MOLECULAR AND CELLULAR BIOLOGY, Nov. 1986, p. 3711–3721
0270-7306/86/113711-11$02.00/0

Vol. 6, No. 11

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

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Received March 1986/Accepted 23 June 1986

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

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

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

The single β-tubulin gene of *S. cerevisiae* has been isolated, sequenced, and shown to be essential for growth (36). All of the tubulin genes of *Schizosaccharomyces pombe* have been isolated and sequenced, including one β-tubulin gene (24) and two functional α-tubulin genes (69). All of these genes show striking homologies to tubulin genes from other species (for reviews see references 15 and 50). In this report we complete the identification and sequence analysis of tubulin genes from *S. cerevisiae*. We report the isolation of the two functional α-tubulin genes, the complete sequence of both genes, and the identification of their protein products in yeast microtubules. In an accompanying paper (57), we report on the genetic analysis of null mutations in these two genes, which we have named *TUB1* and *TUB3*.

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

RESULTS

Isolation of two α-tubulin genes. To identify the α-tubulin genes of *S. cerevisiae*, two fragments of the *Schizosaccharomyces pombe NDA2* α-tubulin gene (69) (generously provided by T. Toda and M. Yanagida) were used as hybridization probes. Gel transfer hybridization experiments with genomic *S. cerevisiae* DNA (64) with these probes at low stringency showed two independent sets of strongly hybridizing bands (data not shown). Molecular cloning experiments yielded two genes, henceforth called *TUB1* and *TUB3* (the *TUB2* gene encodes β-tubulin). The *TUB3* gene was isolated from a library of partially *Sau*3A-digested genomic DNA in the vector λBF101 by plaque hybridization (7) and subcloned into the vector pPL7 to make plasmid pRB300. The *TUB1* gene was isolated as described in Materials and Methods and subcloned into pBR322 to make plasmid pRB306. The restriction maps of the yeast DNA inserts of pRB300 and pRB306 are shown in Fig. 1.

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

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

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

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

TABLE 1. α-Tubulin protein sequence comparisons

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

[a] Percentages of identical amino acid residues are given based on the alignments shown in Fig. 4 of the porcine (45), *Schizosaccharomyces pombe* (69), and *Chlamydomonas reinhardi* α1 (62) α-tubulin sequences.

