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Insertions of up to 17 Amino Acids into a Region of α-Tubulin Do Not Disrupt Function In Vivo

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Microtubules in yeasts are essential components of the mitotic and meiotic spindles and are necessary for nuclear movement during cell division and mating. The yeast *Saccharomyces cerevisiae* has two α-tubulin genes, *TUB1* and *TUB3*, either of which alone is sufficient for these processes when present in a high enough copy number. Comparisons of sequences from several species reveals the presence of a variable region near the amino terminus of α-tubulin proteins. We perturbed the structure of this region in *TUB3* by inserting into it 3, 9, or 17 amino acids and tested the ability of these altered proteins to function as the only α-tubulin protein in yeast cells. We found that each of these altered proteins was sufficient on its own for mitotic growth, mating, and meiosis of yeasts. We conclude that this region can tolerate considerable variation without losing any of the highly conserved functions of α-tubulin. Our results suggest that variability in this region occurs because it can be tolerated, not because it specifies an important function for the protein.

The α,β-tubulin heterodimer is the major structural component of microtubules, which are intimately involved in eucaryotic cell structure and motility (47). The ultrastructure of microtubules is highly conserved in a wide variety of species. The sequences of α- and β-tubulins are also highly conserved (7, 35). These observations lead to the hypothesis that many of the mechanisms that regulate microtubule structure and function are conserved as well. We are studying microtubules in yeasts by using a combination of genetic and biochemical techniques.

Microtubules in yeasts are elements of structures involved in chromosome and nuclear movement (2, 6, 18, 20, 27, 29). Studies with antimicrotubule drugs and with conditional lethal tubulin mutants have confirmed the role of microtubules in the mitotic and meiotic spindles of yeast, as well as in nuclear movement during cell division and mating (14, 33, 34, 37, 50–53, 56, 57). Tubulin has been purified from yeast and shown to have biochemical properties similar to tubulin from higher eucaryotes (17). Two very different yeasts have been used in genetic studies, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Results of genetic and molecular cloning experiments have shown that both yeasts have one essential β-tubulin gene and two α-tubulin genes (14, 28, 39, 40, 51). Within each species, the two α-tubulin genes differ substantially from each other in sequence and differ markedly in their importance for normal cell growth. In both cases, one of the genes is genetically essential and the other is not (1, 40). In both species, however, either gene alone can perform all of the functions that are normally performed by the pair if present in the cell in sufficient copy number (40; M. Yanagida, personal communication). Thus, the differences between the genes are apparently due to the level of expression and not to functional differences between the encoded proteins.

Several approaches have been used to study the relation between the primary sequence of isolated tubulin proteins and their tertiary structure and function. Cleavage of both α- and β-tubulin to remove carboxy-terminal fragments alters their ability to assemble into microtubules in vitro (38, 42). The carboxyl termini have also been identified as sites of high-affinity calcium binding (43). Using proteolytic fragments of tubulin, several groups of investigators have identified potential sites of interaction between tubulin and microtubule-associated proteins (24, 41). Specific amino acid residues have been identified that are important for microtubule assembly in vitro (3). These sorts of analyses can now be pursued in vivo. The in vivo consequences of variation in the sequence of β-tubulin have been investigated through the expression of a chicken-yeast chimeric β-tubulin in mouse cells. The results indicate that the variant sequence places no restrictions on the ability of the protein to assemble into all microtubules in the cells (4). Results of a recent study have demonstrated that naturally occurring tubulin variants in mammalian cells show no restriction in their ability to assemble into a wide variety of microtubular structures (23a). The functional consequences of several kinds of sequence variation in the chicken-yeast chimeric β-tubulin have been described in the accompanying paper (12).

