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Regulation of Tubulin Levels and Microtubule Assembly in *Saccharomyces cerevisiae*: Consequences of Altered Tubulin Gene Copy Number

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Microtubule organization in the cytoplasm is in part a function of the number and length of the assembled polymers. The intracellular concentration of tubulin could specify those parameters. *Saccharomyces cerevisiae* strains constructed with moderately decreased or increased copy numbers of tubulin genes provide an opportunity to study the cellular response to a steady-state change in tubulin concentration. We found no evidence of a mechanism for adjusting tubulin concentrations upward from a deficit, nor did we find a need for such a mechanism: cells with no more than 50% of the wild-type tubulin level were normal with respect to a series of microtubule-dependent properties. Strains with increased copies of both alpha- and beta-tubulin genes, or of alpha-tubulin genes alone, apparently did down regulate their tubulin levels. As a result, they contained greater than normal concentrations of tubulin but much less than predicted from the increase in gene number. Some of this down regulation occurred at the level of protein. These strains were also phenotypically normal. Cells could contain excess alpha-tubulin protein without detectable consequences, but perturbations resulting in excess beta-tubulin genes may have affected microtubule-dependent functions. All of the observed regulation of levels of tubulin can be explained as a response to toxicity associated with excess tubulin proteins, especially if beta-tubulin is much more toxic than alpha-tubulin.

Among the factors thought to regulate microtubule assembly and organization in the cytoplasm is the assembly reaction itself. In contemporary analyses of this reaction, the elements which appear to be crucial are the thermodynamic polarity of the microtubules themselves, the number of microtubules, the presence and number of nucleating sites, and the concentration of tubulin (16, 17). These analyses demonstrate that microtubules probably do not behave according to steady-state models but rather as if their ends are present in two states, growing and shrinking (18, 19). This property, taken together with the placement of nucleating centers, can explain not only quantitative but also qualitative elements of microtubule organization. The notion that total tubulin levels are important to this mechanism is supported by in vitro experiments (5), by model building (20), and by demonstrations that cells appear to have mechanisms for regulating tubulin levels in response to changes in microtubule assembly (2, 7). Other factors are likely to be involved as well. For example, some changes in cell shape which depend upon assembled microtubules do not require de novo protein synthesis (30).

Testing the role of tubulin levels in vivo has generally involved the application of antimicrotubule drugs to increase or decrease the levels of unassembled subunits, but the effects of these drugs are extreme compared with the situations normally encountered by resting or proliferating cells. In addition, microtubule depolymerization can have secondary consequences (12, 13, 32). An alternative experimental approach would be to change the cellular level of tubulin by altering the complement of tubulin genes. These sorts of manipulations are not technically feasible in the cells of metazoa. Those organisms typically contain many genes for both alpha- and beta-tubulin (6), and gene disruption by homologous integration can be accomplished only with very low efficiency. In contrast, the yeast *Saccharomyces cerevisiae* is ideally suited for analyses of this sort. *S. cerevisiae* has only a single beta-tubulin gene (*TUB2*) and two alpha-tubulin genes (*TUB1* and *TUB3*), and site-directed alterations in the chromosomal copies of these genes are easily and efficiently made. Analyses of phenotypes involving microtubule structure and function are further simplified because in yeast cells the microtubule organelles are relatively simple and well defined throughout the cell cycle (24).

We have increased and decreased the number of copies of alpha- and beta-tubulin genes in *S. cerevisiae*. In strains with extra copies of tubulin genes, the levels of the tubulin polypeptides are reduced to close to the levels of wild-type strains. In contrast, there is no compensatory overexpression to restore wild-type tubulin levels in response to a decrease in the number of beta-tubulin genes. The generation of phenotypically normal strains with decreased levels of tubulin demonstrates that yeast cells can assemble normal microtubule arrays from substantially reduced pools of tubulin heterodimer, indicating the existence of nontubulin factor(s) controlling the extent of microtubule assembly in vivo. Our results, and those in the accompanying paper (34), also indicate that the consequences of relative excesses of alpha- and beta-tubulin differ: a modest excess of alpha-tubulin over beta-tubulin has no phenotypic effect, but the presence of beta-tubulin in excess of alpha-tubulin cannot be tolerated.

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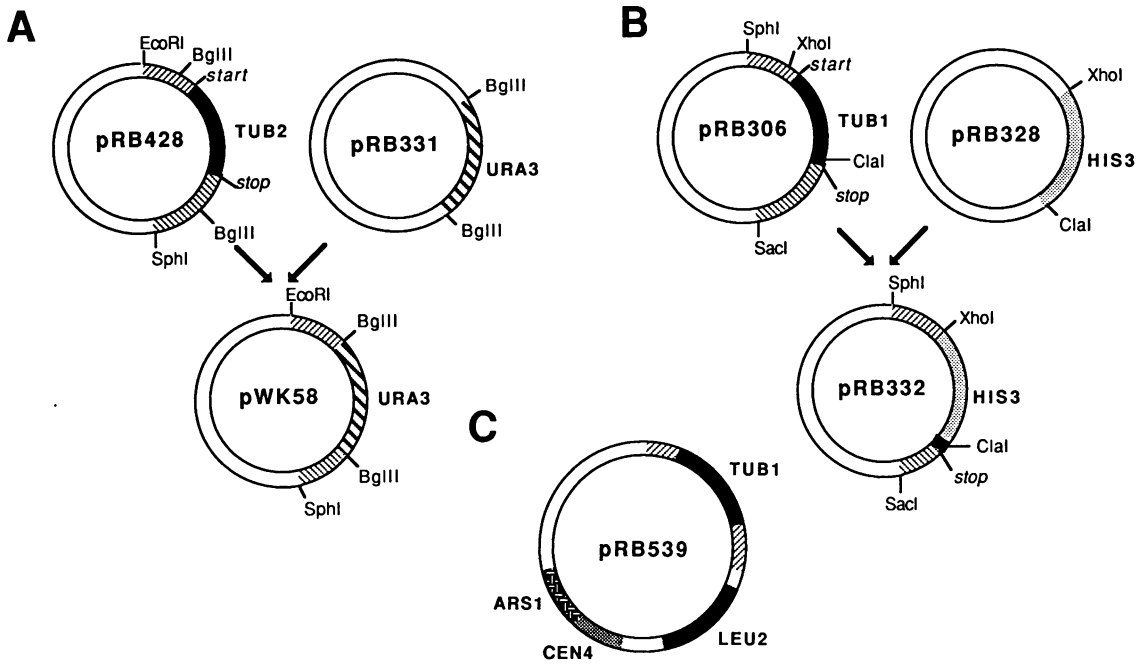