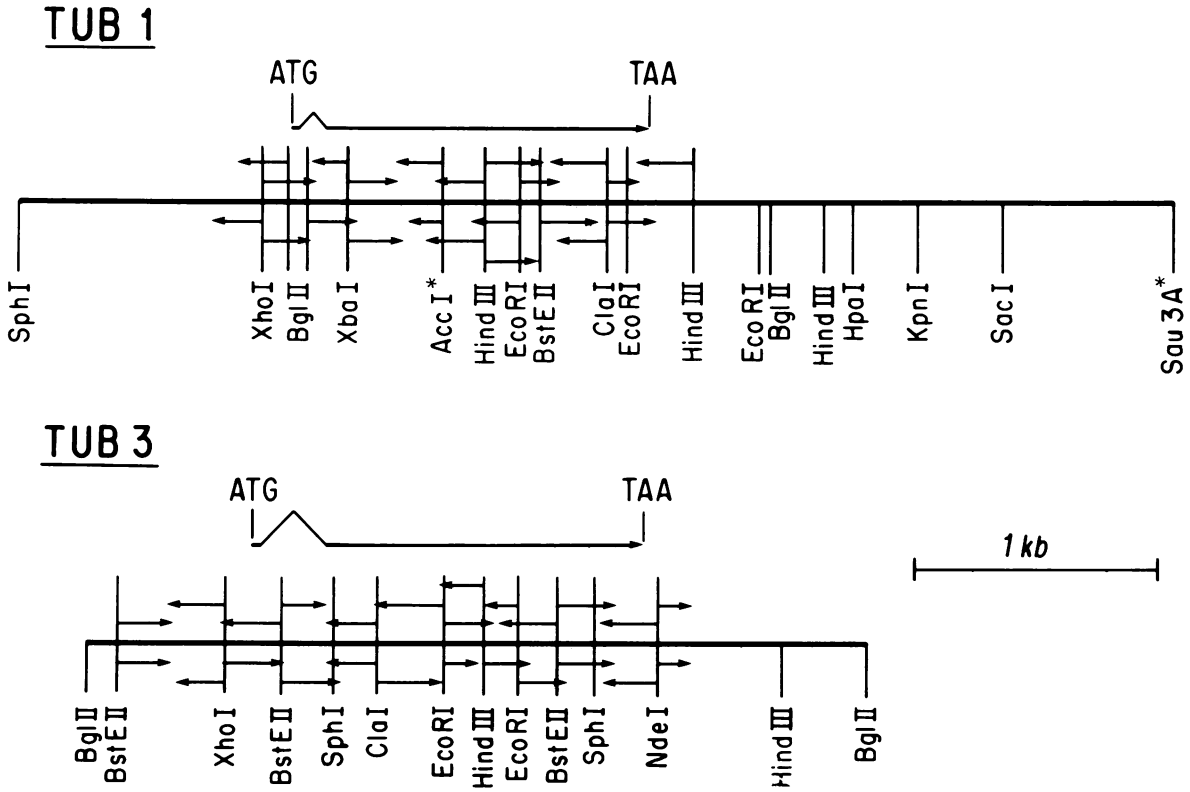


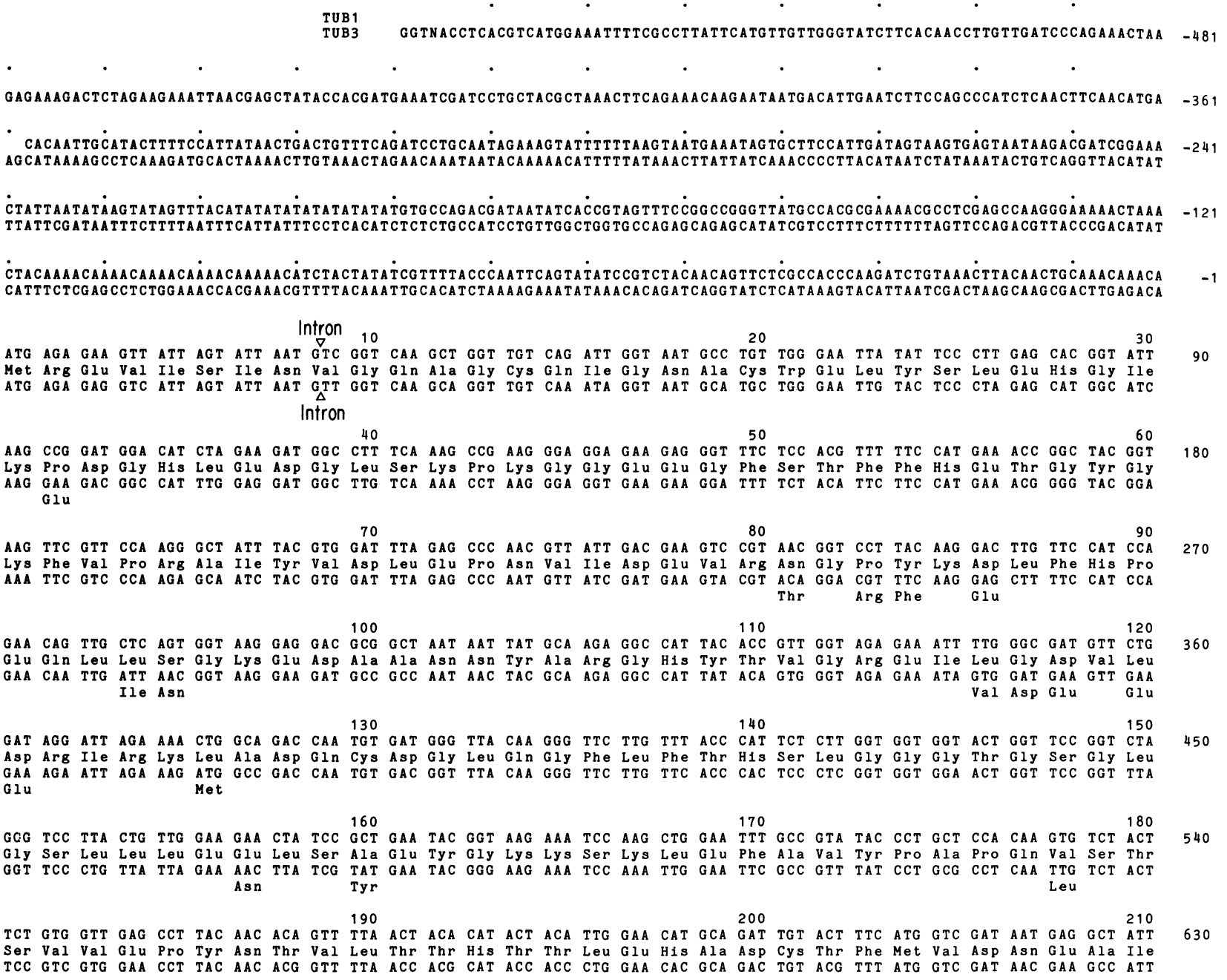
FIG. 1. Restriction enzyme maps, sequencing strategies, and open reading frames in the α-tubulin gene regions. Fragments of *S. cerevisiae* DNA containing the *TUB1* and *TUB3* genes are shown. The *TUB1* fragment was cloned between the *Sph*I and *Bam*HI sites of pBR322 to make plasmid pRB306. The *TUB3*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

