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

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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

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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 constructions 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 *Bam*HI 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

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.

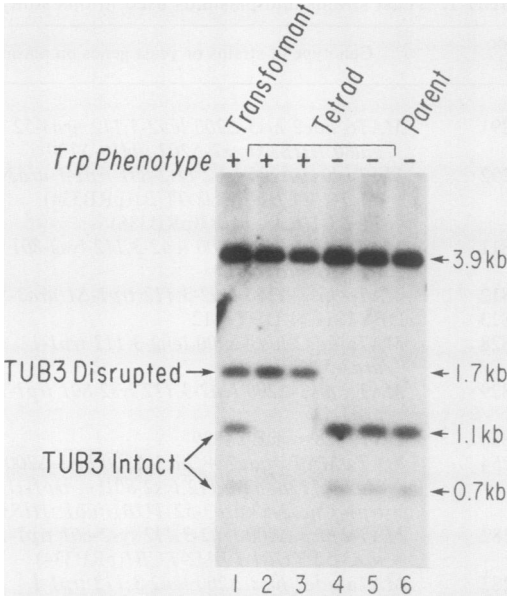

FIG. 1. Gel transfer hybridization of total yeast DNA from strains transformed with the *tub3*::*TRP1* insertion-deletion construction. Total yeast DNA was purified from a diploid transformant (lane 1), from four progeny spores from the diploid (lanes 2 through 5), and from the untransformed parent diploid (lane 6). The DNA was digested with *Bst*EII, 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 *Bgl*II 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.

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 *Bgl*II fragment in the *GAL80* gene with a *Bam*HI 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

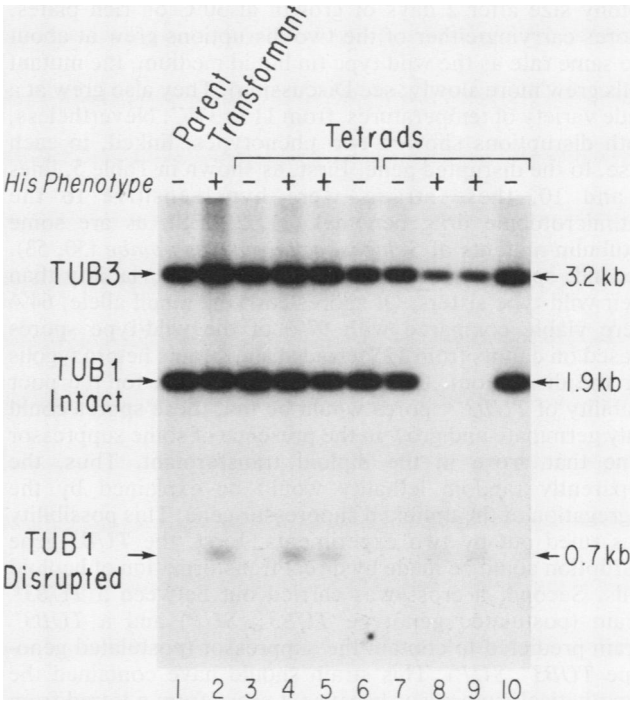

FIG. 2. Gel transfer hybridization of total yeast DNA from strains transformed with the *tub1*::*HIS3* insertion-deletion construction. Total yeast DNA was purified from the untransformed parent diploid (lane 1), from a diploid transformant (lane 2), and from four progeny spores from each of two tetrads 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* gene product in microtubules (35) show that the *TUB3* gene is expressed.

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.

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

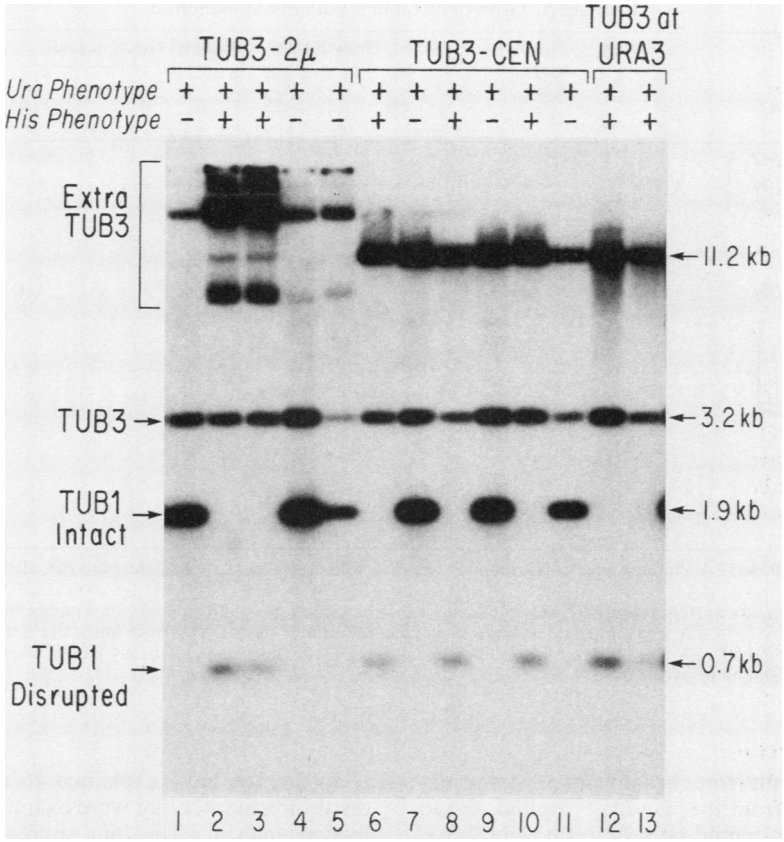

FIG. 3. Gel transfer hybridization of total yeast DNA from strains heterozygous for the *tub1*::*HIS3* insertion-deletion construction transformed with plasmids containing extra copies of *TUB3*. Total yeast DNA was purified from four spores of a tetrad from the diploid transformed with a plasmid (pRB316) 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×	+++	++	+	+/−	−	−	−	−
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) containing *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 *Bgl*II 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 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.

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