FIG. 1. Construction of vectors for preparing *tub2*::*URA3* (A) and *tub1*::*HIS3* (B) transplacement fragments and for introducing extrachromosomal *TUB1* in low copy number (C). Restriction sites within the coding regions are omitted for clarity. Symbols: ■, *TUB1* or *TUB2* coding sequence; ▨, *TUB1* or *TUB2* flanking sequence; ▭, *URA3* gene; ▩, *HIS3* gene; ▩, *LEU2* gene; *ARS1* and *CEN4* sequences are as indicated; □, irrelevant plasmid sequences. (A) Construction of pWK58; (B) construction of pRB332; (C) structure of pRB539.

MATERIALS AND METHODS

Plasmid constructions. DNA manipulations were performed by standard techniques (25). Figure 1 shows the relevant yeast genes and plasmids.

pRB428 (alias pJT71 [33]; Fig. 1A) is a YIp5 derivative containing the *TUB2* gene plus approximately 0.3 kilobase pairs (kb) of 5′ noncoding sequence and 1.3 kb of 3′ noncoding sequence. To delete the *TUB2* coding region, we digested pRB428 with *Bgl*II and isolated the largest fragment. This fragment lacks the entire *TUB2* coding sequence, from 80 base pairs upstream of the translational start to approximately 900 base pairs downstream from the termination codon. To create pWK58, we gel purified this fragment and ligated it to the *URA3* fragment isolated from a *Bgl*II digest of pRB331. The transplacement fragment used to eliminate one genomic copy of *TUB2* was generated by digesting pWK58 with *Eco*RI and *Sph*I.

pRB306 (Fig. 1B) is a pBR322 derivative containing the entire *TUB1* gene as well as approximately 1 kb of 5′ noncoding and 2 kb of 3′ noncoding DNA (27). pRB332 was constructed by digesting pRB306 with *Cla*I and *Xho*I and replacing the 1.4-kb fragment released by this digestion with a 1.3-kb *HIS3 Cla*I-*Xho*I fragment from pRB328. This replaces all but the last 115 base pairs of the *TUB1* coding region with the *HIS3* gene. The transplacement fragment used to eliminate one genomic copy of *TUB1* was generated by digesting pRB332 with *Sac*I and *Sph*I.

The genomic duplication of *TUB2* was generated by transforming cells with pRB428 linearized at a *Kpn*I site within the *TUB2* coding sequence, to direct integration of the plasmid to the chromosomal *TUB2* locus.

pRB539 (Fig. 1C), the vector used to introduce low-copy-copy extrachromosomal *TUB1*, was constructed as described previously (29).

Strains and media. Genotypes of the strains used are listed in Table 1. All culture of strains on either liquid or solid medium was performed with selection for plasmid or integration maintenance. For harvest of protein, RNA, and DNA and for growth curves, strains were grown in synthetic complete medium lacking either uracil, histidine, leucine, or some combination thereof. Synthetic complete medium contained, per liter, 6.7 g of Difco yeast nitrogen base without amino acids and 2 g of a powdered supplement mix contain-

TABLE 1. Yeast strains used

Strain	Genotype
EJL374	*MATa/MATα ade2-101/ade2-101 can1*^{r}*/CAN1*^{s} cyh2^r/*CYH2*^{s} *his4/HIS4 Δleu2/Δleu2 lys2-801/lys2-r2 trp1*::*LEU2/TRP1 ura3-52/ura3-52*
FSY120[a]	*MATa/MATα his4-619/HIS4 leu2-3,112/leu2-3,112 lys2-801/LYS2 ura3-52/ura3-52*
FSY185	*MATa/MATα ade2/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52*
FSY188, FSY189	*MATa/MATα his4-619/HIS4 leu2-3,112/leu2-3,112 lys2-801/LYS2 ura3-52/ura3-52 tub2*::*URA3/TUB2*
FSY190, FSY191	*MATa/MATα ade2/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52 TUB2*::*URA3*::*TUB2/TUB2* pRB539
FSY200	*MATa/MATα his4-619/HIS4 leu2-3,112/leu2-3,112 lys2-801/LYS2 ura3-52/ura3-52 TUB2*::*URA3*::*TUB2/TUB2* pRB539
FSY208, FSY209	*MATa/MATα ade2-101/ade2-101 can1*^{r} *CAN1*^{s} *cyh2*^{r}*/CYH2*^{s}*:his4/HIS4 Δleu2/Δleu2 lys2-801/lys2-r2 trp1*::*LEU2/TRP1 ura3-52/ura3-52 tub2*::*URA3/TUB2*

[a] Construction is described in reference 14.

ing 2 g of each of 18 amino acids (leucine and histidine were omitted) and adenine plus 0.2 g each of *p*-aminobenzoic acid and *myo*-inositol. Glucose was added to 2% as a carbon source after autoclaving of the other constituents for 30 min, to make SCD medium. Uracil, leucine, and histidine were added to 100 mg/liter as required. Plates were prepared by separately autoclaving equal volumes of 3% agar and 2× synthetic complete media for 30 min and mixing after cooling to approximately 55°C. All medium constituents were obtained from Sigma Chemical Co. (St. Louis, Mo.) except for Difco yeast nitrogen base without amino acids and Difco agar, which were obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Other media and procedures were as described by Sherman et al. (31).