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

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

Expression of *TUB1* and *TUB3*. Gel transfer hybridization with whole-cell RNA from exponentially growing cultures of haploid cells showed that both genes gave rise to an mRNA of approximately 1.6 kilobases (kb). Transfers were also done with RNA from an *rna2 rna8* temperature-sensitive splicing mutant (54, 67) at the permissive and restrictive temperatures. The results of the analysis with a *TUB3* probe are shown in Fig. 5. At the restrictive temperature (35°C), putative mRNA precursors to both the *TUB1* and *TUB3* sequences were observed, each larger by approximately the amount predicted from the size of the proposed introns. The *TUB1* gene appeared to be expressed at a higher level than the *TUB3* gene, but this could not be estimated quantitatively because of cross-hybridization of the probes from each gene with the mRNA from the other. The fact that both genes expressed a 1.6-kb mRNA was confirmed by using RNA from null mutants of each gene, which are described in the accompanying paper (57). When the entire *TUB1* region shown in Fig. 1 was used as a probe, a transcript of 1.9 kb also hybridized. The *TUB3* fragment shown in Fig. 1 hybridized to additional transcripts of 1.1 and 2.2 kb. We have not investigated further the origin of these transcripts.

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


TUB1
TUB3 GGTNACCTCACGTCATGGAAATTTTCGCCTTATTCATGTTGTTGGGTATCTTCACAACCTTGTTGATCCCAGAAACTAA -481
GAGAAAGACTCTAGAAGAAATTAACGAGCTATACCACGATGAAATCGATCCTGCTACGCTAAACTTCAGAAACAAGAATAATGACATTGAATCTTCCAGCCCATCTCAACTTCAACATGA -361
CACAATTGCATACTTTTCCATTATAACTGACTGTTTCAGATCCTGCAATAGAAAGTATTTTTTAAGTAATGAAATAGTGCTTCCATTGATAGTAAGTGAGTAATAAGACGATCGGAAA -241
AGCATAAAAGCCTCAAAGATGCACTAAAACTTGTAAACTAGAACAAATAATACAAAAACATTTTTATAAACTTATTATCAAACCCCTTACATAATCTATAAATACTGTCAGGTTACATAT
CTATTAATATAAGTATAGTTTACATATATATATATATATGTGCCAGACGATAATATCACCGTAGTTTCCGGCCGGGTTATGCCACGCGAAAACGCCTCGAGCCAAGGGAAAAACTAAA -121
TTATTCGATAATTTCTTTTAATTTCATTATTTCCTCACATCTCTCTGCCATCCTGTTGGCTGGTGCCAGAGCAGAGCATATCGTCCTTTCTTTTTTAGTTCCAGACGTTACCCGACATAT
CTACAAAACAAAACAAAACAAAACAAAAACATCTACTATATCGTTTTACCCAATTCAGTATATCCGTCTACAACAGTTCTCGCCACCCAAGATCTGTAAACTTACAACTGCAAACAAACA -1
CATTTCTCGAGCCTCTGGAAACCACGAAACGTTTTACAAATTGCACATCTAAAAGAAATATAAACACAGATCAGGTATCTCATAAAGTACATTAATCGACTAAGCAAGCGACTTGAGACA
Intron
Intron
ATG AGA GAA GTT ATT AGT ATT AAT GTC GGT CAA GCT GGT TGT CAG ATT GGT AAT GCC TGT TGG GAA TTA TAT TCC CTT GAG CAC GGT ATT 90