We used one of the two α-tubulin genes of the budding yeast *Saccharomyces cerevisiae*, *TUB3* (39, 40), to study structure-function relationships in a region of α-tubulin that is extremely variable in cross-species comparisons by both length and sequence. We perturbed the structure of this region of the *TUB3* protein by inserting into it up to 17 additional amino acids. We show that yeast strains containing only these mutant genes show no detectable defect in growth, mating, or sporulation.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* HB101 was used for bacterial transformation and plasmid growth. Bacterial media were made as described by Davis et al. (9). Media for yeast growth and sporulation were made as described by Sherman et al. (44), 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 & Co., Inc. (Wilmington, Del.). It was kept as a 10-mg/ml stock in dimethyl sulfoxide at 4°C and was added to warm YPD medium with swirling imme-

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TABLE 1. Yeast strains used in this study

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

diately before the medium was poured into the plates. The yeast strains used in this study were derived from a set of essentially isogenic S288C strains originally provided by G. R. Fink. The strains used are listed in Table 1.

Gel electrophoresis and nucleic acid preparation. Restriction enzymes, DNA polymerase I, DNA polymerase I large fragment, polynucleotide kinase, T4 DNA ligase, and terminal transferase were purchased from New England BioLabs, Inc. (Beverly, Mass.) and used in the buffers described by Maniatis et al. (25). Agarose gel electrophoresis and plasmid DNA isolation were performed as described by Davis et al. (9). Small-scale preparations of yeast DNA were prepared by the method described by Holm et al. (15).

Hybridization methods. DNA fragments were transferred from agarose gels to a Zetapore membrane (AMF Inc.) by the method described by Southern (48) with 20× SSPE (25). Hybridizations were done at 42°C in the buffer described by Wahl et al. (55) with nick-translated probes (36). After hybridization, the filters were washed in 2× SSPE–0.5% sodium dodecyl sulfate (SDS) at 50°C.

Plasmid constructions. An extensive map and the sequence of the *TUB3* gene have been published elsewhere (39). The *Bam*HI linkers were purchased from Collaborative Research, Inc. (Waltham, Mass.) and consisted of the sequences CGGATCCG, CGGGATCCCG, and CGCGGATC CGCG. *Bam*HI linker insertions were made in plasmid pRB325 (40) (see Fig. 2). pRB325 was digested with *Mst*II, and the 5′ overhangs were filled with the large fragment of DNA polymerase I. Linkers were added to the blunt ends, and the molecules were recircularized with T4 DNA ligase.

The *TUB3* linker insertion mutations were transferred to the yeast plasmid YEp24 (5) by an in vivo recombination technique described in detail elsewhere (H. Ma, S. Kunes, P. J. Schatz, and D. Botstein, Gene, in press). Briefly, the pRB325 derivatives were digested with *Sal* and *Pst*I. The 4.6-kilobase (kb) fragments, containing the modified *TUB3* genes plus flanking pBR322 sequences, were purified on an agarose gel. These fragments were mixed with *Bam*HI-digested YEp24 DNA and were used to transform yeast strain DBY2254 with selection for *Ura3*$^+$. The resulting plasmids were recovered from yeast and transformed into *E. coli*. All had the expected structure (Fig. 2).

DNA sequence analysis. The sequences of the linker insertions were determined by the method described by Maxam and Gilbert (26) by using the pRB325 derivatives described above (see Fig. 2). The plasmids were digested with *Cla*I and labeled at their 3′ ends by filling the 5′ overhangs with the large fragment of DNA polymerase I and [α-^{32}P]dCTP. The labeled plasmids were recut with *Xho*I before gel purification of the 0.64-kb fragment for sequencing. The complementary strand was sequenced from the opposite side by cutting with *Nsi*I and labeling the 3′ end with [α-^{32}P]ddATP and terminal transferase as described by Yousaf et al. (58). The labeled plasmids were recut with *Eco*RI before gel purification of the 0.45-kb fragment for sequencing.

Genetic techniques and transformation. Methods of yeast mating, sporulation, and tetrad analysis were as described by Sherman et al. (44). Yeast cells were transformed by the lithium acetate method described by Ito et al. (16), as modified by Kuo and Campbell (22), with 1 to 4 μg of plasmid DNA and 50 μg of sonicated chicken blood DNA (Sigma Chemical Co., St. Louis, Mo.) as carrier. Transformants were plated on SD medium (44) supplemented with the appropriate nutrients to select cells with the plasmid.