Analysis of RNA levels. DNA, RNA, and protein were isolated in parallel from the same culture. For RNA isolation, cells were lysed by vortexing with glass beads in RNA extraction buffer (0.5 M NaCl, 0.2 M Tris hydrochloride [pH 7.6], 0.01 M EDTA, 1% sodium dodecyl sulfate) plus an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The extracts were precipitated, and the pellets were washed twice with 3 M sodium acetate (pH 6). The RNA was run on morpholinepropanesulfonic acid (MOPS)-formaldehyde gels, and Northern (RNA) blot analysis was performed according to standard techniques (25). The probe was a gel-purified 1.6-kb fragment from pRB428, extending from the 5′ *Eco*RI site to an *Eco*RI site within the coding region. The *TUB1* and *TUB3* probes were prepared by digesting pRB306 and pRB300 (27), respectively, with *Bgl*II. The dolichol-phosphate mannose synthase (*DPM1*) probe used for normalization was a 0.6-kb *Nsi*I fragment obtained from P. Orlean (Massachusetts Institute of Technology [MIT]) (22). Probes were labeled with [^{32}P]dATP by the random-priming method (8).

Analysis of protein levels. Preparation of anti-beta-tubulin antibodies, protein harvests, and Western immunoblot analysis were done as described by Katz and Solomon (14). The preparation of antibodies against the TUB1 and TUB3 proteins is described by Schatz et al. (26). The anti-phosphoglycerate kinase antibody was provided by J. Thorner (University of California, Berkeley) (1). The anti-glutamyl-tRNA synthetase (GTS) antibody was provided by P. Schimmel (MIT). Quantitation was as described above. The results were similar whether anti-GTS or anti-phosphoglycerate kinase was used to normalize blots.

Southern blot analysis. Total cellular DNA was isolated from cells harvested from the same culture and at the same time as those taken for protein and RNA analysis. DNA was prepared as previously described (10). DNA was digested with *Bgl*II for analysis of *TUB1* and *TUB3* and with *Eco*RI for analysis of *TUB2*. Digested DNA was run on 1% agarose TAE gels and blotted according to standard techniques (25). Probes were as described above.

Quantitation of autoradiograms. Autoradiograms of Northern blots and of Southern and Western blots were quantitated either by laser densitometry using an Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.; mRNA in Table 2 and DNA in Table 5) or by using a digital BA-100 Bio-Image analyzer (Fujix; protein in Table 2 and Fig. 3).

Growth rate analysis. Cultures were inoculated with cells in logarithmic growth. Samples were taken periodically during growth and counted in duplicate on a hemacytometer. The doubling times shown are the averages from three experiments.

Immunofluorescence. Cells were fixed, permeabilized, and stained as described by Katz and Solomon (14). Cells were photographed on a Zeiss Axioplan, using Hypertech film (Microfluor, Stony Brook, N.Y.).

RESULTS

Strains with reduced tubulin gene dosage. (i) Strains hemizygous for *TUB2* show normal segregation. Figure 1A shows the construction used to delete one copy of the *TUB2* gene in diploids (see Materials and Methods). The diploid strain FSY120 was transformed with the *tub2*::*URA3* transplacement fragment, and transformants were selected by their ability to grow on medium lacking uracil. Neff et al. (21) previously established that *TUB2* is an essential gene by showing that sporulation of strains hemizygous for *TUB2* (having only one copy of the gene in a diploid background) produce two viable and two inviable spores. We obtained the same results from sporulation of two transformants of FSY120, designated FSY188 and FSY189. Of the 32 tetrads, 30 gave two viable spores and two inviable spores. Two tetrads (from separate strains) gave three viable spores and one inviable spore. Of the 52 viable spores tested, 51 were Ura$^-$, indicating that they lacked the *TUB2*-disrupting fragment. The single Ura$^+$ spore came from a tetrad with three viable spores and therefore most likely resulted from a rearrangement or gene conversion.

(ii) Tubulin mRNA levels reflect gene number in *TUB2* hemizygotes, but alpha-tubulin protein levels are reduced. The levels of the *TUB1* and *TUB3* alpha-tubulin and *TUB2* beta-tubulin mRNAs were determined in the *TUB2* hemizygotes and wild-type parental strains. Total cellular RNA was loaded in three twofold increments for Northern blot analysis. The filters were probed with tubulin-specific sequences and then reprobed for the amount of the message encoding dolichol-phosphate mannose synthase (DPM) (22) as a control for loading. We did not anticipate that the DPM message would be affected by changes in the tubulin genes. Laser densitometric scans were performed on the resulting autoradiograms. The level of *TUB2* message was reduced by about 50% in the hemizygous strains compared with wild-type diploids (Table 2). The levels of *TUB1* and *TUB3* messages in the wild-type and hemizygous strains were indistinguishable. The message levels of beta-tubulin and of the two alpha-tubulins directly reflected their gene number.