Met Arg Glu Val Ile Ser Ile Asn Val Gly Gln Ala Gly Cys Gln Ile Gly Asn Ala Cys Trp Glu Leu Tyr Ser Leu Glu His Gly Ile
ATG AGA GAG GTC ATT AGT ATT AAT GTT GGT CAA GCA GGT TGT CAA ATA GGT AAT GCA TGC TGG GAA TTG TAC TCC CTA GAG CAT GGC ATC
AAG CCG GAT GGA CAT CTA GAA GAT GGC CTT TCA AAG CCG AAG GGA GGA GAA GAG GGT TTC TCC ACG TTT TTC CAT GAA ACC GGC TAC GGT 180
Lys Pro Asp Gly His Leu Glu Asp Gly Leu Ser Lys Pro Lys Gly Gly Glu Glu Gly Phe Ser Thr Phe Phe His Glu Thr Gly Tyr Gly
AAG GAA GAC GGC CAT TTG GAG GAT GGC TTG TCA AAA CCT AAG GGA GGT GAA GAA GGA TTT TCT ACA TTC TTC CAT GAA ACG GGG TAC GGA
Glu
AAG TTC GTT CCA AGG GCT ATT TAC GTG GAT TTA GAG CCC AAC GTT ATT GAC GAA GTC CGT AAC GGT CCT TAC AAG GAC TTG TTC CAT CCA 270
Lys Phe Val Pro Arg Ala Ile Tyr Val Asp Leu Glu Pro Asn Val Ile Asp Glu Val Arg Asn Gly Pro Tyr Lys Asp Leu Phe His Pro
AAA TTC GTC CCA AGA GCA ATC TAC GTG GAT TTA GAG CCC AAT GTT ATC GAT GAA GTA CGT ACA GGA CGT TTC AAG GAG CTT TTC CAT CCA
Thr Arg Phe Glu
GAA CAG TTG CTC AGT GGT AAG GAG GAC GCG GCT AAT AAT TAT GCA AGA GGC CAT TAC ACC GTT GGT AGA GAA ATT TTG GGC GAT GTT CTG 360
Glu Gln Leu Leu Ser Gly Lys Glu Asp Ala Ala Asn Asn Tyr Ala Arg Gly His Tyr Thr Val Gly Arg Glu Ile Leu Gly Asp Val Leu
GAA CAA TTG ATT AAC GGT AAG GAA GAT GCC GCC AAT AAC TAC GCA AGA GGC CAT TAT ACA GTG GGT AGA GAA ATA GTG GAT GAA GTT GAA
Ile Asn Val Asp Glu Glu
GAT AGG ATT AGA AAA CTG GCA GAC CAA TGT GAT GGG TTA CAA GGG TTC TTG TTT ACC CAT TCT CTT GGT GGT GGT ACT GGT TCC GGT CTA 450
Asp Arg Ile Arg Lys Leu Ala Asp Gln Cys Asp Gly Leu Gln Gly Phe Leu Phe Thr His Ser Leu Gly Gly Gly Thr Gly Ser Gly Leu
GAA AGA ATT AGA AAG ATG GCC GAC CAA TGT GAC GGT TTA CAA GGG TTC TTG TTC ACC CAC TCC CTC GGT GGT GGA ACT GGT TCC GGT TTA
Glu Met
GGG TCC TTA CTG TTG GAA GAA CTA TCC GCT GAA TAC GGT AAG AAA TCC AAG CTG GAA TTT GCC GTA TAC CCT GCT CCA CAA GTG TCT ACT 540
Gly Ser Leu Leu Leu Glu Glu Leu Ser Ala Glu Tyr Gly Lys Lys Ser Lys Leu Glu Phe Ala Val Tyr Pro Ala Pro Gln Val Ser Thr
GGT TCC CTG TTA TTA GAA AAC TTA TCG TAT GAA TAC GGG AAG AAA TCC AAA TTG GAA TTC GCC GTT TAT CCT GCG CCT CAA TTG TCT ACT
Asn Tyr Leu
TCT GTG GTT GAG CCT TAC AAC ACA GTT TTA ACT ACA CAT ACT ACA TTG GAA CAT GCA GAT TGT ACT TTC ATG GTC GAT AAT GAG GCT ATT 630
Ser Val Val Glu Pro Tyr Asn Thr Val Leu Thr Thr His Thr Thr Leu Glu His Ala Asp Cys Thr Phe Met Val Asp Asn Glu Ala Ile
TCC GTC GTG GAA CCT TAC AAC ACG GTT TTA ACC ACG CAT ACC ACC CTG GAA CAC GCA GAC TGT ACG TTT ATG GTC GAT AAC GAA GCC ATT

