Isolation and Characterization of Conditional-Lethal Mutations in the *TUB1* α-Tubulin Gene of the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Microtubules in yeast are functional components of the mitotic and meiotic spindles and are essential for nuclear movement during cell division and mating. We have isolated 70 conditional-lethal mutations in the *TUB1* α-tubulin gene of the yeast *Saccharomyces cerevisiae* using a plasmid replacement technique. Of the 70 mutations isolated, 67 resulted in cold-sensitivity, one resulted in temperature-sensitivity, and two resulted in both. Fine-structure mapping revealed that the mutations were located throughout the *TUB1* gene. We characterized the phenotypes caused by 38 of the mutations after shifts of mutants to the nonpermissive 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 at least one 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. There were no obvious correlations between these phenotypes and the map positions of the mutations. Greater than 90% of the mutants examined were hypersensitive to the antimicrotubule drug benomyl. Mutations that suppressed the cold-sensitive phenotypes of two of the *TUB1* alleles occurred in *TUB2*, the single structural gene specifying β-tubulin.

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, MOOR and ROBINOW 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; SHEIR-NEISS, LAI and MORRIS 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, POGSON and GULL 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, TODA and YANAGIDA 1984; HUFFAKER, THOMAS and BOTSTEIN 1988; ROY and FANTES 1983; STEARNS and BOTSTEIN 1988; THOMAS, NEFF and BOTSTEIN 1985; TODA *et al.* 1983, 1984). These mutants also display defects in meiotic spindles and in nuclear fusion during mating (HIRAOKA, TODA and YANAGIDA 1984; HUFFAKER, THOMAS and BOTSTEIN 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 (SCHATZ *et al.* 1986). 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 (SCHATZ, SOLOMON and BOTSTEIN 1986). 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

TABLE 1

Yeast strains used in this study

Strain	Genotype of strain
DBY1034	*MAT***a**, *his4-539, lys2-801, ura3-52*
DBY1035	*MATα, ade2, his4-539, ura3-52*
DBY1384	*MAT***a**, his4, ura3-52, tub2-104
DBY1385	MATα, ade2, ura3-52, tub2-104
DBY1828	MAT**a**, *ade2, his3-Δ200, leu2-3, 112, trp1-1, ura3-52*
DBY1829	*MATα, his3-Δ200, leu2-3, 112, lys2-801, trp1-1, ura3-52*
DBY2304	*MAT***a**, *his4-539, lys2-801, ura3-52, tub2-402*
DBY2303	*MATα, ade2-101, ura3-52, tub2-402*
DBY2305	*MAT***a**, *his4-539, lys2-801, ura3-52, tub2-403*
DBY2306	*MATα, ade2-101, lys2-801, ura3-52, tub2-403*
DBY2309	*MAT***a**, *his4-539, lys2-801, ura3-52, tub2-405*
DBY2310	*MATα, ade2-101, ura3-52, tub2-405*
DBY2375	*MAT***a**, *his3-Δ200, leu2-3, 112, lys2-801, ura3-52, tub3::TRP1*
DBY2383	*MAT***a**, *ade4, his3-Δ200, leu2-3, 112, ura3-52, tub1::HIS3, tub3::TRP1, TUB3-URA3-2μm* (pRB316)
DBY2384	*MATα, his3-Δ200, leu2-3, 112, lys2-801, ura3-52, tub1::HIS3, tub3::TRP1, TUB3-URA3-2μm* (pRB316)
DBY2416	*MATα, his3-Δ200, leu2-3, 112, lys2-801, ura3-52, tub1::HIS3, tub3::TRP1, tub1-737-LEU2-CEN4-ARS1* (pRB637)
DBY2422	As DBY2416 except *tub1-746* (pRB646)
DBY2433	As DBY2416 except *TUB1* (pRB539)
DBY4976	MAT**a**, *ade2, his3-Δ200, leu2-3, 112, ura3-52, ACT1:URA3:act 1* (pRB151)
DBY4982	MAT**a**, *his3-Δ200, leu2-3, 112, lys2-801, trp1-1, ura3-52, tub2-551*

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, TODA and YANAGIDA 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 *S. pombe.* The earliest mutants were isolated on the basis of altered sensitivity to benzimidazole antimicrotubule drugs (ROY and FANTES 1983; THOMAS, NEFF and BOTSTEIN 1985; TODA *et al.* 1983; UMESONO *et al.* 1983; YAMAMOTO 1981). 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, NOVICK and BOTSTEIN (1984), HUFFAKER, THOMAS and BOTSTEIN (1988) 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 (MORRIS, LAI and OAKLEY 1979; for review see HUFFAKER, HOYT and BOTSTEIN 1987). A second strategy relies on the *de novo* identification of mutants with phenotypes related to microtubule function (*e.g.*, ROSE and FINK 1987; SCHILD, ANANTHASWAMY and MORTIMER 1981; THOMAS and BOTSTEIN 1986; BAUM, FURLONG and BYERS 1986; BAUM, GOETSCH and BYERS 1986). 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 continued our genetic analysis of the *TUB1* gene. We report here the isolation of 70 conditional-lethal alleles of the *TUB1* gene, using a technique called the "plasmid shuffle" that relies on replacement of one plasmid by another (BOEKE *et al.* 1987). We report on fine structure mapping of these mutations and on detailed studies of the phenotypes

that they display. We also report on suppression analysis of two *TUB1* alleles.