The levels of all three tubulin gene products were also determined by Western blot analysis. We prepared total cell lysates from an equal number of cells of each strain. Samples were loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis in three twofold increments for each strain and transferred to nitrocellulose. Parallel filters were probed with antisera specific for each of the three tubulin polypeptides. To normalize for protein loads between strains and between blots, the filters were simultaneously probed with an antiserum against GTS. Probing with each antiserum separately showed that anti-GTS gave no signal in the tubulin region and vice versa (data not shown). Radioactive bands on the filters were quantitated by using the digital image analyzer. Although the copy numbers and message levels of the alpha-tubulin genes were the same in the *TUB2* hemizygotes and the wild-type strain, the levels of both alpha-tubulin polypeptides were reduced in the *TUB2* hemizygotes to approximately 60% of the wild-type level (Table 2). The level of beta-tubulin was reduced by approximately 50% in the *TUB2* hemizygous strains, commensurate with *TUB2* message levels. With a beta-tubulin complement 50% that of the wild-type strain, these cells cannot contain more than half the normal amount of tubulin heterodimer.

TABLE 2. Levels of *TUB1*, *TUB2*, and *TUB3* message and protein in a wild-type diploid and isogenic *TUB2* hemizygote

Genotype	Level of[a]:					
	mRNA			Polypeptide		
	TUB1	*TUB2*	*TUB3*	TUB1	TUB2	TUB3
TUB2/TUB2	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.12	1.00 ± 0.13	1.00 ± 0.03	1.00 ± 0.09
TUB2/null	1.05 ± 0.08	0.46 ± 0.06	0.97 ± 0.07	0.64 ± 0.12	0.45 ± 0.03	0.52 ± 0.05

[a] Wild-type levels are set at 1.00. Values are averages of either three (mRNA) or four (polypeptide) separate determinations on the same RNA or protein samples.

(iii) Strains hemizygous for *TUB2* show normal growth rates, microtubule morphology, and chromosome segregation. We examined hemizygous strains for four properties associated with microtubule structure and function: growth rate, microtubule morphology, chromosome segregation, and sensitivity to antimicrotubule drugs.

Table 3 shows growth rates at three temperatures for FSY120, -188, and -189. The growth rates of the *TUB2* hemizygotes were indistinguishable from those of the wild-type strain at 28, 12, and 37°C. In addition, the distribution of cells at different stages of the cell cycle (as assayed by bud size) was the same in logarithmically growing cultures of each of the three strains (data not shown).

Figure 2 shows fixed cells stained with an antibody that recognizes beta-tubulin. The microtubule structures in *TUB2* hemizygotes were indistinguishable from those of the wild-type cells, at least at the level of immunofluorescence.

Using the same transplacement fragment described above, we deleted one of the two copies of *TUB2* from EJL374. This diploid strain is designed to assay the frequency of loss or mitotic recombination for chromosomes 5 and 7. Resistance to either canavanine or cycloheximide is conferred by recessive mutations present in the heterozygous state in these strains, so they are normally sensitive to both of these drugs. The cells can become drug resistant if the drug-sensitive allele of the gene encoding the resistance is lost from the cell, either through loss of the chromosome bearing the allele as a result of mitotic error or through mitotic recombination. The frequencies at which drug-resistant cells were generated were the same in the *TUB2* hemizygotes and the untransformed parental strain (Table 4). Therefore, there was no significant elevation in the frequency of mitotic errors in the strains hemizygous for *TUB2*.

We assayed growth on plates in the presence of various concentrations of methyl 2-benzimidazolecarbamate and benomyl. Wild-type strains and *TUB2* hemizygotes grew indistinguishably at MBC concentrations as high as 7.5 μg/ml and at benomyl concentrations as high as 10.0 μg/ml. At high concentrations of benomyl (15 and 20 μg/ml), the *TUB2* hemizygotes appeared to be about twice as sensitive to the drug as were wild-type strains.

The *TUB2* hemizygote is thus indistinguishable from the isogenic wild-type strain by all tests of growth rate, immunofluorescence microtubule morphology, and chromosomal segregation that we have applied, despite a 50% reduction in tubulin dimer levels in this strain. The one phenotype that we do observe, enhanced sensitivity to microtubule-depolymerizing drugs, is quite mild compared with that observed for other mutations in beta-tubulin (11, 14) or for strains bearing a deletion of the minor alpha-tubulin (*TUB3*) gene (28).

TABLE 3. Growth rate of two *TUB2* hemizygotes (FSY188 and FSY189) and an isogenic wild-type strain in selective synthetic complete glucose medium at 12, 28, and 37°C

Tubulin genotype	Doubling time (h) at[a]:		
	12°C	28°C	37°C
TUB2/TUB2	22	1.8	1.8
TUB2/null	18	1.8	1.7
TUB2/null	20	2.0	1.9

[a] Values are averages of three experiments.

(iv) Characterization of putative *TUB1* hemizygotes. We previously showed that attempts to generate *TUB1* hemizygotes produced aneuploid strains containing an extra chromosome 13, which bears both of the alpha-tubulin genes (28). We confirmed that result in FSY120 and FSY185, using plasmid pRB332 (Fig. 1B) to generate the *tub1*::*HIS3* transplacement fragment (see Materials and Methods). Southern blotting of the transformants and segregation of markers upon sporulation demonstrated that they contained chromosomal abnormalities. Given these results, we did not attempt further phenotypic analysis of putative alpha-tubulin hemizygote strains.