Preparation of antibodies. A peptide corresponding to the last 10 amino acids of the *TUB3* sequence was purchased from Peninsula Labs and coupled to keyhole limpet hemo yanin via an amino-terminal cysteine. Two rabbits were injected with 0.5 mg each of the conjugate homogenized in Freund complete adjuvant. The rabbits were boosted twice with 0.5 mg of peptide in Freund incomplete adjuvant at 2-week intervals. At 2 weeks after the last injection the rabbits were bled, and the serum was stored in frozen aliquots.

Protein preparation and Western blotting. Yeast cells were grown to a density of about 10^7 cells per ml, harvested by centrifugation, and washed in 20 mM Tris hydrochloride (pH 7.5). Cells were lysed by boiling for 3 min in 2% SDS–10% glycerol–80 mM Tris (pH 6.8)–1 mM phenylmethylsulfonyl fluoride–0.1 M dithiothreitol–0.001% bromophenol blue (ESB) buffer, vortexed vigorously for 1 min with glass beads (diameter, 0.5 mm), and boiled for 1 more min. The proteins were resolved on an 8.5% SDS-polyacrylamide gel and transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) by electroblotting at 30 V overnight. The nitrocellulose paper was blocked with 5% dry milk (Carnation) in 0.01 M Tris (pH 7.4)–0.15 M NaCl for 1 h and then probed with the α-tubulin monoclonal antibody B-5-1-2 (30) for 1 h in the same solution. The excess antibody was removed by washing four times in the Tris-NaCl solution (without milk) over the course of 1 h. The primary antibody was then labeled with ^{125}I-labeled rabbit anti-mouse immunoglobulin G (IgG) (generously provided by Tom Briner and Malcolm Gefter) in Tris-NaCl for 1 h. Finally, the filter was washed four times during 1 h with Tris-NaCl and exposed to film (XAR; Eastman Kodak Co., Rochester, N.Y.). Blots with the anti-peptide antibody were performed by a similar procedure. The primary antibody (from rabbit 6-346) was used at a 1/1,000 dilution and detected with [^{125}I]protein A at 0.2 μCi/ml.

RESULTS

Comparison of sequences of α-tubulins from several species revealed a region near the amino terminus which was exceptionally variable (Fig. 1). To begin to examine the role

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                    20
Human               WELYCLEHGIQPDG--QMPSDKTIGGGDDSFN---------TFFSETGAGKHVPRAV
Porcine                           --                 ---------
Rat                               --                 ---------
Hamster                           --                 ---------
Mouse 1,2,3                       --                 ---------
Mouse 4                           --                 T---------   C
Chicken testis        F    S      ---TF  PP--SS      A---------   R  SMS Y    I
Drosophila 1,3                    --       V         ---------
Drosophila 2                      --H      V         S---------
Drosophila 4           L     NL   SLKTKEELTASGSSASVGHDTSANDAR   T   N  Q   SI
Trypanosome           F          --A        VE  A    ---------
Stylonychia           F          --                  A ---------        E     C
Physarum                   N     --       SV        A ---------       SS      I
Chlamydomonas                     --                  A ---------                CI
S. pombe 1                 G      FPTEN EVHKNNSYLNDGFG-------       Q  F    SI
S. pombe 2                   N    --Y NP TASQNS GG S---------       Q  Y    SI
TUB1                   S     K    -HLEDGLSKPK  EEG S---------   H   Y  F    I
TUB3                   S     KE   -HLEDGLSKPK  EEG S---------   H   Y  F    I
                                          ▲
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FIG. 1. α-Tubulin sequences from a variety of species in the variable amino-terminal region. α-Tubulin sequences are shown from a variety of species starting with amino acid 20. Dashes indicate insertions to bring the sequences into register. Positions of identity with the human sequence are left blank. The large arrowhead indicates the position of the insertion mutations in *TUB3*. The α-tubulin sequences shown are from human keratinocyte (8), porcine brain (31), rat (23), CHO cells (11), mouse α1-4 (54), chicken testis-specific cα2 (32), *Drosophila melanogaster* α1-4 (49), *Trypanosome brucei* (19), *Stylonychia lemnae* (13), *Physarum polycephalum* (21), *Chlamydomonas reinhardi* (45), *Schizosaccharomyces pombe* (51), and *Saccharomyces cerevisiae TUB1* and *TUB3* (39).