MATERIALS AND METHODS

Strains and media: 5-Fluoroorotic acid (5-FOA) was purchased from PCR Inc. It was used to select for yeast strains without a functional *URA3* gene (BOEKE, LACROUTE and FINK 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. Strains carrying all of the characterized mutations are available upon request.

Plasmid constructions: The plasmid pRB306, a pBR322 derivative carrying the *TUB1* gene, has been described previously (SCHATZ *et al.* 1986). The integrating plasmid pRB337, which was used in attempts to isolate *TUB1* mutations, consisted of the 1.9-kilobase (kb) *Bgl*II fragment from pRB306 inserted into the *Bam*HI site of YIp5 (BOTSTEIN *et al.* 1979). The *Bgl*II fragment contained all of the *TUB1* coding sequences except the first exon of 9 codons and about ¼ 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 *Sph*I to *Bgl*II fragment, containing the entire *TUB1* gene, inserted between the *Sph*I and *Bam*HI sites of YCp402. YCp402 is a replication-competent yeast centromere vector containing the yeast genes *CEN4, ARS1* and *LEU2* (MA *et al.* 1987). pRB539 was constructed using the method described by MA *et al.* (1987) by *in vivo* recombination between the *TUB1-URA3-CEN4-ARS1* plasmid pRB326 (SCHATZ, SOLOMON and BOTSTEIN 1986) and a *LEU2*-containing fragment of pRB327 (SCHATZ, SOLOMON and BOTSTEIN 1986).

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 (SCHATZ, SOLOMON and BOTSTEIN, 1986).

***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 unit/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 β-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 unit/μl), and T4 DNA ligase (2.5 units/μl), in 60 mM Tris (pH 8.0), 20 mM β-mercaptoethanol, 1 mM Mg-acetate, 2 mM $MnCl_2$, 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 *Escherichia 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*−, *leuB*6, *pyrF74*::*Tn*5, *proA*2, *recA*13, *hsdS*20 (*r*⁻, *m*⁻), *ara*-14, *lacY*1, *galK*2, *rpsL*20 (*Sm*ʳ), *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. DB6507 from four independently mutagenized pools of pRB337 yielded an average of 1.5% Ura⁻ colonies (20/1356). To check for loss of *LEU2* function on plasmid pRB539, we used strain HB101 (*F*⁻, *leuB*6, *proA*2, *recA*13, *hsdS*20 (*r*⁻, *m*⁻), *ara*-14, *lacY*1, *galK*2, *rpsL*20 (*Sm*ʳ),*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 four misincorporation reaction mixtures. The level of mutagenesis was 5.8% (29/501).

The four gap-filling reaction mixtures were divided into eight 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.

Hydroxylamine mutagenesis of pRB539 was carried out using the method described by ROSE and FINK (1987).

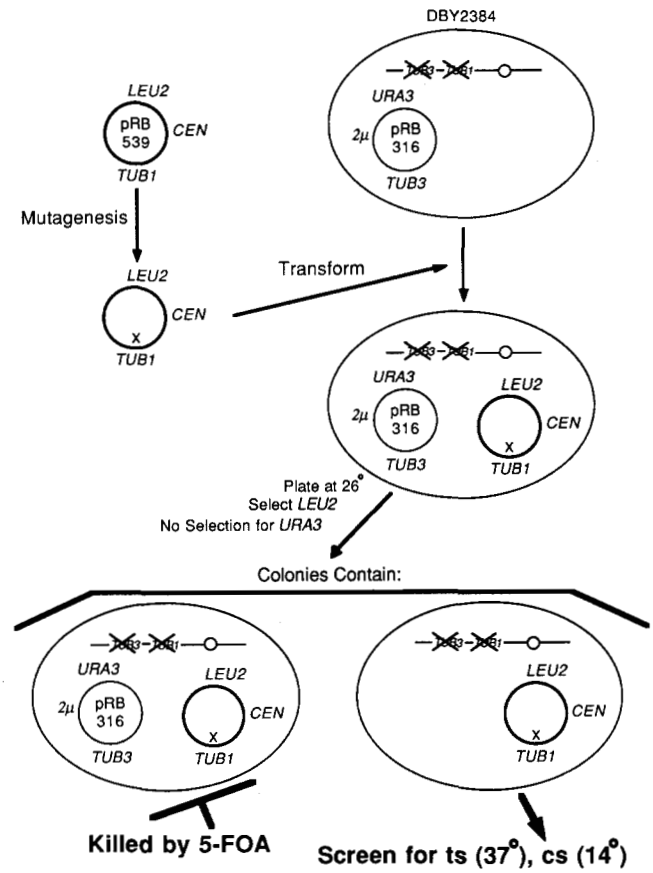

FIGURE 1.—"Plasmid shuffle" strategy for isolating *TUB1* mutations. The starting strain, DBY2384, contained deletions of both *TUB1* and *TUB3* on the chromosome and contained *TUB3* on the plasmid pRB316. It was transformed to Leu⁺ with the *TUB1* plasmid pRB539, that had been mutagenized *in vitro*. 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.

Aliquots of 0.35 g of hydroxylamine hydrochloride and 0.09 g of NaOH were dissolved in water to make 5 ml of solution. An aliquot of 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 level of mutagenesis in pool 9, determined as described above, was 2% (17/861).