Strains with extra copies of tubulin genes. (i) Construction of strains with extra copies of *TUB1* and *TUB2*. Figure 1A shows PRB428, the plasmid used to introduce a single extra copy of *TUB2* at the chromosomal *TUB2* locus. Additional extrachromosomal *TUB1* genes were introduced on the CEN vector pRB539 (Fig. 1C; 29).

We transformed FSY120 or FSY185 with pRB539 and *Kpn*I-linearized pRB428 and selected Leu$^+$ Ura$^+$ transformants. Independent 2+:2− segregation of both the uracil and leucine auxotrophies was seen in four tetrads from FSY120 transformants. In tetrads from three FSY185 transformants, only the uracil auxotrophy segregated 2+:2−. The leucine auxotrophy segregated 4+:0− in each case. Southern blot analysis indicated that the *TUB1* plasmid was maintained at a higher copy number in FSY185 transformants than in FSY120 transformants (data not shown). This probably accounts for the strain differences in the *LEU*-marked plasmid segregation. The results reported for strains containing extra copies of *TUB1* with (extra *TUB1+2*) or without (extra *TUB1*) an extra copy of *TUB2* are for haploid segregants derived from transformants of FSY185. Segregants with the same genotypes from FSY120 transformants gave similar but less dramatic results. Because of the 4+:0− segregation of the *TUB1*-bearing plasmid from FSY185 transformants, the strains containing only an extra copy of *TUB2* (extra *TUB2*) were derived from FSY120 transformants.

Growth rates at 25°C of extra *TUB1*, extra *TUB1+2*, and extra *TUB2* haploids were indistinguishable from those of congenic wild-type strains. However, colonies of the extra *TUB2* haploid showed a highly variable size on synthetic complete glucose plates.

(ii) Determination of DNA, RNA, and total protein. Cells

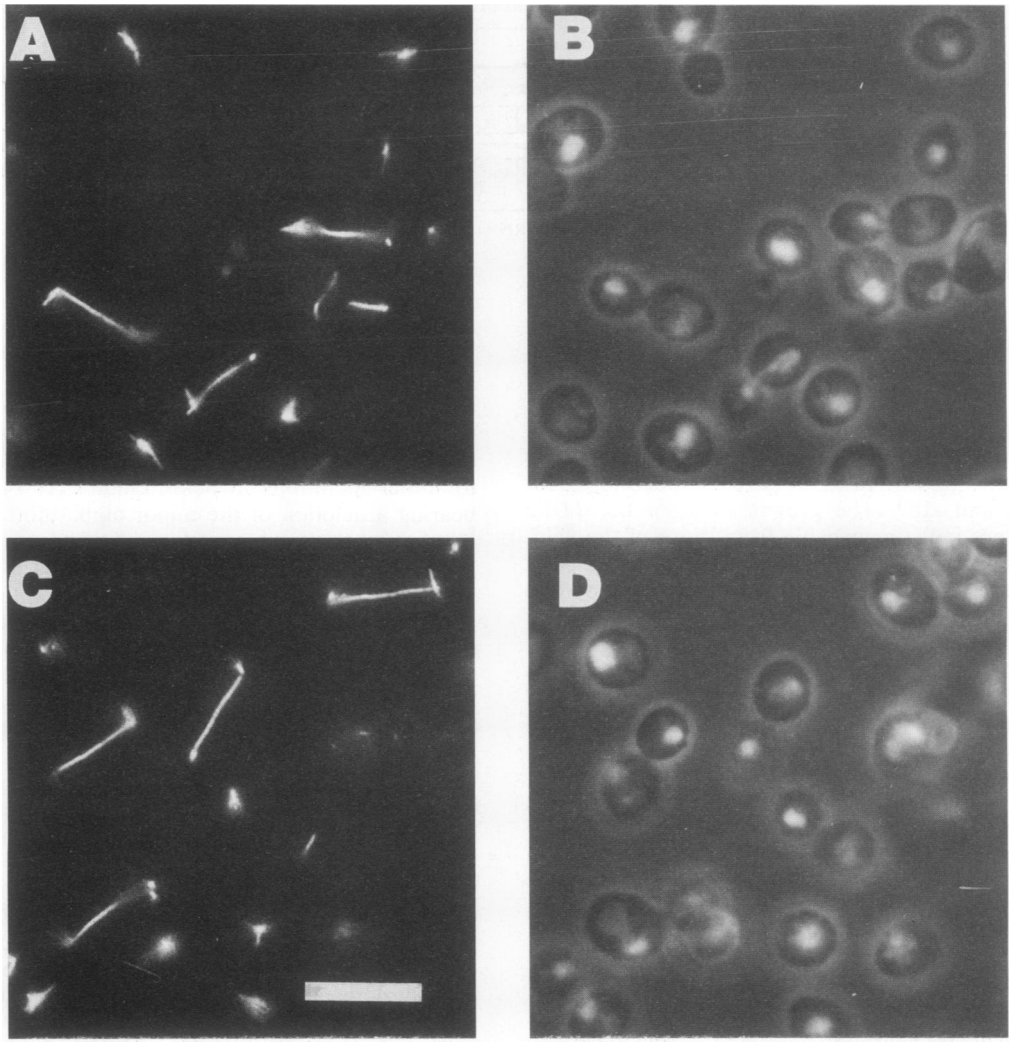

FIG. 2. *TUB2* hemizygotes containing normal microtubules. Wild-type cells (FSY120; A and B) and *TUB2* hemizygotes (FSY189; C and D) were stained with antitubulin and 4,6-diamidino-2-phenylindole. (A and C) Antitubulin immunofluorescence; (B and D) 4,6-diamidino-2-phenylindole staining of the same fields, plus phase microscopy. Bar = 10 μm.

from liquid cultures in logarithmic growth were collected and used for the simultaneous preparation of DNA, RNA, and total protein for Southern, Northern, and Western blot analysis, respectively. The results of these analyses for the FSY185 transformant strains are described below.