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                                        220                                 230                                 240
TAC GAC ATG TGC AAA AGA AAC TTG GAT ATC CCA AGA CCA AGC TTT GCA AAC TTA AAC AAC CTA ATT GCT CAA GTG GTA TCA TCT GTT ACA   720
Tyr Asp Met Cys Lys Arg Asn Leu Asp Ile Pro Arg Pro Ser Phe Ala Asn Leu Asn Asn Leu Ile Ala Gln Val Val Ser Ser Val Thr
TAC GAT ATA TGC AAG AGG AAC TTG GGA ATT TCT AGA CCA AGC TTC AGC AAC TTG AAC GGG TTG ATT GCC CAA GTG ATA TCA TCT GTT ACA
        Ile                     Gly     Ser                 Ser             Gly                     Ile

                                        250                                 260                                 270
GCA TCA TTG AGA TTC GAC GGT TCA TTA AAT GTA GAT TTG AAC GAA TTT CAA ACC AAT TTG GTT CCA TAT CCA AGA ATT CAT TTC CCC TTA   810
Ala Ser Leu Arg Phe Asp Gly Ser Leu Asn Val Asp Leu Asn Glu Phe Gln Thr Asn Leu Val Pro Tyr Pro Arg Ile His Phe Pro Leu
GCC TCT TTG AGG TTC GAT GGT TCA TTA AAC GTG GAT TTG AAC GAA TTT CAG ACC AAC TTG GTA CCA TAT CCA AGA ATT CAT TTC CCT TTG


                                        280                                 290                                 300
GTT TCA TAT TCT CCA GTC TTA TCC AAA TCA AAG GCA TTC CAT GAG TCC AAC TCT GTG TCA GAA ATT ACA AAC GCT TGT TTT GAA CCT GGT   900
Val Ser Tyr Ser Pro Val Leu Ser Lys Ser Lys Ala Phe His Glu Ser Asn Ser Val Ser Glu Ile Thr Asn Ala Cys Phe Glu Pro Gly
GTT TCC TAC GCA CCC ATC TTG TCC AAG AAG AGG GCC ACC CAT GAA TCC AAC TCC GTG TCA GAA ATC ACA AAC GCT TGT TTC GAA CCG GGC
            Ala     Ile             Lys Arg     Thr

                                        310                                 320                                 330
AAC CAG ATG GTC AAG TGT GAT CCA AGA GAT GGT AAA TAC ATG GCT ACT TGT CTG TTA TAC AGG GGT GAT GTG GTA ACG GGA GAT GTT CAA   990
Asn Gln Met Val Lys Cys Asp Pro Arg Asp Gly Lys Tyr Met Ala Thr Cys Leu Leu Tyr Arg Gly Asp Val Val Thr Gly Asp Val Gln
AAT CAA ATG GTT AAG TGT GAC CCA ACA AAG GGG AAG TAC ATG GCT AAC TGT TTG TTA TAC AGA GGT GAC GTG GTG ACC AGA GAT GTC CAA
                        Thr Lys                         Asn                                 Arg

                                        340                                 350                                 360
AGA GCT GTC GAG CAG GTG AAA AAC AAG AAG ACC GTC CAA TTG GTT GAT TGG TGT CCA ACT GGT TTC AAG ATC GGT ATT TGC TAC GAA CCT  1080
Arg Ala Val Glu Gln Val Lys Asn Lys Lys Thr Val Gln Leu Val Asp Trp Cys Pro Thr Gly Phe Lys Ile Gly Ile Cys Tyr Glu Pro
AGA GCC GTC GAA CAG GTG AAG AAT AAA AAA ACA GTA CAA ATG GTG GAT TGG TGT CCA ACA GGA TTT AAG ATT GGT ATT TGT TAT GAG CCA
                                        Met

                                        370                                 380                                 390
CCA ACT GCC ACA CCA AAC TCA CAA TTG GCC ACT GTG GAT AGG GCC GTC TGT ATG TTG TCA AAT ACC ACA TCC ATT GCT GAG GCT TGG AAG  1170
Pro Thr Ala Thr Pro Asn Ser Gln Leu Ala Thr Val Asp Arg Ala Val Cys Met Leu Ser Asn Thr Thr Ser Ile Ala Glu Ala Trp Lys
CCA AGT GTG ATA CCA AGT TCC GAA TTA GCC AAT GTG GAT AGA GCT GTC TGC ATG CTA TCC AAC ACC ACT GCC ATC GCG GAC GCT TGG AAG
    Ser Val Ile     Ser     Glu         Asn                                         Ala         Asp