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).

"Plasmid shuffle" mutant isolation: The nine pools of mutagenized plasmid pRB539, eight from misincorporation mutagenesis and one 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. Aliquots of 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 fourfold. 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 14° and/or 37°, 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°. Of these strains, 160 were chosen that failed to grow on 5-FOA plates at any temperature. These strains, carrying presumptive *TUB1 nonconditional* 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°, but not at 14° and/or 37° on 5-FOA plates, and (2) grow at all temperatures on control plates. The two mutations that resulted in both temperature-sensitivity (*i.e.*, warm-sensitive, called Ts) and cold-sensitivity (Cs) were isolated in these initial experiments along with several others that were only Cs.

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 *TUB1-LEU2* plasmids were isolated from all of these strains by transformation of DNA into *E. coli*, and their structures were 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.

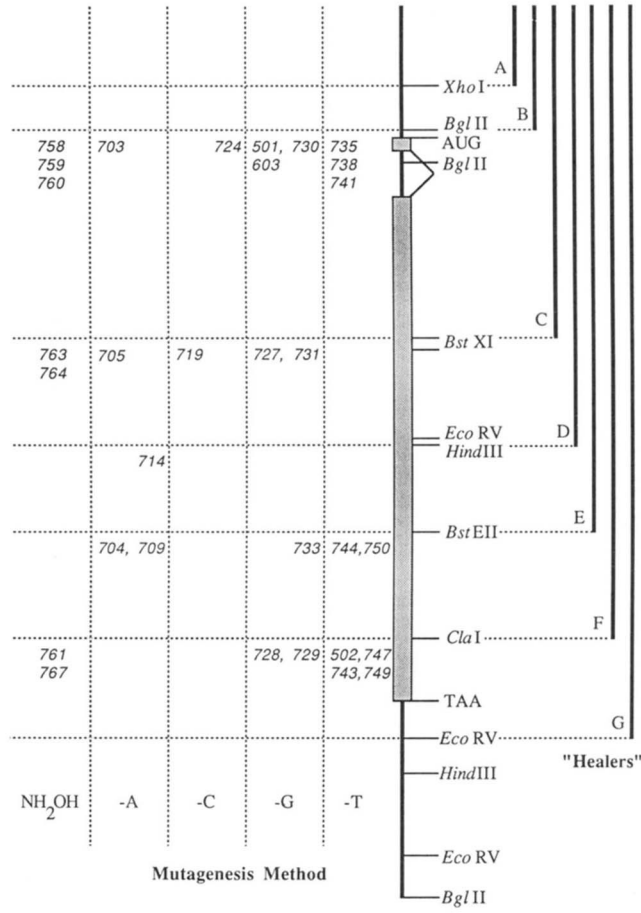

FIGURE 2.—Results for fine structure mapping of *TUB1* mutations. The central line represents the *TUB1* gene, with several restriction sites indicated to the right. The wide shaded region represents the coding sequence of *TUB1*. The left side of the Figure shows the *TUB1* mutations, tabulated according 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 *Xho*I to *Kpn*I fragment. Healer B was a 2.05-kb *Bgl*II to *Kpn*I fragment. Healer C was a 3.1-kb *Bst*XI fragment. Healer D was a 2.85-kb *Hin*dIII to *Kpn*I fragment. Healer E was a 2.9-kb *Bst*EII fragment. Healer F was a 3.25-kb *Cla*I fragment. Healer G was a 3.65-kb *Eco*RV to *Kpn*I partial digestion fragment.

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 *Sph*I, which cut at a single site 1 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 (Figure 2). These fragments overlapped the *Sph*I site of the plasmid and extended into the *TUB1* coding region to different extents. Each of the cut mutant plasmids was 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, BOTSTEIN and FOX 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 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.

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. Only the *tub1-704*, *tub1-705*, and *tub1-719* mutants exhibited growth rates at permissive temperature that were significantly slower than that of the wild type at the same temperature. 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 2–12 hr. After fixation, the cells were centrifuged at 2000 rpm 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 hours. 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 β-mercaptoethanol, 0.1 M KPO_4 (pH 7.5) for 30 min at 30°. The cells were centrifuged at 2000 rpm 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, WRIGHT and MILSTEIN 1982) diluted 1/250 in PBS/BSA. It was used for 1 h at room temperature followed by four washes with PBS. The secondary antibody was fluorescein-labeled goat-anti-rat-IgG (Cappel) diluted 1/100 in PBS. Incubation was for 1 hr at room temperature followed by four 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 four 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 63× 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.

All other methods were as previously described (SCHATZ *et al.* 1986, 1987; SCHATZ, SOLOMON and BOTSTEIN 1986).

RESULTS

Isolation of α-tubulin mutants: In order to explore the functions of α-tubulin in the yeast *S. 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 (SCHATZ, SOLOMON and BOTSTEIN 1986). Because of this functional similarity between the two proteins, we isolated mutants of the more strongly expressed *TUB1* gene in a strain without 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, NOVICK and BOTSTEIN (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, SZOSTAK and ROTHSTEIN 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, NOVICK and BOTSTEIN 1984).