The copy numbers of each of the three tubulin genes were determined by Southern blotting. Total yeast DNA was digested with appropriate restriction enzymes, and the digested DNA was loaded on gels in 1-, 1.5-, and 2-fold increments. The filters were simultaneously probed with tubulin-specific sequences and DPM-specific sequences. The radioactive blots were scanned by using a Fujix BA-100 image analyzer (see Materials and Methods). The results for extra *TUB1* and extra *TUB1+2* strains are shown in Fig. 3.

As expected, the amount of *TUB2* DNA in the extra *TUB1+2* haploids was twice that in the wild-type and extra *TUB1* haploids. Also as expected, additional *TUB1* DNA was detected in the plasmid described above in both the extra *TUB1* and extra *TUB1+2* haploids, at approximately 2.5 copies per chromosomal *TUB1* copy.

Somewhat unexpected results were obtained when the copy numbers of tubulin genes were examined in the extra *TUB2* haploid. This strain contained the predicted single extra copy of the *TUB2* gene, integrated at the *TUB2* locus, and lacks plasmid-borne copies of *TUB1* genes. However, the chromosomal *TUB1* and *TUB3* genes were doubled in this strain (Table 5). Since the *TUB1* and *TUB3* genes are

TABLE 4. Chromosome loss assayed in a wild-type diploid (EJL374; see Table 1) and two isogenic *TUB2* hemizygote strains derived from it

Strain[a]	*TUB2* genotype	Frequency (10^{-6}) of colonies resistant to[b]:	
		Cycloheximide	Canavanine
EJL374	Wild type	3.7 × 10	4.9 × 10
FSY208	Hemizygote	5.5 × 10	7.3 × 10
FSY209	Hemizygote	2.6 × 10	ND

[a] All three strains are heterozygous for recessive drug resistances to canavanine and cycloheximide.

[b] Frequencies at which resistant cells appear per generation of growth. ND, Not done.

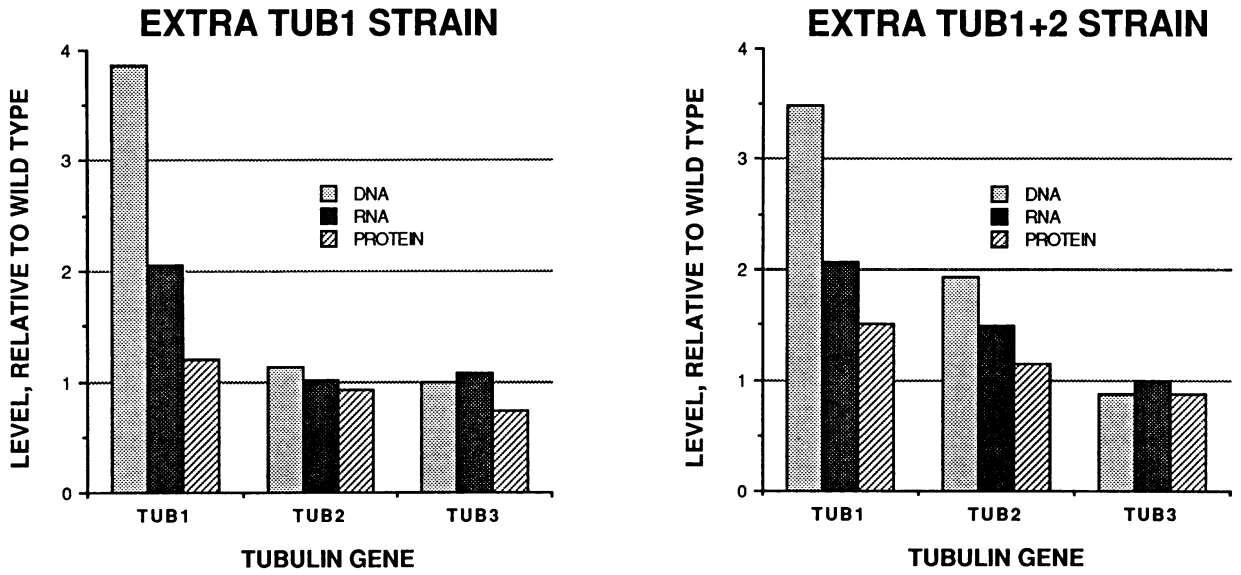

FIG. 3. Graphic representation of tubulin DNA, mRNA, and protein levels for each of the three tubulin genes in extra *TUB1* and extra *TUB1+2* strains. Levels are normalized to DNA, mRNA, and protein values of 1.00 for the wild-type controls. The average standard deviation of these data is 6.7 ± 3.0%.

linked to one another on chromosome 13, this strain most likely has acquired an additional copy of chromosome 13 to compensate for the excess dosage of beta-tubulin, in a manner analogous to the aneuploidy generated in the *TUB1* hemizygote. Because of these chromosomal abnormalities in extra *TUB2* strains, they were not characterized further.

The levels of the tubulin mRNAs in extra *TUB1* and extra *TUB1+2* cultures were also determined. Total cellular RNA was loaded in 1-, 1.5-, and 2-fold increments for Northern blot analysis. The filters were probed with both tubulin-specific and DPM-specific sequences as described above. Radioactive bands were quantitated by using the digital image analyzer. The results for extra *TUB1* and extra *TUB1+2* strains (Fig. 3) show that levels of *TUB1* message were elevated approximately twofold in both the extra *TUB1* and extra *TUB1+2* haploids relative to the wild-type haploid, levels of *TUB2* message were elevated approximately 1.5-fold in the extra *TUB1+2* haploid and appeared to be equivalent in the wild-type and extra *TUB1* haploids, and levels of *TUB3* mRNA in the three strains were essentially the same.