                                        400                                 410                                 420
AGA ATC GAT AGA AAA TTC GAT TTA ATG TAT GCC AAA CGT GCT TTC GTC CAC TGG TAT GTC GGT GAA GGT ATG GAA GAA GGT GAA TTC ACC  1260
Arg Ile Asp Arg Lys Phe Asp Leu Met Tyr Ala Lys Arg Ala Phe Val His Trp Tyr Val Gly Glu Gly Met Glu Glu Gly Glu Phe Thr
AGA ATC GAT CAG AAA TTC GAC CTG ATG TAT GCC AAA CGT GCT TTC GTC CAT TGG TAT GTC GGT GAA GGT ATG GAA GAA GGT GAG TTC ACC
            Gln

                                        430                                 440                         447
GAA GCT AGA GAA GAT TTG GCT GCT TTA GAA AGA GAT TAC ATC GAA GTG GGT GCC GAC TCA TAC GCT GAG GAA GAG GAA TTT TAA GGT TCA  1350
Glu Ala Arg Glu Asp Leu Ala Ala Leu Glu Arg Asp Tyr Ile Glu Val Gly Ala Asp Ser Tyr Ala Glu Glu Glu Glu Phe End
GAA GCT AGA GAA GAT TTA GCT GCC TTA GAA AGA GAT TAT ATT GAA GTG GGT GCC GAT TCT TAC GCT GAG GAG TTC TAA GAA AGT GGC TCT
                                                                                    Phe End

.       .       .       .       .       .       .       .       .       .       .       .       .       .       .
CTCTCGCCCCCAACCTCCTTATCCTTTCTATATTTCAAGTATATTGTAAAAAAAAAAAATTCCCCCTACTGTTATATCTCTTTGGTGATATCTTATCTATAATCTACTTATTATATTTCTC  1470
GATCCCGATACCCACTTATAGATTTATATTTACTTAATGTCCATATGTATATACATATATATATATATATATATATACGCTTATATATATATATGCATATTCTATCCAAATAAAGATACC

.       .       .       .       .       .       .       .
TAATATCTATGTACAAAAAATAAAAAAAAAAACCATTCTCCTTCTCTAACCTGCATTTGATGAAAGCTT  1539
TCTAATTATCGAATTATTTTTCACTTACCTTTAGGTGTACAGTTAGAACAATAGCTTAATTAAATTTAT
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FIG. 2. Sequences of coding and flanking regions. The nucleotide sequence of the *TUB1* gene is shown above the *TUB3* sequence, numbered to the right of each line. Translation of the *TUB1* coding sequence is shown below its sequence line. Only the differences in the *TUB3* amino acid sequence from that of *TUB1* are shown. Amino acids are numbered above the coding sequence lines. The positions of the introns (see Fig. 3) are shown by arrowheads.

TUB1 INTRON:

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   1  GTATGTTCGA TTTGCCCGTC CAGGCTAGAT CTTTTTTTGT TAGTTCATTT
  51  TGGCGTTTCA TCTTTCATTA CTAACTTCAA CGCAAAATTT TTTTTTTTTT
 101  GATTTCTCTT TACAG
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TUB3 INTRON:

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   1  GTATGTATGC GTTCCTTTTT TTGTTCAATA TTCGCAACCA ATGGCACCTG
  51  TGGGACAGGG AAAGAAGTTT GATCTGATCT GGTTTGATTC ATTCCCAATT
 101  GGTCACCATC TGGTTGATTT ACGGCAAATA ATTTGACTTG TACCAGCACA
 151  GTTTACTAAC AGTTTCTTTT TCTCCATTTT TTCTGGGCAT ACTCGGACGA
 201  AAAAGCTCAT AATTGACCTC ATTACATGGG GAGTGATTTT TGTGTCTTCT
 251  TCTTCGGAGG ATTGCTGGAA CTTTTGTTAT TTTTCTTTTT TACAACAG
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FIG. 3. Intron sequences. The nucleotide sequences of the putative *TUB1* and *TUB3* introns are shown. Consensus 5′, internal, and 3′ splicing sequences are enclosed by boxes.