After screening 40,000 colonies of strain DBY2375 transformed with mutagenized plasmid pRB337, we picked 46 conditional 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* mutants. They probably arose during growth of the strain or during the transformation procedure. Such an outcome of this method has been observed previously (SHORTLE, NOVICK and BOTSTEIN 1984), although the reason is not understood. Therefore, 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.* 1987; see Figure 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 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. We isolated 70 conditional-lethal alleles of *TUB1*. These experiments revealed a definite bias in the ability of *TUB1* to mutate to temperature (warm)- *vs.* cold-sensitivity: 67 of the alleles resulted in a Cs phenotype, 1 resulted in a Ts phenotype, and 2 displayed Ts/Cs phenotypes. 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 strain 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 mutations were mapped as described in MATERIALS AND METHODS; the results are shown in Figure 2. Most (31 of 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 of 38) produced phenotypes so leaky that they proved impossible to map.

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 within the resolution of the mapping procedure, the same mutation caused both phenotypes.

Characterization of *TUB1* mutants: Previous results have suggested that tubulin mutants are defective in nuclear division and in nuclear migration to the neck between the mother and bud (THOMAS, NEFF and BOTSTEIN 1985; STEARNS and BOTSTEIN 1988; HUFFAKER, THOMAS and BOTSTEIN 1988). 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 first determined the optimum permissive growth temperature of the strains by comparing their growth at 20°, 26° and 30° to that of a similar strain containing wild-type *TUB1* on the *CEN* plasmid. Compared to the wild type, all of the Cs strains grew most quickly at the warmest temperature (30°); 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

TABLE 2

Properties of the TUB1 mutants and wild-type (WT) controls

TUB1 allele	Growth on benomyl	Microtubule and nuclear phenotype	Plasmid No.
WT (11°)	++++	Normal	539
Class 1:			
704	+	Very few or no MTs, enormous cells with large buds, single nucleus in random location with respect to the neck, nuclei sometimes disorganized	604
709	+		609
724	+		624
728	+		628
729	+		629
738	+		638
744	+		644
750	+		650
759	+		659
760	+		660
767	+		667
Class 2:			
714	+	Many extra MTs, especially cytoplasmic ones, enormous cells with large buds, single nucleus usually in or near neck, nuclei sometimes disorganized	614
730	+		630
733	++		633
741	+		641
758	+		658
Class 3:			
703	++	Disorganized MTs, large cells, disorganized nuclei	603
705	+		605
735	++		635
764	++++		664
713	+	Fewer disorganized MTs, large cells, disorganized nuclei	613
717	+		617
731	+		631
743	+		643
749	+		649
763	+		663
719	++	More disorganized MTs, large cells, disorganized nuclei	619
742	+++		642
716	+	Fairly normal MTs, some MTs and nuclei disorganized	616
723	+		623
727	+		627
747	+		647
737	++++	Large budded cells with bright, medium length intranuclear spindle, normal cytoplasmic MTs	637
746	++		646
761	++++	ND	661
Ts and Ts/Cs:			
WT (37°)	++++	Normal	539
603 (37°)	+	Disorganized MTs, large cells, disorganized nuclei	598
WT (11°)	++++	Normal	539
501 (11°)	+	Very few MTs, large cells, disorganized nuclei	594
501 (37°)	+	Disorganized MTs, large cells, disorganized nuclei	594
502 (11°)	+	Fairly normal, some cells with less MTs	595
502 (37°)	+	Fewer disorganized MTs, large cells, disorganized nuclei	595

Growth of the mutants at permissive temperature was scored at various concentrations of benomyl on a scale of + to ++++. The microtubule (MT) 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°. The plasmid numbers refer to pRB numbers of the plasmids containing the TUB1 mutations.

permissive temperature. These mutants included both of the Ts/Cs mutants (501 and 502), the Ts mutant (603), and 35 of the Cs mutants. Most of these mutants grew at about the same rate as the wild type in liquid medium at permissive temperature.

Sensitivity to benomyl: Hypersensitivity to benzim-

TABLE 3

Properties of the TUB1 mutants and wild-type (WT) controls

TUB1 allele	Cell count increase at nonpermissive temperature	Half-life (hr) at nonpermissive temperature	Cell division cycle position			
			Unbudded	Small bud	Large bud	Multibud
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
735	1.5	12	16	5	64	15
764	2.5	14	21	6	70	3
713	2.5	ND	21	9	51	19
717	3.0	ND	26	7	49	18
731	2.1	8.0	19	10	53	18
743	3.0	16	18	12	60	10
749	4.5	24	14	9	70	7
763	3.0	24	24	8	56	12
719	5.1	ND	31	16	45	8
742	7.0	Poor growth	34	17	39	10
716	8.0	ND	22	12	55	11
723	4.5	13	30	17	45	8
727	3.5	15	23	16	52	9
747	4.4	39	29	17	49	5
737	3.9	15	11	9	76	4
746	3.0	13	13	11	71	5
761	ND	ND	ND	ND	ND	ND
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

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. ND = not determined.

idazole antimicrotubule compounds is a common phenotype of both α- and β-tubulin mutants in a variety of species (SCHATZ, SOLOMON and BOTSTEIN 1986; ADACHI *et al.* 1986; HUFFAKER, THOMAS and BOTSTEIN 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 (SHEIR-NEISS, LAI AND MORRIS 1978; THOMAS, NEFF and BOTSTEIN 1985; UMESONO *et al.* 1983). Because our *TUB1* mutants were isolated only on the basis of temperature conditional lethality,