These results suggest that message levels do increase in the presence of extra copies of tubulin genes, although not in direct proportion to the total number of genes. For example, the levels of *TUB1* mRNA increased about 2-fold, whereas the gene copy number increased by about 3.5-fold. The message level of genes present in single copy did not rise or fall in response to the changes in levels of the other tubulin genes.

TABLE 5. Relative chromosomal content of *TUB1*, *TUB2*, and *TUB3* DNA in extra *TUB2* and congenic wild-type haploid

Tubulin genotype	Relative chromosomal DNA content[a]		
	TUB1	*TUB2*	*TUB3*
Wild type	1.00 ± 0.15	1.00 ± 0.10	1.00 ± 0.05
Extra *TUB2*	2.19 ± 0.23	2.09 ± 0.21	2.14 ± 0.46

[a] Wild-type levels are set at 1.00. Values are averages of three separate determinations on the same DNA preparations.

The steady-state levels of the *TUB1*, *TUB2*, and *TUB3* gene products were assayed by Western blot analysis. Protein samples were loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 1-, 1.5-, and 2-fold increments. After transfer to nitrocellulose, parallel filters were probed with antisera specific to each of the three tubulin polypeptides. To normalize for protein loads between strains and between blots, the filters were probed simultaneously with anti-GTS. Radioactive bands were quantitated by using the digital image analyzer. The results (Fig. 3) show that levels of TUB1 protein were increased to 150% of the wild-type level in the extra *TUB1+2* strains and to 119% of the wild-type level in the extra *TUB1* strains, levels of TUB2 protein were 115% of wild-type level in the extra *TUB1+2* strains and 92% of the wild-type level in the extra *TUB1* strains, and levels of TUB3 protein were 88% of the wild-type level in the extra *TUB1+2* strains and 74% of the wild-type level in the extra *TUB1* strains.

Thus, all strains bearing extra *TUB1* genes, and therefore expressing extra *TUB1* mRNA, showed increased levels of TUB1 protein. However, the rise in protein levels was not directly proportional to the rise in mRNA levels. The levels of *TUB1* mRNA were elevated 2-fold in both extra *TUB1* and extra *TUB1+2* strains, but the levels of the TUB1 protein were elevated only 1.2-fold and 1.5-fold, respectively. As a result, the ratio of TUB1 protein to TUB2 protein in both strains was only 1.3, compared to 1.0 in wild-type strains. This relative excess of TUB1 protein to TUB2 protein is quantitatively similar to that displayed by the *TUB2* hemizygotes described above.

DISCUSSION

In vitro experiments suggest that tubulin concentration may specify both qualitative and quantitative aspects of microtubule assembly (16, 17). Such a mechanism is supported by results from cultured animal cells connecting tubulin synthesis with level of assembly (7). To test this model more directly, we have examined quantitative aspects of tubulin function in yeast cells by constructing *S. cerevi-*

siae strains with increased or decreased numbers of tubulin genes. Our results show that diploid strains hemizygous for *TUB2* (beta-tubulin) and containing 50% of normal steady-state levels of tubulin heterodimer grow normally and are almost indistinguishable from isogenic wild-type strains with respect to microtubule-dependent functions. This outcome suggests that yeast cells have no mechanism for increasing tubulin levels when cells are confronted with a chronic deficit, nor is there any need for such a mechanism for normal microtubule function. Instead, yeast cells apparently can control the extent of microtubule assembly independently of tubulin concentration, at least over about a twofold range.

A quantitatively different result is obtained with strains containing excess *TUB1* (alpha-tubulin) genes, either in the presence or in the absence of an additional copy of the *TUB2* gene. These strains also grow normally, but the levels of *TUB1* and *TUB2* mRNAs and proteins do not vary in proportion to gene dosage. As a consequence, the levels of tubulin heterodimer in these strains are no more than 115% of that found in wild-type cells. This outcome suggests that yeast cells do have mechanisms for reducing excess levels of tubulin mRNAs and proteins.

What is apparently intolerable to yeast cells is an excess of the *TUB2* gene or its protein product. The toxicity of excess beta-tubulin has been previously suggested (4, 33). When the *TUB2/TUB1* gene copy ratio is made less than 1, either by introducing a single extra copy of *TUB2* or by making diploid cells hemizygous with respect to *TUB1*, the strains become aneuploid for chromosome 13 and so increase the alpha-tubulin gene dosage. In contrast, a *TUB2/TUB1* gene copy ratio greater than 1 does not result in an increase in *TUB2* gene dosage. These results are reinforced in the study reported in the accompanying paper (34), in which alpha- and beta-tubulin were inducibly overexpressed in yeast cells. In that study, dramatic loss of viability and loss of microtubules were observed upon overproduction of beta-tubulin but not upon overproduction of alpha-tubulin or of beta-tubulin in the presence of cooverproduced alpha-tubulin.

