth without uracil and found to be Ura⁺, indicating that the recombinant plasmids had retained the original YCp50 marker, *URA3*, as expected. The resulting *LYS2 URA3 CEN4 ARS1* plasmid, subsequently recovered in *E. coli*, was named YCp401 (Fig. 4). It is worth noting that this product presumably could have been recovered by selecting for transformants for the original plasmid marker, *URA3*, instead of *LYS2*, since the repair event necessarily incorporates the latter into the product. The possibility of recovering the recombinant plasmid without selection for the fragment-borne gene is explored in section b, below.

(2) Substitution of a new marker for the original marker

It is often desirable to delete the original selectable marker while simultaneously introducing a new marker. This can be accomplished by using a DNA fragment that contains the new marker to repair a linearized plasmid cut within the original marker. In the example shown in Fig. 3b, YCp50 was linearized at the *Sma*I site in *URA3*, and introduced to a *leu2 ura3* strain along with the *Sph*I, *Pvu*II fragment of YEp21 that contains a *LEU2* gene insert. All of the resulting Leu⁺ transformants examined had lost the *URA3* gene. Plasmids from four of the yeast transformants were recovered in *E. coli* and shown by restriction analysis (not shown) to have the expected struc-

ture, designated YCp402 (Fig. 4). This outcome was as expected, because for a circular product containing the *LEU2* gene to form, recombination must occur in the overlapping intervals I and III (Fig. 3b). Consequently, the *Sma*I-generated *URA3* ends are lost. It is worth noting that the overlapping homology of interval I is only 89 bp long, indicating that a region of homology between the linearized plasmid and the DNA fragment can be very short and still sufficient for homologous recombination. Furthermore, while repair of *Sma*I-linearized YCp50 by recombination with the chromosomal *URA3* locus is a possibility, products that had retained *URA3* were not recovered by the Leu⁺ selection. The circular products of recombination with the chromosome presumably do not efficiently recombine with and incorporate the fragment-borne *LEU2* gene.

(3) Insertion or substitution

When introducing a new marker to a plasmid, one can recover products with and without the original plasmid marker if the plasmid is linearized outside of the original marker and the DNA fragment spans both the plasmid's break and the original marker's position. The construction depicted in Fig. 3c illustrates this case. Plasmid YCp50, linearized by *Bam*HI cleavage, was introduced to a *his3 ura3* strain along with the YEp6 *Eco*RV-*Pvu*II fragment bearing a *HIS3* gene insert. Repair of the linearized YCp50 requires a crossover within the overlapping homologous interval I, and in either homologous interval II or III (Fig. 3c). A crossover in interval II would retain the original *URA3* marker of YCp50 in the product, while a crossover in interval III would exclude *URA3* from the product. As expected, a high frequency of His⁺ transformants resulted, containing either of these two possible plasmid products, designated YCp403 and YCp404 (Fig. 4). Of 100 His⁺ transformants tested, 98 were Ura⁺. Thus recombination between the linearized plasmid and DNA fragment in interval III was rare, an observation that might bear on the recombination mechanism.

Through the use of the three basic strategies outlined above, we constructed an extended series of plasmid derivatives of YCp50 containing in several combinations the generally useful selectable markers *HIS3*, *LEU2*, *LYS2*, *TRP1* and *URA3*. These plasmids and their derivatives are described in Table I. Their structures are shown in Fig. 4.

TABLE I

New plasmids and their derivations

New plasmid[a]	Linearized plasmid		Homologous fragment		Selection[f]
	Plasmid[b]	Digested with[c]	Plasmid[d]	Digested with[e]	
YCp401	YCp50	EcoRI, BamHI	pRB506	PvuI, SalI	Lys⁻
YCp402	YCp50	SmaI	YEp21	SphI, PvuII	Leu⁻
YCp403, YCp404	YCp50	BamHI	YEp6	EcoRV, PvuI, PvuII	His⁻
YCp405	YCp401	SalI	pBR322	EcoRI, PvuII	Lys⁻
YCp406	YCp50	EcoRI, BamHI	pPL7	PvuI, SalI	Ura⁻
YCp407, YCp408	YCp50	EcoRI, BamHI	pRB328	PvuI, PvuII	His⁻
YCp409	YCp402	EcoRI, BamHI	pPL7	PvuI, SalI	Leu⁺
YCp410, YCp411	YCp50	EcoRI, SalI	pRB315	PvuI, PvuII	Trp⁺
YEp421, YEp422	YEp420	BamHI	YEp6	EcoRV, PvuI, PvuII	His⁺
YEp423, YEp424	YEp420	SalI	YEp21	HindIII, PstI, PvuII	Leu⁺
YEp425	YEp420	EcoRI, BamHI	pRB506	PvuI, SalI	Lys⁺
YEp426	YEp425	SalI	pBR322	BamHI, PvuII	Lys⁺
YEp427, YEp428	YEp420	EcoRI, BamHI	YRp7	PvuI, PvuII	Trp⁺
YEp429	YEp420	EcoRI, BamHI	pPL7	PvuI, SalI	Ura⁺
YEp430, YEp431	YEp420	EcoRI, BamHI	pRB328	PvuI, PvuII	His⁺
YEp432, YEp433	YEp420	EcoRI, BamHI	pRB315	PvuI, PvuII	Trp⁺
YEp434, YEp435	pGM65	SalI	YEp21	HindIII, PstI, PvuII	Leu⁺
YEp436	pSI4	BamHI, SalI	YIp5	EcoRI, PvuII	Ura⁺
YEp437	pSI4	BamHI, SalI	YEp6	EcoRV, PvuI, PvuII	His⁺
YRp441	YRp7	BamHI	YEp6	EcoRV, PvuI, PvuII	His⁺
YRp442	YRp7	SalI	YEp21	HindIII, PstI, PvuII	Leu⁺

[a] Each new plasmid was constructed by homologous recombination in yeast between the indicated linearized plasmid and homologous DNA fragments. The restriction maps of these plasmids are shown in Figs. 4, 6 and 7.