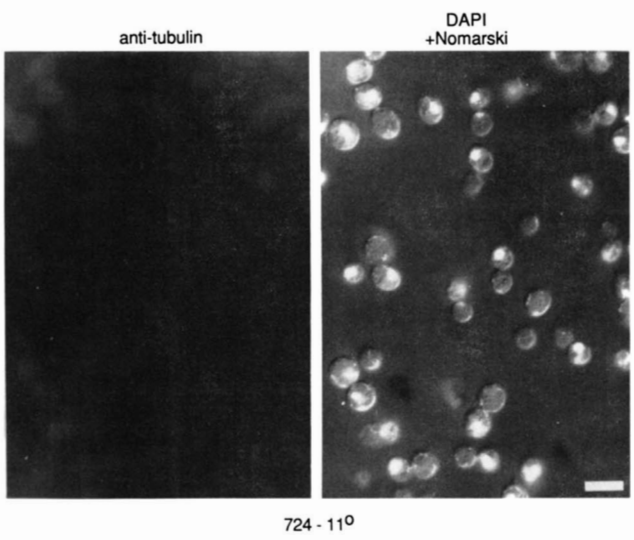

FIGURE 3.—Microscopic analysis of the *tub1-724* mutant. The left panel shows anti-tubulin immunofluorescence analysis of a field of *tub1-724* mutant cells after two generations (24 hr) at the nonpermissive temperature (11°). The right panel shows the same cells viewed with Nomarski optics in addition to DAPI epifluorescence (to view DNA). In this panel, the white areas correspond to the DAPI staining regions. The bar represents 10 μm.

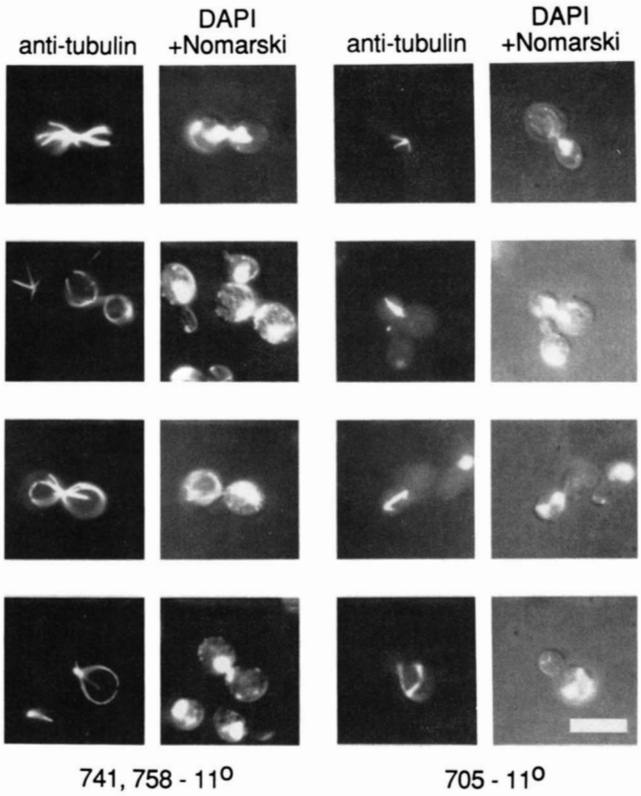

FIGURE 4.—Microscopic analysis of *TUB1* mutants. The left panel of each pair shows anti-tubulin immunofluorescence and the right panel shows a combination of Nomarski optics and DAPI epifluorescence. (*Left*) Images of the *tub1-741* and *tub1-758* mutants after 2 generations (24 hr) at the nonpermissive temperature (11°). The first and third pairs from the top are photographs of the *tub1-741* mutant and the other are of the *tub1-758* mutant. (*Right*) Similar images of the *tub1-705* mutant. The bar represents 10 μm.

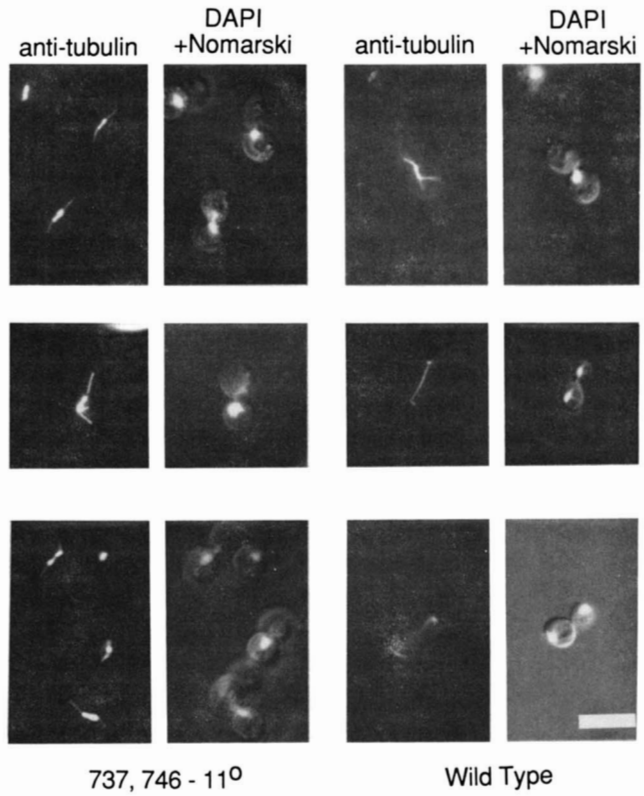

FIGURE 5.—Microscopic analysis of *TUB1* mutants. The left panel of each pair shows anti-tubulin immunofluorescence and the right panel shows a combination of Nomarski optics and DAPI epifluorescence. (*Left*) Images of the *tub1-737* and *tub1-746* mutants after 2 generations (24 hr) at the nonpermissive temperature (11°). The top photograph is of the *tub1-737* mutant and the bottom two are of the *tub1-746* mutant. (*Right*) Similar images of the wild-type control for comparison. The bar represents 10 μm.