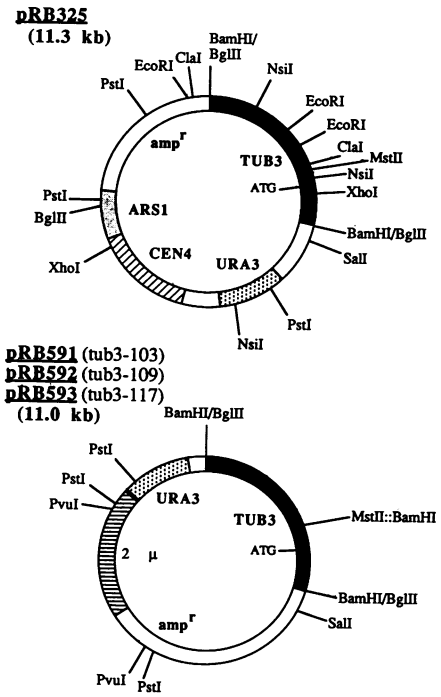

FIG. 2. Restriction maps of plasmids. The construction of plasmid pRB325 has been described previously (40). The *TUB3* coding and intron sequence are darkly shaded, while the *TUB3* flanking sequences have medium shading. Unshaded regions were derived from pBR322, and the other sequences on the plasmid are labeled. *Bam*HI linker insertions were made into the single *Mst*II site. The *tub3-103*, *tub3-109*, and *tub3-117* alleles were used to construct plasmids pRB591, pRB592, and pRB593, respectively, by the method described in the text. They are analogous to pRB316 (40), which carries the wild-type *TUB3* gene.

of this region in the function of yeast α-tubulin, we perturbed its structure by inserting extra amino acids into the region. The alterations consisted of 8, 10, or 12-base-pair *Bam*HI linkers inserted into a blunted *Mst*II site in the *TUB3* α-tubulin gene of the yeast *Saccharomyces cerevisiae* (39). The mutations were initially constructed and examined in the plasmid pRB325 (Fig. 2), which carries a yeast centromere (CEN4), replication origin (ARS1), and selectable marker (URA3). Four of each type of linker insertion were examined in detail.

To determine the structure of the mutations, we sequenced all 12 of the plasmids in the region of the inserts. The sequences revealed that some of the mutations resulted from the insertion of more than one linker. Variation of a single base pair was also observed in the sequences of the ligation sites, perhaps because of incomplete filling of the *Mst*II overlapping ends or to exonuclease digestion of the ends before ligation. Three of the mutations (Fig. 3) preserved the reading frame of *TUB3*, while the rest shifted the frame. The in-frame insertions added 3, 9, or 17 amino acids after the normal amino acid number 43 (Fig. 1). These mutations were designated *tub3-103*, *tub3-109*, and *tub3-117*, respectively.

Tests of function. We first assayed the altered genes for their ability to complement one of the phenotypes of a null mutation in *TUB3*, hypersensitivity to the antimicrotubule drug benomyl (40). We transformed strain DBY2375 (*tub3*$^-$) with centromere plasmids carrying the linker insertions (Fig. 2). The transformed derivatives were then tested for growth on several concentrations of benomyl. A plasmid carrying intact *TUB3* served as a positive control, and the centromere plasmid with no insert served as a negative control. Results of the experiments revealed that all of the insertion mutant alleles that had preserved the reading frame of *TUB3* retained the ability to complement the benomyl sensitivity of the *TUB3* null mutant as effectively as did the positive control. None of the insertions that changed the reading frame of *TUB3* retained the ability to complement.