[b] Plasmids used to generate the linearized plasmid substrates for homologous recombination in yeast. Their restriction maps are shown in Figs. 1, 4 (YCp401, YCp402), and 6 (YEp425).

[c] The restriction enzyme(s) used to generate the linearized plasmid.

[d] Plasmids that provided the homologous DNA fragments for recombination with the linearized plasmid. Their restriction maps are shown in Figs. 1 and 2, except for pBR322.

[e] The restriction enzymes used to generate the homologous DNA fragments.

[f] Selection for transformants was applied on SD medium lacking the corresponding nutrient.

[a] The new yeast vectors constructed in this paper. Their restriction maps are shown in Figs. 4, 6 and 7. Table I shows their derivations.

[b] The selectable yeast markers residing on the indicated plasmid. In addition, the presence of the GAL1-Gal10 promoter region is also indicated when applicable.

[c] The known unique restriction sites of the vectors. The underlined sites are not in the coding or promoter regions of yeast genes, the CEN4, ARS1 and 2-μm sequences, or the Amp^R gene. They may be used for cloning purposes. The information is compiled from the maps of pBR322 and YIp5 and the reported sequences of URA3 (Rose et al., 1984), HIS3 (Struhl, 1985b), LEU2 (Andreadis et al., 1982; 1984), CEN4 (Mann and Davis, 1986), TRP1 ARS1 (Tschumper and Carbon, 1980), the GAL1-GAL10 promoter (Yocum et al., 1984) and the 2-μm circle (Hartley and Donelson, 1980). The LYS2 restriction map was provided by Dr. G. Simchen. A restriction map of the LYS2 locus is also available in Barnes and Thorner, 1986.

[d] The HIS3 gene has an additional ClaI site that overlaps a dam methylation site. Therefore, this site will not be cut if the plasmid DNA is prepared from dam⁺ E. coli host.

TABLE II

New plasmids and their features

Plasmid [a]	Yeast genes [b]	Unique restriction sites [c]
YCp401	*LYS2 URA3*	*ClaI*, *HindIII*, *SalI*, *SmaI*, *SphI*, *XbaI*
YCp402	*LEU2*	*AatII*, *BamHI*, *BglII*, *BspMII*, *BstEII*, DraIII, *HindIII*, *NdeI*, *NruI*, PvuI, *SalI*, *SpeI*, *SphI*, XhoI, *XmaIII(EagI)*
YCp403	*HIS3*	*AatII*, *BspMII*, *BssHII*, *ClaI* [d], DraIII, *EcoRI*, *EcoRV*, HpaI, *NsiI(AvaIII)*, *NruI*, PvuI, *SalI*, SpeI, *SphI*
YCp404	*HIS3 URA3*	*AatII*, ApaI, *BspMII*, *BssHII*, *ClaI* [d], *EcoRI*, HpaI, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SpeI, *SphI*
YCp405	LYS2	*ClaI*, *HindIII*, *SalI*, *SphI*, *XbaI*
YCp406	URA3	*AatII*, ApaI, *BamHI*, *BspMII*, BstXI, *ClaI*, *EcoRI*, EcoRV, *HindIII*, HpaI, KpnI, NcoI, *NruI*, *NsiI(AvaIII)*, PvuI, *SalI*, *SmaI*, SpeI, *SphI*, *XbaI*, XhoI, *XmaIII(EagI)*
YCp407	*HIS3*	*AatII*, *BspMII*, *BssHII*, *ClaI* [d], DraIII, *EcoRI*, HpaI, *NsiI(AvaIII)*, *NruI*, PvuI, *SalI*, SpeI, *SphI*, *XbaI*
YCp408	*HIS3 URA3*	*AatII*, ApaI, *BspMII*, *BssHII*, *ClaI* [d], *EcoRI*, EcoRV, HpaI, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SpeI, *SphI*, *XbaI*
YCp409	*LEU2*	*AatII*, *BamHI*, *BspMII*, BstEII, DraIII, EcoRV, *HindIII*, *NdeI*, *NruI*, PvuI, *SalI*, SpeI, *SphI*, *XbaI*, XhoI, *XmaIII(EagI)*
YCp410	*TRPI*	*AatII*, *BamHI*, BglII, *BspMII*, DraIII, *EcoRI*, EcoRV, HindIII, HpaI, KpnI, MstII, *NdeI*, *NruI*, PvuI, *SalI*, SpeI, *SphI*, XbaI, XhoI, *XmaIII(EagI)*
YCp411	*TRPI URA3*	*AatII*, ApaI, *BamHI*, BglII, *BspMII*, *EcoRI*, HindIII, HpaI, KpnI, MstII, NcoI, *NruI*, *NsiI(AvaIII)*, PvuI, *SalI*, *SmaI*, SpeI, *SphI*, XbaI, XhoI, *XmaIII(EagI)*
YEp421	*HIS3*	*AatII*, *BspMI*, *BspMII*, *BssHII*, BstXI, *ClaI* [d], *EcoRI*, *EcoRV*, KpnI, *NruI*, PvuI, *SalI*, ScaI, SnaBI, SpeI, *SphI*, *SrfI*, *Tth111I*, XbaI, XhoI
YEp422	*HIS3 URA3*	*AatII*, ApaI, *BspMII*, *BssHII*, BstXI, *ClaI* [d], *EcoRI*, KpnI, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, *SpeI*, *SphI*, StuI, *Tth111I*, XbaI, XhoI
YEp423	*LEU2*	*AatII*, *BamHI*, BclI, *BsmI*, *BspMII*, BstEII, BstXI, HpaI, *HindIII*, KpnI, *NheI*, *NruI*, PstI, PvuI, ScaI, *SalI*, SnaBI, SpeI, *SphI*, XbaI, *XmaIII(EagI)*
YEp424	*LEU2 URA3*	*AatII*, ApaI, *BamHI*, BclI, *BspMII*, BstEII, BstXI, HpaI, *HindIII*, KpnI, NcoI, *NheI*, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, StuI, XbaI, *XmaIII(EagI)*
YEp425	*LYS2 URA3*	*ClaI*, *HindIII*, HpaI, *SalI*, *SmaI*, *SphI*, XhoI
YEp426	*LYS2*	*ClaI*, *HindIII*, HpaI, *SalI*, *SphI*, StuI, XhoI
YEp427	*TRPI*	*AatII*, *BamHI*, BclI, BglII, *BsmI*, *BspMII*, BstXI, *ClaI*, DraIII, MstII, *NruI*, PvuI, *SalI*, ScaI, SnaBI, SpeI, *SphI*, StuI, *SrfI*, *Tth111I*, *XmaIII(EagI)*
YEp428	*TRPI URA3*	*AatII*, ApaI, *BamHI*, BclI, BglII, *BspMII*, BstXI, *ClaI*, MstII, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, *Tth111I*, *XmaIII(EagI)*
YEp429	*URA3*	*AatII*, ApaI, *BamHI*, BclI, *BglII*, *BspMII*, *ClaI*, *EcoRI*, EcoRV, *HindIII*, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, StuI, *Tth111I*, *XmaIII(EagI)*
YEp430	*HIS3*	*AatII*, *BspMI*, *BspMII*, *BssHII*, BstXI, *ClaI* [d], *EcoRI*, KpnI, NheI, *NruI*, PvuI, *SalI*, ScaI, SnaBI, SpeI, *SphI*, *SrfI*, *Tth111I*, *XhoI*
YEp431	*HIS3 URA3*	*AatII*, ApaI, *BspMII*, *BssHII*, BstXI, *ClaI* [d], *EcoRI*, EcoRV, KpnI, NcoI, NheI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, StuI, *Tth111I*, *XhoI*
YEp432	*TRPI*	*AatII*, *BamHI*, BclI, *BsmI*, *BspMII*, BstXI, *EcoRI*, EcoRV, HindIII, MstII, *NruI*, PvuI, *SalI*, ScaI, SnaBI, SpeI, *SphI*, *SrfI*, *Tth111I*, *XmaIII(EagI)*
YEp433	*TRPI URA3*	*AatII*, ApaI, *BamHI*, BclI, *BspMII*, BstXI, *EcoRI*, HindIII, MstII, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, StuI, *Tth111I*, *XmaIII(EagI)*
YEp434	*LEU2* $P_{GAL1-10}$	*AatII*, *BamHI*, BclI, *BsmI*, *BspMII*, BstEII, BstXI, ClaI, DraIII, EcoRV, HpaI, KpnI, *NruI*, PstI, PvuI, *SalI*, SnaBI, SpeI, *SphI*, XbaI, *XmaIII(EagI)*
YEp435	*LEU2 URA3* $P_{GAL1-10}$	*AatII*, ApaI, *BamHI*, BclI, *BspMII*, BstEII, BstXI, ClaI, HpaI, KpnI, NcoI, *NruI*, PvuI, *SalI*, *SmaI*, SnaBI, SpeI, *SphI*, StuI, XbaI, *XmaIII(EagI)*
YEp436	*LEU2-d URA3*	*AatII*, *BamHI*, BclI, *BspMII*, BstEII, BstXI, KpnI, MstII, *NheI*, *SalI*, *SmaI*, SnaBI
YEp437	*LEU2-d HIS3*	*AatII*, ApaI, *BspMII*, *BssHII*, BstEII, MstII, NcoI, *SalI*, ScaI, SnaBI
YRp441	*TRPI HIS3*	*AatII*, BclI, *BspMII*, *BssHII*, *ClaI* [d], DraIII, KpnI, MstII, *NruI*, NsiI(AvaIII), PvuI, *PvuII*, *SalI*, ScaI, *SphI*, SspI, StuI, StyI, *Tth111I*, XbaI, *XhoI*
YRp442	*TRPI LEU2*	*AatII*, *BalI*, *BamHI*, BglII, *BsmI*, *BspMII*, BstEII, DraIII, HpaI, KpnI, MstII, *NdeI*, *NruI*, PvuI, *PvuII*, *SalI*, ScaI, *SphI*, StuI, XbaI, *XmaIII(EagI)*