we were interested in determining the generality of the drug sensitivity phenotype. As shown in Table 2, 35 of the 38 mutants exhibited hypersensitivity to the benzimidazole benomyl when grown on a series of different concentrations of the drug from 5 μg/ml to 35 μg/ml. The other three mutants showed the same resistance to the drug as the wild-type control. None of the mutants was more resistant than the control. Thus, benomyl hypersensitivity is a nearly universal phenotype of our conditional-lethal α-tubulin mutants.

Analysis of mutants at nonpermissive temperature: We grew the mutants to a density of 10^6 cells/ml at permissive temperature in liquid medium, shifted them to nonpermissive temperature, and observed several phenotypes over a time course: (1) To determine the rates 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 3 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 3 shows the number of cells

TABLE 4

Properties of *TUB2* suppressors of *TUB1* alleles

TUB2 allele	Suppressed *TUB1* allele	Phenotype of suppressor in the presence of:		
		TUB1 and *TUB3*	*tub1-737* or *tub1-746*	*TUB3* only
tub2-551	*tub1-737*	Ts^+, Cs^-, Ben^r	Ts^+, Cs^+, Ben^r	Ts^-, Cs^-, Ben^r
tub2-552	*tub1-737*	Ts^+, Cs^-, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^-, Cs^-, Ben^{hs}
tub2-553	*tub1-737*	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^-, Ben^{hs}
tub2-554	*tub1-737*	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}
tub2-555	*tub1-737*	Ts^+, Cs^-, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^-, Ben^{hs}
tub2-556	*tub1-737*	Ts^+, Cs^-, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^-, Cs^-, Ben^{hs}
tub2-557	*tub1-737*	Inviable	Ts^+, Cs^+, Ben^{hs}	Inviable
tub2-558,559	*tub1-737*	Very sick, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Very sick, Ben^{hs}
tub2-560	*tub1-737*	Ts^-, Cs^-, Ben^r	Ts^+, Cs^+, Ben^r	Ts^-, Cs^-, Ben^r
tub2-561	*tub1-737*	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Very sick, Ben^{hs}
tub2-562	*tub1-746*	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}	Ts^+, Cs^+, Ben^{hs}

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 3, 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. Table 2 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 3, 4 and 5.

The mutants shown in Tables 2 and 3 can be grouped according to the morphologies of their microtubules after 2 generations at nonpermissive temperature. Two classes of the mutants present clear-cut phenotypes. Class 1, represented by the *tub1-724* mutant, had few or no microtubular structures (Figure 3). 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 (Figure 4). 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. The remaining mutants (class 3) displayed several different aberrant microtubule morphologies. Those morphologies are described in Table 2, and examples of some are shown in Figure 4.

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 3). Six of the mutants accumulated more than 85% large-budded cells. By this criterion, *TUB1* qualifies as a *CDC* gene as defined by HARTWELL, CULOTTI and REID (1970). 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. That several of the mutant cultures contained an excess of multi-budded cells with single nuclei supports this hypothesis (Table 3).

Mutants in each of these classes have other properties in common as well. The mutants with gross defects in microtubule assembly (class 1 with no microtubules and class 2 with many) showed tighter 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 than class 3 mutants (2.1-fold *vs.* 3.8-fold). Thus mutants with the strongest microtubule assembly defects had the tightest growth and cell cycle phenotypes. In general, the class 1 mutants lost viability more quickly than the other groups (11 hr half-life *vs.* 18 hr), 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 4, which contains several photographs of the *tub1-705* mutant. The top set of pictures shows a cell with incorrectly oriented 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 daugh-

ter 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 unique step necessary for progression of the cell cycle.

Of all of the mutants, only two, containing *tub1-737* and *tub1-746*, had a spindle that looked normal at arrest. Both arrested with medium-length intranuclear spindles and a normal complement of cytoplasmic microtubules (Figure 5). The intranuclear spindles appeared to be as bright in the center as the wild type, but they were much brighter at the ends. 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.

Suppressor analysis of *tub1-737* and *tub1-746*: To identify genes whose products interact with α-tubulin, we isolated suppressors of two of the α-tubulin Cs^- mutations. The alleles *tub1-737* and *tub1-746* were chosen because of the apparent defect in a specific stage of spindle function. Strains carrying the mutations, DBY2416 and DBY2422, were plated for single colonies at 30° on YPD. After three days of growth, 25 colonies of each strain were resuspended in water and spread on each of 25 YPD plates. After incubation at 11° for 6 weeks, revertants were colony purified at 26° and retested for growth at 11°.