Why are *TUB2* hemizygotes not sick? Our results indicate that the amount of total tubulin in yeast cells is ordinarily at least twice the level required for normal growth, since the phenotypically normal beta-tubulin hemizygotes contain 50% or less of the normal level of tubulin heterodimer. This fact could be explained most readily on one of two grounds. The first possibility is that the hemizygotes contain as little as half the normal diploid complement of assembled tubulin but that this reduction does not damage the cells. By this argument, the microtubules of the hemizygotes would be shorter or fewer than those of wild-type diploids. This possibility could be tested by biochemical fractionation of the assembled and unassembled tubulin pools (23), but at present this assay is not sufficiently quantitative in yeast cells. Even without the direct measurement, however, this explanation seems unlikely. The cells of *TUB2* hemizygotes are not distinguishable from wild-type cells on the basis of size or of the apparent length of either their nuclei or their microtubules. In addition, there does not seem to be much flexibility in microtubule number in *S. cerevisiae*, at least with respect to the spindle, which may contain only one kinetochore fiber for each chromosome (15).

A possible second explanation is that the hemizygotes have assembled their normal complement of microtubules from only half the normal complement of tubulin. In that case, the ratio of unassembled to assembled tubulin in these cells would be significantly altered compared with wild type. If this is true, it would suggest that some factor other than tubulin concentration is extent limiting for assembly. Evidence from other work has shown that the ability of cells to form microtubules is not saturated under normal circumstances. For example, wild-type yeast cells are able to faithfully segregate extra centromeres introduced on CEN plasmids (9). A mutant CHO cell line has also been reported which appears to contain lower than normal levels of tubulin (3), although detailed tests of microtubule function have not been performed on these cells. In any case, the flexibility that yeast cells exhibit in assembling apparently normal microtubule arrays from a substantially reduced pool of tubulin heterodimers would help to explain the apparent absence of a mechanism to compensate for a genetic deficiency of beta-tubulin.

Moderate excess of tubulin. Cells bearing extra copies of the *TUB1* gene do not show a proportionate increase in the *TUB1* protein, suggesting that yeast cells apparently respond to excess alpha-tubulin by modulating levels of either mRNA or protein or both. Control at the protein level is supported by the observation that modest overproduction of the TUB1 protein leads to lower levels of the TUB3 protein but does not affect *TUB3* mRNA. Furthermore, in strains hemizygous for beta-tubulin, the levels of alpha-tubulin protein are reduced but not the levels of alpha-tubulin message. Finally, the amount of excess alpha-tubulin over beta-tubulin is the same irrespective of the number of *TUB2* genes present, implying that there is a level of excess alpha-tubulin that can be tolerated by yeast cells without phenotypic effect. Other mechanisms could also contribute. For example, transcription from the plasmid copies of the *TUB1* gene may be less efficient than from the chromosomal copies, although the plasmid does contain 1 kb of 5′ noncoding sequence. The plasmid-produced message may be less translatable than that produced from the chromosome, although the *TUB1* mRNA synthesized from the plasmid is the same size as that synthesized from the chromosome (data not shown).

Our results do not indicate whether yeast cells might have an analogous mechanism for reducing a less than twofold excess level of beta-tubulin. We have been unable to produce stable strains in which beta-tubulin is produced in excess of alpha-tubulin. In the accompanying paper (34), we show that a 40% increase in beta-tubulin levels causes loss of all microtubules. Putative *TUB1* hemizygotes acquire extra copies of *TUB1* and *TUB3* by chromosomal duplication. Haploid segregants which contain an extra copy of the *TUB2* gene but no plasmid copies of the *TUB1* gene show evidence of similar chromosomal abnormalities. When the *TUB1* hemizygotes were originally characterized (28), it was not clear whether the inability to maintain cells with reduced levels of alpha-tubulin was the result of inadequate levels of tubulin heterodimer or because of a particular toxicity associated with too little alpha-tubulin or too much beta-tubulin. We now know, from the *TUB2* hemizygotes, that the defect in the *TUB1* hemizygotes is not ascribable to a deficiency in total tubulin levels. If the levels of tubulins were produced in proportion to their gene dosages in both hemizygotes, then heterodimer levels would be 50% of wild type in the *TUB2* hemizygote and 60% of wild type in the *TUB1* hemizygote (since *TUB1* accounts for approximately 80% of the alpha-tubulin in a wild-type diploid yeast cell).

Tubulin regulation in yeast and animal cells. Burke et al. (4) proposed that yeast cells might balance the levels of alpha-tubulin and beta-tubulin subunits if alpha-tubulin were synthesized in excess and then degraded until only het-

erodimeric tubulin remains. Results reported here support predictions of that model. First, increased copy number of alpha-tubulin genes should not result in comparable increases in levels of its gene product. We show here that yeast cells down regulate the total alpha-tubulin protein to a nearly wild-type level when the gene dosage of beta-tubulin is normal but the number of *TUB1* genes is increased. Both the *TUB1* and *TUB3* alpha-tubulin gene products are down regulated even though only *TUB1* gene dosage and mRNA level are elevated. Second, decreased copy number of the beta-tubulin gene should result in comparable decreases in levels of both alpha-tubulin and beta-tubulin proteins. We show here that the reduced levels of beta-tubulin protein in the *TUB2* hemizygote directly reflect its reduced gene dosage, and alpha-tubulin levels are reduced as well. There is an obvious rationale for this mechanism: synthesis of excess alpha-tubulin would ensure that a harmful excess of beta-tubulin could not accumulate.

The experiment described here, using changes in gene copy number to produce changes in tubulin levels, does not identify a mechanism to up regulate tubulin levels in response to lower intracellular concentrations. Although we do see a response to modest overproduction, a significant part of that response is at the level of protein rather than at the level of mRNA. Thus, the steady-state response of yeast cells is significantly different from the acute response postulated in animal cells (7). Our results suggest that regulation of tubulin monomer and dimer levels in yeast cells is a response to problems of toxicity rather than problems of assembly. This issue is further explored in the accompanying paper (34).

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