In a similar manner, we constructed derivatives of several yeast episomal plasmids (YEp; autonomous due to the presence of a portion of the yeast plasmid 2-μm circle) and the *TRP1 ARS1* containing the yeast-replicating plasmid YRp7 (Botstein et al., 1979; Fig. 1). These new plasmids and their derivations are listed in Table I, and their structures are shown in Fig. 6 and 7. In Table II, we have listed the yeast markers of the new plasmids, and the known unique restriction sites of each plasmid.

(b) Transfer of a cloned gene to a different plasmid

It is often desirable to move a gene from one kind of plasmid to another. A case that frequently arises is the transfer of a cloned gene onto a 2-μm plasmid to achieve overexpression of the gene by virtue of the plasmid's high copy number. If the gene to be transferred is amenable to direct selection in yeast transformation, then the transfer can be done as described in RESULTS AND DISCUSSION, section a. This is, however, often not the case. We show here that a cloned gene on a DNA fragment can be incorporated into a plasmid by selecting the linearized plasmid's marker.

This case was demonstrated in an experiment in which *HIS3* was chosen as the fragment-borne gene

to be transferred without selection. As shown in Fig. 5, a DNA fragment containing the *HIS3* gene was used to repair the 2-μm plasmid, YEp420, linearized by cleavage with *Eco*RI, *Bam*HI, or *Sal*I. With *Eco*RI or *Bam*HI cleavage, the *HIS3* insert on the fragment spans the break in the linearized plasmid, and thus recombinational repair must incorporate the *HIS3* gene. In the other hand, the *Sal*I site of YEp420 corresponds to a position outside of the *HIS3* insert of the fragment. As a result, recombination with the *HIS3* fragment could generate products containing or lacking the *HIS3* insert via recombination in interval I or interval II, respectively (Fig. 5).