Strains that retested were crossed to DBY2383 to create strains that carried the *tub1-LEU2-CEN4-ARS1* plasmid from the revertant strain, the *TUB3-URA3-2μ* plasmid from DBY2383, and no chromosomal copies of α-tubulin genes. To characterize the nature of the reversion events, we dissected and analyzed tetrads from these diploids. Because both plasmids were maintained at fairly high copy number in these strains, most of the progeny of these crosses contained both the *TUB1* and *TUB3* plasmids. Since the *TUB1* mutations are suppressed by *TUB3*, we detected the presence of suppression of the original Cs^- phenotype in progeny grown on 5-FOA to select for loss of the *TUB3-URA3* plasmid. Intragenic reversion events were indicated by crosses in which suppression of cold-sensitivity was linked to the *tub1-LEU2* plasmid. In the case of both alleles, several intragenic reversion events were revealed by these crosses (*i.e.*, all of the progeny were Cs^+). These strains were not studied further.

Extragenic suppressors were indicated by crosses in which suppression of cold-sensitivity segregated 2:2, as expected for a chromosomal mutation. Such extragenic suppressor strains were examined for additional phenotypes including conditional lethality and altered sensitivity to benomyl. Almost all of the strong suppressors cosegregated with an altered sensitivity to benomyl, two with benomyl resistance (Ben^r) and ten with benomyl hypersensitivity (Ben^{hs}). None of the other suppressors caused a detectable conditional phenotype. The benomyl phenotypes were used in subsequent crosses to detect the presence of the suppressor mutations. Suppressors that did not have additional phenotypes were not studied further.

In addition to altered sensitivity to benomyl, many of the suppressors of *tub1-737* had Cs^- and Ts^- phenotypes of their own when they were separated from the original *tub1-737* allele. These phenotypes could be observed in the progeny of the crosses described above that inherited only the *TUB3-URA3* plasmid. Both mutations caused conditional lethality on their own. When together, however, each mutation suppressed the conditional lethality caused by the other. In the case of one suppressor (#557), this mutual suppression was necessary to the viability of strains containing the suppressor mutation. Strains that contained this suppressor without *tub1-737* were inviable.

To confirm that the Ben^r and Ben^{hs} mutations would suppress the starting *tub1-737* and *tub1-746* alleles, we crossed strains of genotype *sup, TUB3-URA3-2μ, tub1::HIS3, tub3::TRP1* to the starting strains DBY2416 and DBY2422. The resulting diploids were grown overnight on 5-FOA plates to select for loss of the *TUB3-URA3-2μ* plasmid, leaving the *tub1-LEU2-CEN4* plasmid as the only source of functional α-tubulin. The diploids were then sporulated and progeny tetrads were scored for suppression. In all of these crosses, suppression cosegregated with the altered benomyl sensitivity phenotypes. For the case of #557, this test was impossible to perform because strains of the appropriate starting genotype were inviable.

For three reasons, we suspected that some of the suppressors might be alleles of the *TUB2 β-tubulin gene (Neff *et al.* 1983). First, α- and β-tubulin associate very tightly to form a heterodimer; second, previously isolated alleles of *TUB2* have exhibited Ben^r and Ben^{hs} phenotypes (Huffaker, Thomas and Botstein 1988; Thomas, Neff and Botstein 1985); third, suppression of β-tubulin alleles by α-tubulin alleles has been previously observed in *Aspergillus nidulans* (Morris, Lai and Oakley 1979). We first crossed the suppressor-carrying strains to DBY1828 or DBY1829 to produce progeny with wild-type chromosomal α-tubulin genes. These strains were then crossed to one of a series of strains (*e.g.*, DBY4976) marked by the integration of a plasmid (pRB151) at the *ACT1* locus, which is closely linked to the *TUB2* locus (Shortle, Novick and Botstein 1984; pRB151 was mistakenly referred to as pRB147 in this paper). In every case, the Ben^r or Ben^{hs} phenotype was tightly linked to the *URA3* marker on the plasmid.

We concluded that the suppressors were all tightly linked to *TUB2*.

To demonstrate conclusively that the suppressors were alleles of *TUB2*, we carried out complementation tests with known alleles of *TUB2*. The Ben^r suppressors were tested for complementation of the recessive Ben^r alleles *tub2-104* and *tub2-402* (strains DBY1384, 1385, 2304, 2303); the Ben^{hs} suppressors were tested for complementation of the recessive Ben^{hs} alleles *tub2-403* and *tub2-405* (strains DBY2305, 2306, 2309, 2310). The strains carrying the suppressors (*e.g.*, DBY4982) were crossed to the tester strains and to wild-type controls (DBY1034 and 1035) and then tested for growth on a variety of different concentrations of benomyl. In all cases the suppressor mutations were recessive and failed to complement the benomyl phenotypes of the tester strains. We conclude that they are all alleles of *TUB2*.

The suppressors were given allele designations of *tub2-551* through *tub2-562*. The phenotypes of these suppressors are listed in Table 4, according to the α-tubulin genotype of the cell. In all cases the benomyl phenotypes persisted independent of the α-tubulin genotype. All of the Ts or Cs phenotypes of the suppressors, however, disappeared when the cell contained only the *tub1* mutant allele. The Ts and Cs phenotypes did vary in some cases depending on whether the cell contained only wild-type *TUB3* plasmid or both wild-type *TUB1* and *TUB3* plasmid. For example, *tub2-552* caused both Ts$^-$ and Cs$^-$ phenotypes in the presence of only *TUB3* plasmid, but caused only a Cs$^-$ phenotype in the presence of *TUB1* and *TUB3* plasmid. Whether this difference is due to quantitative or qualitative differences remains to be determined.