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

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

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

tub3-117    TTGTCAAAACCTaacgcggatccgcgcgcggatccgcgcgcggatccgcgcgcggatccgcgtAAGGGAGGTGAA
 (1)        LeuSerLysProAsnAlaAspProArgAlaAspProArgAlaAspProArgAlaAspProArgLysGlyGlyGlu
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FIG. 3. Sequences of *TUB3 Bam*HI linker insertion mutations. The sequences of wild-type *TUB3* and the linker insertion mutations are shown with translations below each line. The numbers in parentheses below each allele number indicate the number of independent isolates of each mutation. Arrows above the lines indicate sites where single base pairs were lost during the linker insertion procedure. The added nucleotides are indicated with lowercase letters. *tub3-103* was the product of insertion of an 8-base-pair *Bam*HI linker; *tub3-109* and *tub3-117* were the products of insertion of a 12-base-pair linker.

Complementation of benomyl hypersensitivity is only a limited test of the ability of the altered gene products to function as α-tubulins. We therefore decided to test their ability to support growth as the only α-tubulin gene in the cell. Our previous results (40) indicate that the *TUB3* gene in a high enough copy number can support mitotic growth, mating, and sporulation of yeasts in the absence of the *TUB1* α-tubulin gene. We performed the test in two steps. First, we assayed the mutant genes for their ability to complement a *TUB1* null mutation, and then we crossed them to construct strains without wild-type α-tubulin genes.

To ensure sufficient copy number of the altered *TUB3* genes, we transferred the mutant alleles to high-copy-number vectors containing the 2μm plasmid origin of replication. The transfer was performed by using in vivo recombination between a DNA fragment carrying the altered genes and the 2μm plasmid YEp24 (Fig. 2; see above). The in vivo recombination occurred during transformation of strain DBY2254 (Table 1), a diploid strain that is heterozygous for a disruption of the *TUB1* gene (marked with the yeast *HIS3* gene). The resulting diploids were sporulated, and the tetrads were dissected.

The *TUB1* disruption is normally a recessive lethal mutation. In the presence of extra copies of functional α-tubulin genes, however, the lethality is suppressed (40). To determine whether the mutant alleles could suppress the lethality, the progeny spores were examined for the presence of spores containing the *tub1* disruption, a single copy of wild-type chromosomal *TUB3*, and one of the plasmids. Such spores would have two obvious phenotypes; they would be $His3^+$ because the *HIS3* gene is the marker of the *TUB1* disruption; they would be $Ura3^+$ because the *URA3* gene is the marker on the plasmid. In addition, they would show an absence of slow growth, which has previously been correlated with abnormalities of chromosome 13, on which both α-tubulin genes map (40). Experiments were done with all three in-frame insertion mutations, along with an out-of-frame mutation as a negative control. Experiments with all of the in-frame insertions yielded spores of the expected phenotype, while the negative control yielded no such spores. These results indicate that the insertion mutations complement the lethality that is normally associated with the *TUB1*::*HIS3* gene disruption in normal haploid cells (40).

The strains from the experiment described above contained wild-type *TUB3* on the chromosome and mutant alleles of *TUB3* on the plasmid. To show that the growth of yeasts could be supported by the mutant *TUB3* alleles alone, these strains were crossed to strains DBY2287 or DBY2288, which contain a disruption of the *TUB3* gene (marked with the yeast *TRP1* gene). In these crosses, both the *HIS3* marker and the *TRP1* marker segregated 2:2 and the plasmid marker segregated 4:0 in most cases. Strains were recovered that contained each of the plasmids and both the *TUB1* and *TUB3* disruptions. In these strains, the mutated *TUB3* genes were presumably the only α-tubulin genes in the cells.

To confirm the absence of wild-type α-tubulin genes, we carried out gel transfer hybridization experiments (Fig. 4).