A *ura3 his3* yeast strain was transformed with each linearized plasmid in the presence of the *HIS3*-containing fragment, with selection for the *URA3* marker of the linearized plasmid. With YEp420 linearized by either *Eco*RI or *Bam*HI cleavage, nearly all (>96%) of the Ura⁺ transformants were His⁺ and contained a recombinant plasmid that had incorporated the fragment-borne *HIS3* gene. With *Sal*I-linearized YEp420, the fraction of the Ura⁺ transformants that were His⁻ was several-fold larger. The increase in the His⁻ fraction in this case is consistent with a small portion of the repair products having enjoyed recombination in interval II

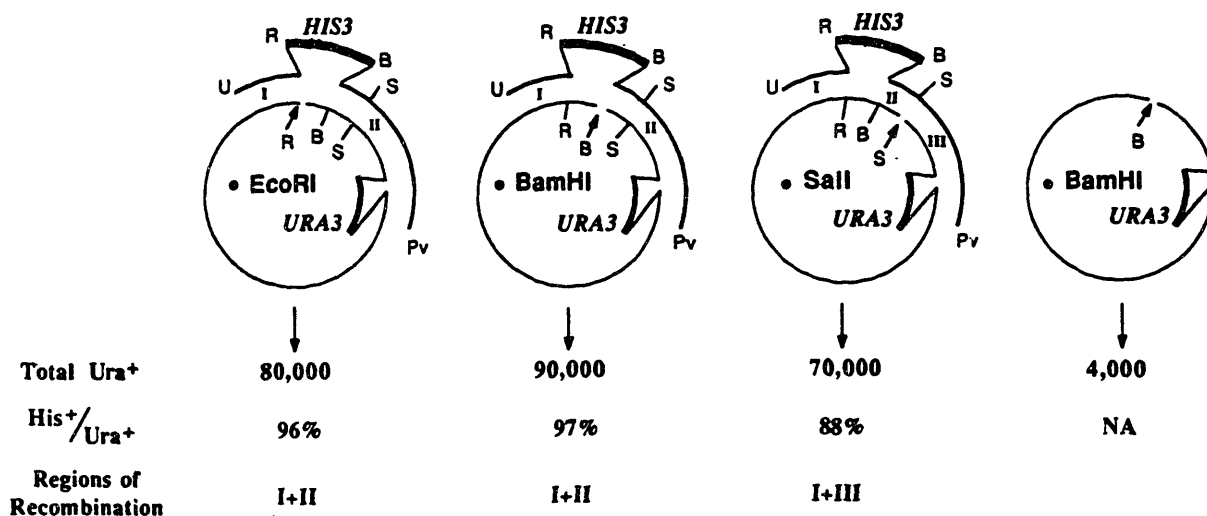|	EcoRI	BamHI	SalI	BamHI
Total Ura⁺	80,000	90,000	70,000	4,000
His⁺/Ura⁺	96%	97%	88%	NA
Regions of Recombination	I+II	I+II	I+III	

Fig. 5. Transfer of *HIS3* to a new plasmid. A *ura3 his3* strain was transformed using YEp420 (see Fig. 1 for structure) linearized at either the *Eco*RI, or *Bam*HI or *Sal*I site with or without (only the *Bam*HI cut) the *Pvu*I-*Pvu*II fragment containing the *HIS3* gene from pRB328 (see Fig. 2 for structure). Ura⁺ transformants were selected on SD medium containing histidine. Total Ura⁺ indicates number of transformants per μg of plasmid DNA. The His phenotype was scored by replica-plating more than 500 transformants from each transformation to SD medium lacking histidine. Restriction enzyme codes: B, *Bam*HI; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; U, *Pvu*I. NA, not applicable.
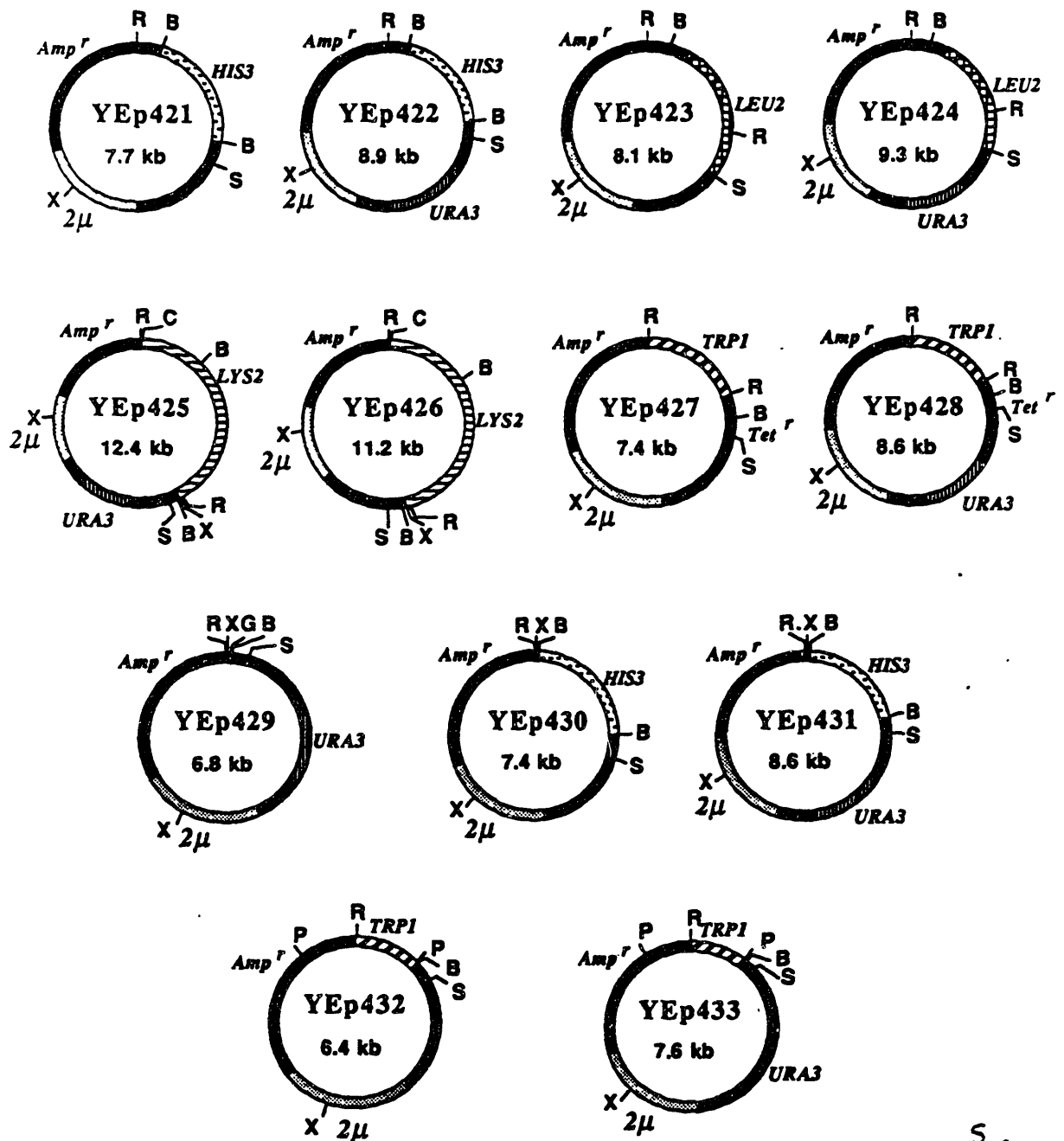
Fig. 6. Restriction maps of YEp420 derivatives with selectable markers useful for cloning in different auxotrophic background. For each plasmid depicted, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B, *Bam*HI; C, *Cla*I; G, *Bgl*II; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I. See map of YEp420 in Fig. 1 and maps of various plasmids providing fragments in Figs. 1 and 2 for positions of other sites. The yeast sequences are indicated and the solid black regions are from pBR322.