DISCUSSION

We have isolated 70 conditional-lethal mutations in the *TUB1* α-tubulin gene of the yeast *S. 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 Cs *vs.* Ts phenotypes. 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 with uniform morphology) (HARTWELL, CULOTTI and REID 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, TODA and YANAGIDA 1984; HUFFAKER, THOMAS and BOTSTEIN 1988; PRINGLE *et al.* 1986; QUINLAN, POGSON and GULL 1980; ROY and FANTES 1983; STEARNS and BOTSTEIN 1988; THOMAS, NEFF and BOTSTEIN 1985; TODA *et al.* 1983, 1984; 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, THOMAS and BOTSTEIN 1988). Nuclear movement to the bud neck in tubulin mutants has been correlated with the pre sence of cytoplasmic microtubules (HUFFAKER, THOMAS and BOTSTEIN 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, NEFF and BOTSTEIN 1985; T. HUFFAKER, personal communication; our unpublished results). This mutant retains an intranuclear spindle at nonpermissive temperature while losing almost all cytoplasmic microtubules (HUFFAKER, THOMAS and BOTSTEIN 1988). The persistence of an intranuclear spindle may be important in preserving the viability of yeast cells blocked in spindle elongation.

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.

Significance of benomyl hypersensitivity: Benomyl is a member of a set of compounds called benzim-

idazoles, that cause fairly specific defects in microtubule assembly (DAVIDSE and FLACH 1977; OAKLEY and MORRIS 1980; SHEIR-NEISS, LAI and MORRIS 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, NEFF and BOTSTEIN 1985). Of 38 *TUB1* α-tubulin mutants that we tested, 35 were hypersensitive to benomyl and none was resistant. Such hypersensitivity is a common phenotype of both α- and β-tubulin mutants in a variety of species (SCHATZ, SOLOMON and BOTSTEIN 1986; ADACHI *et al.* 1986; HUFFAKER, THOMAS and BOTSTEIN 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 (SHEIR-NEISS, LAI and MORRIS 1978; THOMAS, NEFF and BOTSTEIN 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 five that were examined by HUFFAKER, THOMAS and BOTSTEIN (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 functions in the interaction between the tubulin dimer 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, T. STEARNS and D. BOTSTEIN, personal communication). Thus, benomyl hypersensitivity may be a very useful criterion for identifying genes involved in many aspects of microtubule function in yeast.

Suppressor analysis of two alleles of *TUB1*: 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, HOYT and BOTSTEIN 1987). 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 have used this approach to isolate extragenic suppressors of *tub1-737* and *tub1-746*, two alleles that cause the accumulation of fairly normal spindles at the nonpermissive temperature. Most of the revertants characterized contained extragenic suppressors, many of which resulted in altered benomyl sensitivity in addition to suppression. When separated from the original *tub1* allele, many also resulted in temperature conditional lethality. In addition, the phenotype of suppressor strains varied depending on whether they contained wild-type *TUB3* plasmid only, or both *TUB3* plasmid and *TUB1*. Clearly these properties indicate the presence of a strong interaction between the product of the suppressor gene and α-tubulin.

As revealed by both linkage and complementation tests, all of the strong suppressor mutations occurred in the *TUB2* β-tubulin gene. Considering the strong interaction between α- and β-tubulin, we would expect that the *TUB2* locus would be a major source of suppressors of *TUB1* mutations. Suppression of β-tubulin mutations by α-tubulin mutations has been demonstrated previously in *Aspergillus nidulans* (MORRIS, LAI and OAKLEY 1979). It remains to be determined whether other genes will yield suppressors of *TUB1* alleles. Of the 70 alleles that we isolated, only 2 have been examined by this approach. Although these two alleles exhibited very similar phenotypes (Figure 5), they yielded different spectra of revertants. Most of the *tub1-737* reversions were caused by *TUB2* suppressors. In contrast, most of the *tub1-746* reversions were caused by intragenic events; only one weak *TUB2* suppressor was identified. Given the diverse phenotypes caused by the 70 alleles of *TUB1* that we have described, it is likely that further suppression analysis will identify new genes involved in microtubule function in yeast. Such analysis is currently in progress.

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, NOVICK and BOTSTEIN 1984). As described above, this approach did not yield mutations in the *TUB1* gene, although clearly it has been successfully applied to other genes (*e.g.*, HOLM *et al.* 1985). In contrast, a plasmid replacement method, commonly called the "plasmid shuffle" (BOEKE *et al.* 1987), yielded 70 authentic *TUB1* conditional-lethal mutants.

There are several possible explanations for the different success rates with the two methods. In our experiments, 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 theoretical problems with the integrating plasmid strategy. Because the method relies on recombination between plasmid-borne and chromosomal copies of the gene, the recombination event produces a bias towards mutagenesis of one end of the gene. Reconstruction experiments with known mutations 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). Finally, 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 rely on recombination between mutagenized and unmutagenized copies of the gene, so there is no bias towards one end of the gene. As used here, the method also had a built-in suppression test to distinguish *TUB1* mutations from random chromosomal mutations 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.

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