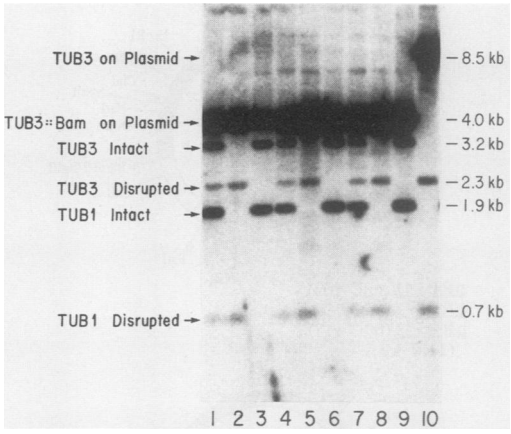

FIG. 4. Gel transfer hybridization of total yeast DNA from strains transformed with plasmids bearing the *TUB3* linker insertion mutations. DNA was purified from diploid strains heterozygous for the *tub3*::*TRP1* and *tub1*::*HIS3* disruption alleles (40) containing pRB591, pRB592, and pRB593 (lanes 1, 4, and 7, respectively). DNA was also purified from progeny spores (from these diploids) carrying the plasmids plus both disruption alleles (lanes 2, 5, and 8) or from their meiotic sisters carrying the plasmids plus wild-type *TUB3* and *TUB1* (lanes 3, 6, and 9). The DNA in lane 10 was from strain DBY2384, which carries wild-type *TUB3* on plasmid pRB316 and the two disruption alleles. In all cases the strains were grown under conditions that selected for maintenance of the plasmids. The DNA was digested with *Bam*HI, *Bgl*II, and *Pvu*I; and the fragments were separated on an agarose gel. The DNA was transferred to a membrane (Zetapore) 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.

DNA was prepared from parental diploids, from His^+ Trp^+ Ura^+ spores, which should contain only the altered tubulins, and also from their His^- Trp^- Ura^+ sisters, which should contain wild-type chromosomal genes. The DNA was digested with *Bam*HI, *Pvu*I, and *Bgl*II; run on an agarose gel; transferred to filter paper; and analyzed with a *TUB1* probe. Under these digestion conditions, the intact *TUB1* gene yielded a hybridizing fragment of 1.9 kb, while the disruption allele yielded a fragment of 0.7 kb. Because of extensive homology between *TUB1* and *TUB3*, the probe also hybridizes to a band of 3.2 kb from intact *TUB3* and a band of 2.3 kb from the *TUB3* disruption allele (40). Plasmid-borne *TUB3* was expected to yield a band of 4.0 kb if a *Bam*HI linker were present or a band of 8.5 kb if no *Bam*HI linker were present. As expected, the diploids (Fig. 4, lanes 1, 4, and 7) showed both sets of chromosomal bands, the His^+ Trp^+ spores (Fig. 4, lanes 2, 5, and 8) showed only the disruption-specific bands, and the His^- Trp^- spores (Fig. 4, lanes 3, 6, and 9) showed the bands from the intact genes. All of the strains presumed to be carrying the plasmid-borne *TUB3* insertion mutations showed the expected 4.0-kb band. A control strain, with wild-type *TUB3* on a similar plasmid, showed the expected 8.5-kb band (Fig. 4, lane 10).

We conclude that the *TUB3* insertion mutations specify α-tubulins that are sufficient for germination and mitotic growth of yeasts in the absence of other α-tubulins.

To confirm that these strains contained only the altered α-tubulin proteins, we examined the mobility of the α-tubulins in SDS-polyacrylamide gels by Western blotting. Because the mutant alleles encoded from 3 to 17 extra amino acids, their products might be expected to have a slightly lower mobility than that of the wild-type *TUB3* protein. Two antibodies were used. The first was a widely cross-reactive monoclonal anti-α-tubulin antibody made against sea urchin tubulin (30; kindly provided by G. Piperno). The second was a rabbit polyclonal antibody made against the peptide encoded by the carboxyl terminus of *TUB3* (see above). Both antibodies gave similar results. The results obtained by probing whole-cell protein extracts with the anti-*TUB3* peptide antibody are shown in Fig. 5. Lanes 1, 4, 7, and 10 (Fig. 5) show the results from an extract made from a strain (DBY2384) that contained only wild-type *TUB3*. Lanes 2, 5, and 8 (Fig. 5) contained extracts from strains carrying only the *tub3-103*, *tub3-109*, and *tub3-117* alleles, respectively. Lanes 3, 6, and 9 (Fig. 5) show the results obtained when extracts for the three mutants were mixed with wild-type extracts. As expected, the altered proteins migrated more slowly in the gel than did the wild-type protein.