(Fig. 5). The plasmids present in a total of 20 His⁻ Ura⁺ transformants from these three transformation experiments were recovered in *E. coli* and subjected to restriction analysis. These plasmids were indistinguishable from the original plasmid, YEp420, with the original site of plasmid cleavage intact. The recovery of transformants containing these plasmids may be due to circular molecules contaminating the preparation of linearized plasmid DNA. Alternatively, these plasmids may be the pro-

ducts of recircularization of a linearized plasmid molecule by ligation after transformation, which has been observed previously (Orr-Weaver and Szostak, 1983; Suzuki et al., 1983).

We thus conclude that nearly all of the transformants recovered with selection for the marker of the linearized plasmid contain plasmids repaired by recombination with the DNA fragment. Consistent with the prominence of recombinational repair is the result of transforming with the linearized YEp420 plasmid alone. In this case, the yield (4000 per µg of DNA) of Ura$^+$ transformants is about 5% of the yield (80 000 per µg of plasmid DNA) obtained in the presence of the HIS3-containing fragment. The observation that about 4% of the Ura$^+$ transformants obtained in the presence of the fragment were His$^-$ is consistent with these relative yields. The

background of nonrecombinant plasmids is consistently low provided that a sufficient amount (10–20-fold in excess of the quantity of linearized plasmid DNA) of the homologous DNA fragment is present during the transformation.

The yield of approx. 1000 Ura$^+$ transformants with closed circular YEp420 (not shown in the figure) is several-fold less than the yield with the BamHI-linearized plasmid alone, and about 80-fold less than the yield with the linearized plasmid in the presence of the HIS3-containing fragment. This difference in the transformation yields of closed circular DNA and linearized plasmid DNA is generally observed and may reflect a difference in the ability of yeast made competent by the LiAc (Ito et al., 1983) to incorporate linear molecules and circular molecules. With yeast made competent by the spheroplasting
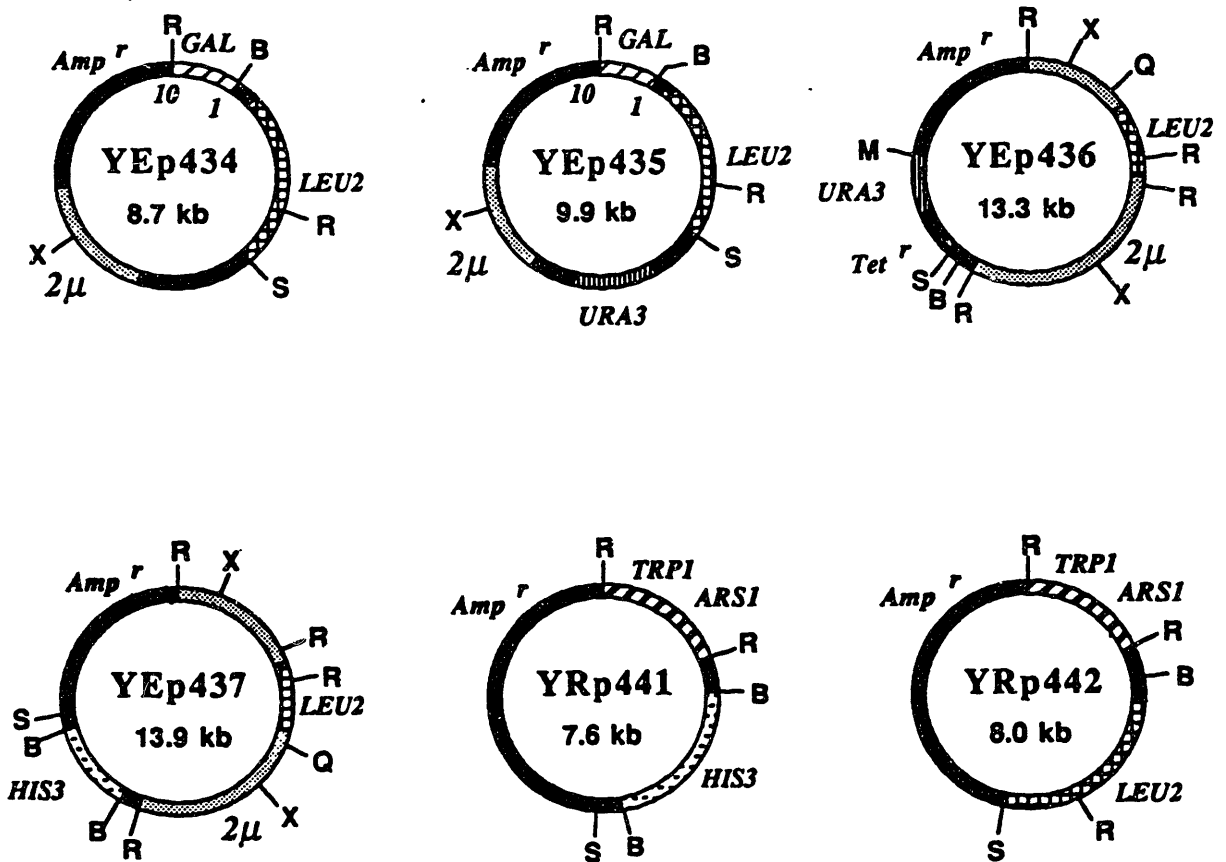