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

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

DISCUSSION

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

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

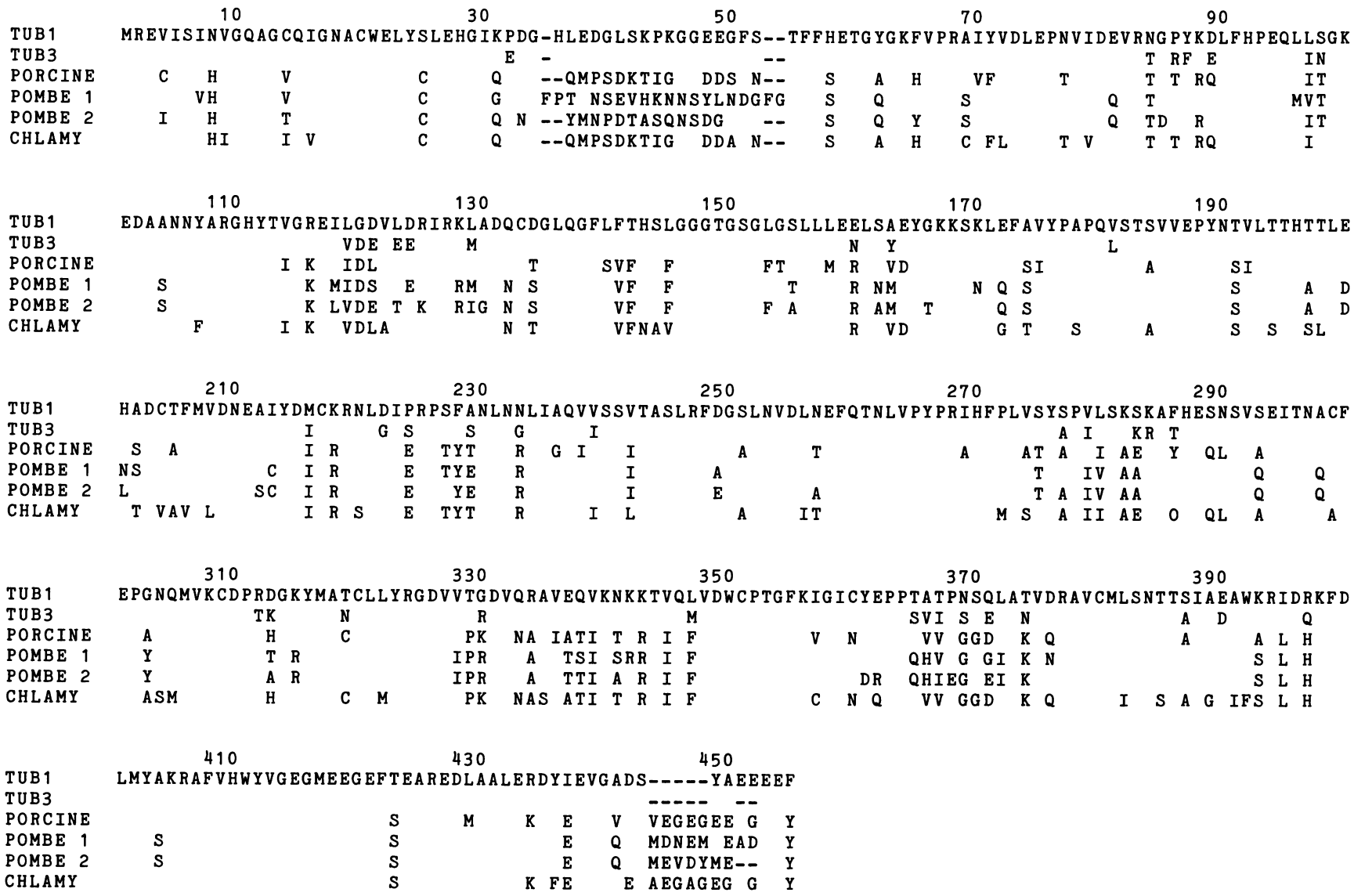

FIG. 4. α-Tubulin sequence comparisons. The *TUB1* amino acid sequence is shown in one-letter code and compared with that of *TUB3*, porcine (45), *Shizosaccharomyces pombe* (69), and *C. reinhardi* (62) α-tubulins. Since the two *C. reinhardi* proteins differ at only two residues, only the α1 gene product is shown. The numbers correspond to the sequence of *Schizosaccharomyces pombe* α1 tubulin, with dashes inserted into the other sequences to bring them into register. Positions of identity to the *TUB1* sequence are left blank.