We tested the strains carrying only *tub3-103*, *tub3-109*, or *tub3-117* for any obvious defects. All of them grew well at temperatures from 14 to 37°C. Based on observations of colony size after 2 days of growth at 30°C, mutant strains grew at about the same rate as the wild type. To extend the analysis to other known microtubule functions in yeasts, the *TUB3* mutations were tested for their ability to support mating and sporulation of yeast. All three mutations allowed strains to mate and sporulate with about the same efficiency as the wild-type controls (data not shown). We conclude that the insertions show no detectable phenotype in any of the known microtubule-dependent processes in yeasts.

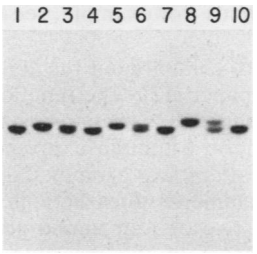

FIG. 5. Western blot analysis of proteins from the *TUB3* mutant alleles. Total cell protein was prepared from strain DBY2384, which contained only wild-type *TUB3* (lanes 1, 4, 7, and 10), and from strains DBY2392 (*tub3-103*), DBY2387 (*tub3-109*), and DBY2389 (*tub3-117*) in lanes 2, 5, and 8, respectively.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

DISCUSSION

We have shown that insertion of 3, 9, or 17 amino acids into a variable region near the amino terminus of yeast α-tubulin has no detectable effect on the function of the protein. Strains carrying only the altered α-tubulins were indistinguishable from the wild type in mitotic growth, mating, and sporulation, the three processes in yeasts that are known to depend on microtubules (10, 14, 33, 34, 37, 50–53, 56, 57).

Comparisons in this area between α-tubulins from a wide variety of species are shown in Fig. 1. Among eucaryotes from humans to *C. reinhardi* the region is quite well conserved, with several notable exceptions. The chick testis sequence shows considerable divergence by length and by sequence (32). The *D. melanogaster* α-4 and all four yeast α-tubulin sequences of this region have no appreciable homology to the other sequences (39, 49, 51). The divergence of *Schizosaccharomyces pombe* α1 and *D. melanogaster* α-4 is especially noticeable because they contain 4 and 11 additional amino acids, respectively, relative to most of the other sequences. Results of the sequence comparisons plus our mutational analysis strongly suggest that this region can tolerate considerable variation without losing any of the highly conserved functions of α-tubulin. These observations suggest that variability in this region occurs because it can be tolerated, not because it specifies an important function for the protein. Deletion analysis is required to prove that this region has no specific function in yeasts and to determine whether it has any minimum length. It is possible that the other eucaryotes retain a subset of microtubule functions, which are dependent on this region, that yeasts do not have. Definitive tests of such a model will only be possible through mutational analysis of α-tubulin in a eucaryote, such as *D. melanogaster*, that combines the ability to test altered genes in vivo with a wide variety of microtubular structures and functions. Functional analysis of other regions of tubulin, however, will continue to be easily accomplished by using the sophisticated genetics available in yeasts.

The identification of regions of proteins that can tolerate sequence variation has been useful in a number of instances. Notable examples include the phage T4 rIIB gene product and *E. coli* β-galactosidase (46). One possible use for such a region in α-tubulin would be the construction of functional mutant alleles whose products could be detected by probes that are specific to the inserted region. Such proteins could be used for structural studies of tubulin and microtubules. They also could be used as a starting point to construct variant tubulins that could be identified in a background of

normal tubulin proteins (for example, see the accompanying paper [12]).

It is informative to compare our results here with those in the accompanying paper (12). The functional consequences of a number of sequence variations were examined in a chicken-yeast chimeric β-tubulin expressed in mouse cells (12). The sequence alterations examined included a deletion, several additional chimeric constructions, and a number of small insertions of two or four amino acids made through linker insertions. Most of the insertion alleles that were examined encoded tubulins with major functional defects, including an inability to assemble into microtubules. The only mutant alleles whose gene products were able to assemble included an insertion and a deletion at the carboxyl terminus and three of the four chimeric constructions. Most regions of tubulin thus appear to be very sensitive to variations in length. The regions so far identified that are not sensitive to insertions include the variable amino-terminal region of α-tubulin and the carboxyl terminus of β-tubulin (also a region of considerable variation in sequence comparisons).

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