Fig. 7. Restriction maps of new vectors derived from pGM65 (for fusions to GAL1 or GAL10 promoters), pSI4 (high copy), and YRp7 (ARS plasmid). Within each plasmid, the lengths of different regions are proportional to their actual lengths. Restriction enzyme codes: B. BamHI; Q, HpaI; R, EcoRI. S, SalI; X, XbaI. See maps of parental plasmids in Figs. 1 and 2 for the positions of other sites. The solid black regions are from pBR322, and the stippled regions are from the yeast 2-µm circle plasmid.

method (Hinnen et al., 1978; Beggs, 1978), approximately equal transformation yields can be obtained with a closed circular plasmid DNA and a linearized plasmid in the presence of a homologous DNA fragment (Kunes et al., 1985).

(c) Introduction of a new alleles to a plasmid-borne gene

Through the use of in vitro mutagenesis, one can obtain a large number of new alleles of a cloned gene that may then require transfer onto a plasmid useful for their characterization in yeast. This can be accomplished by using a mutant DNA fragment to repair the new plasmid in linearized form. If the gene of interest is absent from the plasmid, the transfer reaction can be carried out as described in RESULTS AND DISCUSSION, section b, by selecting for a marker on the linearized plasmid. If, on the other hand, a copy of the gene is already on the plasmid, one can introduce the new alleles by using a mutant-bearing fragment to repair a gap generated by cleaving twice within the plasmid copy. The gap removes the pre-existing allele while leaving flanking homology to serve as regions for recombination with the fragment. Such gap repair has been used to recover chromosomal mutations onto a plasmid (Orr-Weaver et al., 1983). We have found that a co-introduced DNA fragment also serves as an efficient substrate for the repair of a gapped plasmid (our unpublished observations). A specific case of introducing a new allele in which a disruption allele of the *HXK2* gene, *hxk2*::*URA3*, was replaced with the wild-type *HXK2* gene is shown in Fig. 8. Selection for the *HIS3* marker of the linearized plasmid allowed us to obtain the desired replacement using a fragment of *HXK2* DNA to repair the plasmid linearized by a cut at the junction between the *URA3* and *hxk2* material. It should be pointed out that it is desirable to perform this manipulation in a background where the chromosomal locus has been deleted, so that recombination with the chromosome cannot occur.

(d) Formation of recombinant plasmids using substrates bearing nonhomologous free DNA ends

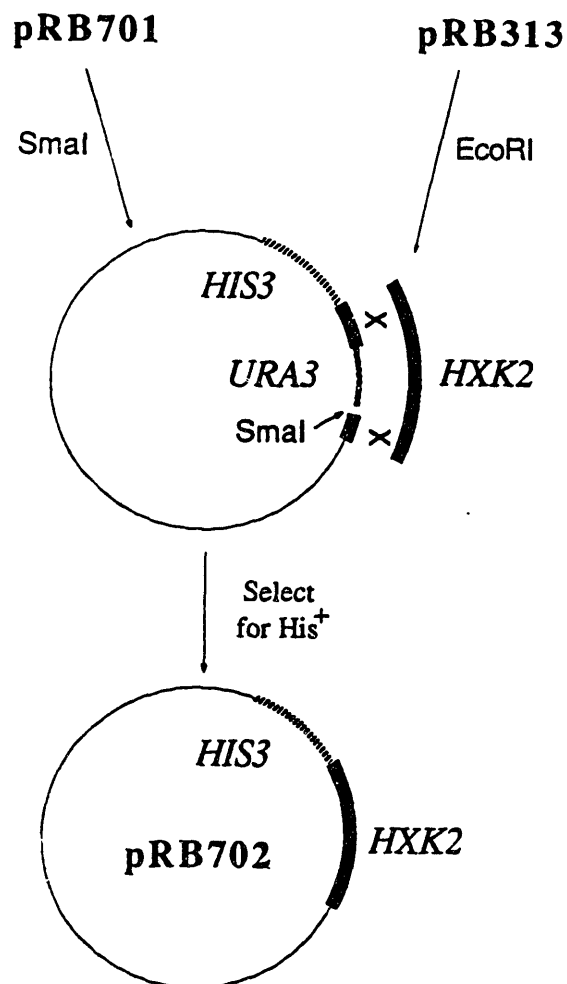In some cases it may be necessary to attempt recombination between a linearized plasmid and a

Fig. 8. Allele replacement of *HXK2* on a plasmid. A *his3 ura3 hxk1 hxk2* strain was transformed with the 3.7 kb *Eco*RI fragment of *HXK2* and pRB701 (a derivative of YEp437, see Fig. 6, with *Eco*RI fragment of *hxk2*::*URA3* between *HIS3* and pBR322 at the *Bam*HI site near the *Sal*I site) linearized at the unique *Sma*I site at the junction of *URA3* and *hxk2* sequences. His⁺ transformants were selected on SD medium containing uracil. Transformants were transferred to SD medium without uracil and to YEP fructose medium. The Ura⁻ Hxk⁺ transformants contained the expected recombinant plasmid. Restriction analysis of plasmids recovered from yeast transformants indicates the products have the expected structure.