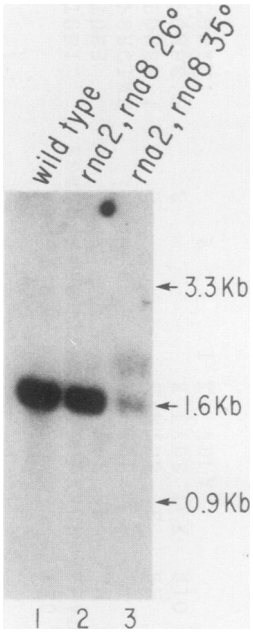

FIG. 5. RNA gel transfer hybridization analysis of the *TUB3* transcripts. Total cellular RNA was made from exponentially growing wild-type cells or an *rna2 rna8* mutant grown at the permissive temperature (26°C) or grown at the permissive temperature and then shifted to the restrictive temperature (35°C) for 30 min. The RNA was run on a formaldehyde-agarose gel, transferred to a GeneScreen membrane, and analyzed with a nick-translated probe made from the *Xho*I-*Nde*I fragment containing the *TUB3* coding sequence. The numbers to the right indicate the positions of size markers of the larger RNA (3.3 kb) and the *URA3* transcript (0.9 kb) along with the calculated size of the *TUB1* and *TUB3* transcripts (1.6 kb).

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

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

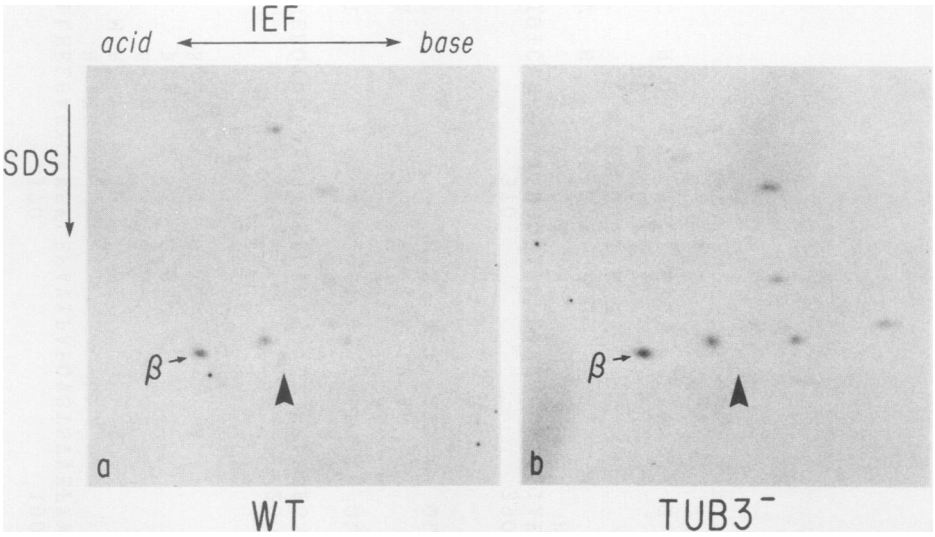

FIG. 6. Two-dimensional gel analysis of yeast tubulin. Yeast cells were labeled with [^{35}S]methionine, and the tubulin was fractionated into unassembled and assembled pools (44). The assembled fraction was mixed with unlabeled carrier calf brain microtubule protein and carried through two cycles of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

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

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

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

ACKNOWLEDGMENTS

We thank T. Toda and M. Yanagida for their generous gift of the *Schizosaccharomyces pombe NDA2* α-tubulin gene. We thank J. Thomas, C. Holm, T. Huffaker, and P. Novick for valuable discussions. We thank D. Schatz, T. Huffaker, A. Hoyt, D. Drubin, and W. Katz for comments on the manuscript.

This work was supported by Public Health Service grants GM-21253 and GM-18973 from the National Institutes of Health, by American Cancer Society grants VC90 (to D.B.) and CD226, and by the National Institutes of Health (PO1-CA26712) (to F.S.). P.J.S. was supported by a Graduate Fellowship from the National Science Foundation and by a Fellowship from the Whitaker Health Sciences Fund. L.P. is a Johnson and Johnson Fellow.

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