DNA fragment bearing nonhomologous segments at their ends, so that the required recombination events must occur within internal homologous regions. This situation would arise if appropriate restriction sites are not available to generate ends within the regions of homology between the two substrates. Another case, described in RESULTS AND DISCUSSION,
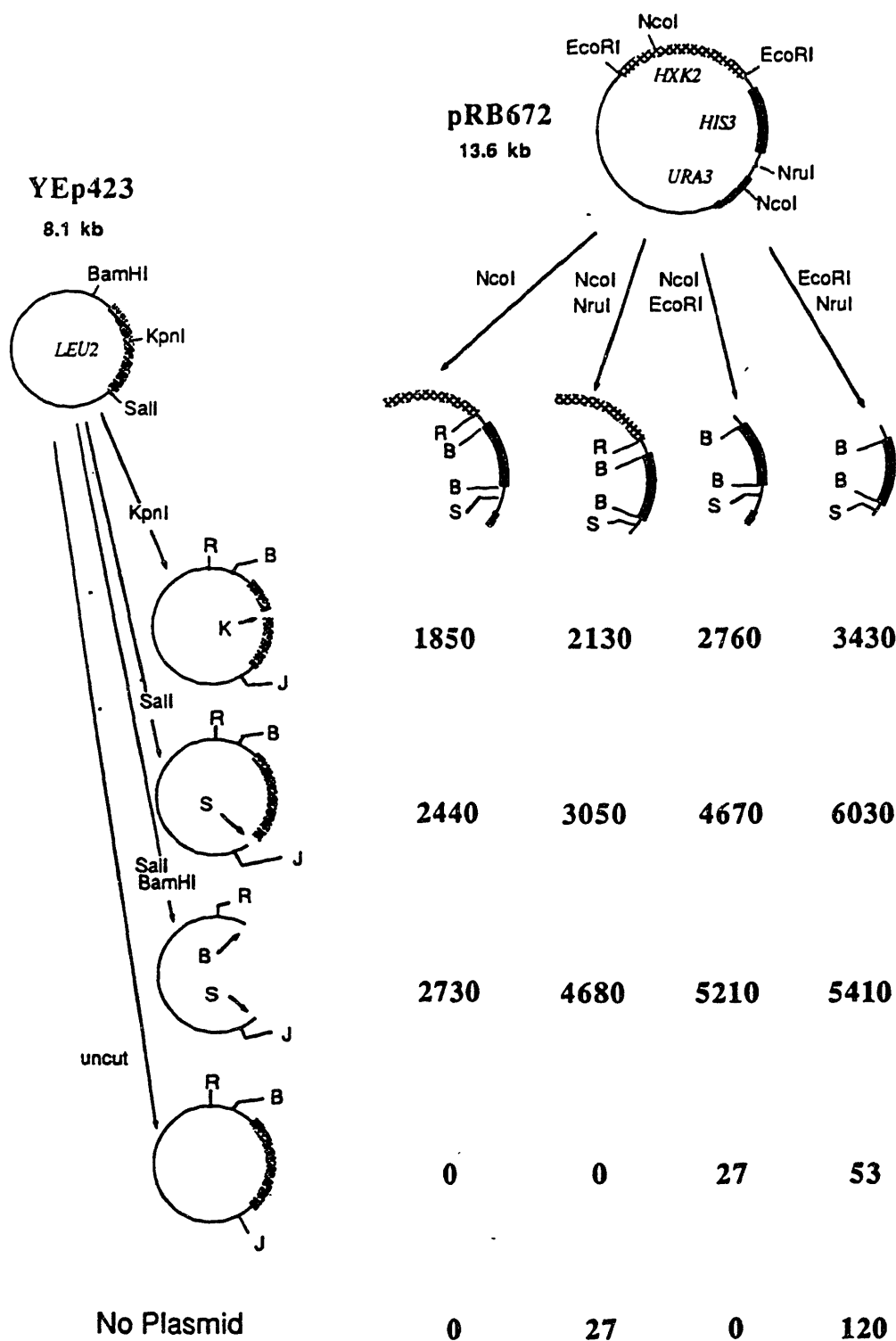
Fig. 9. Effect of nonhomologous free DNA ends on the frequency of recombinational repair of a linearized plasmid. A *his3* strain was transformed with various combinations of a linearized plasmid and a DNA fragment as indicated in the figure, applying selection for His[+] transformants on SD medium lacking histidine. The thin-line regions on the plasmid and fragment are homologous pBR322 sequences, and the various broad-line regions are nonhomologous yeast sequences. The His[+] frequency shown is normalized to the yield for 1 μg of plasmid DNA. Restriction enzyme codes: B, *Bam*HI; J, *Nru*I; K, *Kpn*I; R, *Eco*RI; S, *Sal*I.

section a2, arises when a plasmid is linearized within its original marker so as to facilitate its removal. In such circumstances, the nonhomologous ends might be those of the DNA fragment, or of the linearized plasmid, or possibly of both substrates. Fig. 9 illustrates an experiment designed to determine the effect of the presence of such nonhomologous ends on recombination. Plasmid YEp423 was cut at either the KpnI site, or the SalI site, or both the SalI and BamHI sites to produce, respectively, a linearized plasmid with neither end, one end or both ends homologous to a HIS3-containing DNA fragment derived from pRB672. Similarly, HIS3-containing DNA fragments were generated from pRB672 by digesting with NcoI (neither end homologous), or NcoI + NruI (one end homologous), or NcoI + EcoRI (one end homologous), or EcoRI + NruI (homologous ends). The substrate fragments were purified away from other plasmid fragments by electrophoresis in an agarose gel and recovered by electro-elution. As shown in Fig. 9, even with all four ends of the linearized plasmid and DNA fragment nonhomologous, the frequency of His$^+$ transformants was reduced only modestly, about three-fold, relative to the case where all four ends were homologous. The remaining cases, where either one or two nonhomologous ends were present, resulted in intermediate His$^+$ frequencies. These results indicate that the presence of nonhomologous DNA ends, in the size range of 0.43 to 2.3 kb, has only a modest effect on homologous recombination.

(e) Conclusions

The methods described here provide a new in vivo approach to introducing new selectable markers to plasmids, transferring nonselectable genes between different kinds of plasmids, and replacing the resident allele of a plasmid-borne gene with new ones. This method should prove a useful complement to the existing in vitro methods of plasmid construction.

With direct selection for a gene residing on the DNA fragment used in the repair of a linearized plasmid, the yeast transformants recovered may contain different products, depending on the site at which the plasmid is linearized. If the cut is outside of the plasmid's marker and the DNA fragment spans the marker's position, products that retain or

lack the original marker will both be produced. If the cut is within the plasmid's marker, the marker is not retained in the recombinant product. Because nearly all of the transformants recovered with a linearized plasmid and DNA fragment contain a product of recombination between these two DNAs, direct selection for the fragment-borne gene is not necessary. Last, we have shown that the regions of homology used for recombinational repair need not be at the ends of these DNA molecules. This observation greatly expands the possible sites at which the plasmid's DNAs can be cleaved to generate reactive $\Longleftarrow$ substrates for recombination.

REFERENCES

Andreadis, A., Hsu, Y.-P., Kohlhaw, G.B. and Schimmel, P.: Nucleotide sequence of yeast LEU2 shows 5'-noncoding region has sequences cognate to leucine. Cell 31 (1982) 319–325.

Andreadis, A., Hsu, Y.-P., Hermodson, M., Kohlhaw, G.B. and Schimmel, P.: Yeast LEU2: repression of mRNA levels by leucine and primary structure of the gene product. J. Biol. Chem. 259 (1984) 8059–8062.

Barnes, D.A. and Thorner, J.: Genetic manipulation of Saccharomyces cerevisiae by use of the LYS2 gene. Mol. Cell. Biol. 6 (1986) 2828–2838.

Beggs, J.D.: Transformation of yeast by a replicating hybrid plasmid. Nature 275 (1979) 104–109.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Meyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S.: Construction and characterization of new cloning vehicles, II. A multipurpose cloning system. Gene 2 (1977) 95–113.

Botstein, D. and Davis, R.W.: Principles and practice of recombinant DNA research with yeast. In Strathern, J.N., Jones, E.W. and Broach, J.R. (Eds.), The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Regulation. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, pp. 607–636.

Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K. and Davis, R.W.: Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene 8 (1979) 17–24.

Broach, J.R.: Construction of high copy yeast vectors using 2-μm circle sequences. Methods Enzymol. 101 (1983) 307–325.

Carlson, M., Osmond, B.C., Neigeborn, L. and Botstein, D.: A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics 107 (1984) 19–32.

Davis, R.W., Botstein, D. and Roth, J.R.: A Manual for Genetic Engineering: Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1980.

Hartley, J.L. and Donelson, J.E.: Nucleotide sequence of the yeast plasmid. Nature 286 (1980) 860–865.

Hieter, P., Mann, C., Snyder, M. and Davis, R.W.: Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. Cell 40 (1985) 381–392.

Hinnen, A., Hicks, J.B. and Fink, G.R.: Transformation of yeast. Proc. Natl. Acad. Sci. USA 75 (1978) 1929–1933.

Ito, H., Fukuda, Y., Murada, K. and Kimura, A.: Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153 (1983) 163–168.

Kunes, S., Botstein, D. and Fox, M.S.: Transformation of yeast with linearized plasmid DNA: formation of inverted dimers and recombinant plasmid products. J. Mol. Biol. 184 (1985) 375–387.

Kuo, C.-L. and Campbell, J.L.: Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8*-1 mutation. Mol. Cell. Biol. 3 (1983) 1730–1737.

Mann, C. and Davis, R.W.: Structure and sequence of the centromeric DNA of chromosome 4 in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6 (1986) 241–245.

Orr-Weaver, T.L. and Szostak, J.W.: Yeast recombination: the association between double-strand gap repair and crossing-over. Proc. Natl. Acad. Sci. USA 80 (1983) 4417–4421.

Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J.: Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA 78 (1981) 6354–6358.

Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J.: Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. 101 (1983) 228–245.

Rose, M., Grisafi, P., and Botstein, D.: Structure and function of the yeast *URA3* gene: expression in *Escherichia coli*. Gene 29 (1984) 113–124.

Schatz, P.J., Solomon, F. and Botstein, D.: Genetically essential and nonessential α-tubulin genes specify functionally interchangeable proteins. Mol. Cell. Biol. 6 (1986) 3722–3733.

Sherman, F., Fink, G.R., and Lawrence, C.W.: Laboratory Manual for a Course: Methods in Yeast Genetics (revised ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1978.

Simchen, G., Winston, F., Styles, C.A. and Fink, G.R.: Ty-mediated gene expression of the *LYS2* and *HIS4* genes of *Saccharomyces cerevisiae* is controlled by the same *SPT* genes. Proc. Natl. Acad. Sci. USA 81 (1984) 2431–2434.

Struhl, K.: Naturally occurring poly(dA-dT) sequences are upstream promoter elements of constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA 82 (1985a) 8419–8423.

Struhl, K.: Nucleotide sequence and transcriptional mapping of the yeast *PET56-HIS3-DED1* gene region. Nucl. Acids Res. 13 (1985b) 8587–8601.

Struhl, K., Stinchcomb, D.T., Scherer, S., and Davis, R.W.: High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76 (1979) 1035–1039.

Suzuki, K., Imai, Y., Yamashita, I. and Fukui, S.: In vivo ligation of linear DNA molecules to circular forms in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 155 (1983) 747–754.

Tschumper, G. and Carbon, J.: Sequences of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. Gene 10 (1980) 157–166.

Winston, F., Chumley, F. and Fink, G.R.: Eviction and transplacement of mutant genes in yeast. Methods Enzymol. 101 (1983) 211–228.

Yocum, R.R., Hanley, S., West Jr., R. and Ptashne, M.: Use of *lacZ* fusions to delimit regulatory elements of the inducible divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 4 (1984) 1985–1998.

Communicated by G.R. Fink.