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A Gene Encoding the Major Beta Tubulin of the Mitotic Spindle in *Physarum polycephalum* Plasmodia

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The multinucleate plasmodium of *Physarum polycephalum* is unusual among eucaryotic cells in that it uses tubulins only in mitotic-spindle microtubules; cytoskeletal, flagellar, and centriolar microtubules are absent in this cell type. We have identified a β-tubulin cDNA clone, β105, which is shown to correspond to the transcript of the *betC* β-tubulin locus and to encode β2 tubulin, the β tubulin expressed specifically in the plasmodium and used exclusively in the mitotic spindle. *Physarum* amoebae utilize tubulins in the cytoskeleton, centrioles, and flagella, in addition to the mitotic spindle. Sequence analysis shows that β2 tubulin is only 83% identical to the two β tubulins expressed in amoebae. This compares with 70 to 83% identity between *Physarum* β2 tubulin and the β tubulins of yeasts, fungi, alga, trypanosome, fruit fly, chicken, and mouse. On the other hand, *Physarum* β2 tubulin is no more similar to, for example, *Aspergillus* β tubulins than it is to those of *Drosophila melanogaster* or mammals. Several eucaryotes express at least one widely diverged β tubulin as well as one or more β tubulins that conform more closely to a consensus β-tubulin sequence. We suggest that β-tubulins diverge more when their expression pattern is restricted, especially when this restriction results in their use in fewer functions. This divergence among β tubulins could have resulted through neutral drift. For example, exclusive use of *Physarum* β2 tubulin in the spindle may have allowed more amino acid substitutions than would be functionally tolerable in the β tubulins that are utilized in multiple microtubular organelles. Alternatively, restricted use of β tubulins may allow positive selection to operate more freely to refine β-tubulin function.

Most eucaryotes express multiple genes each for α and β tubulins, the principal protein components of microtubules. The products of these genes usually have distinct sequences. For β tubulins, identity between multiple polypeptides within a single organism varies from 100% for *Chlamydomonas reinhardtii* (38) to 78% for mammals (34). The varied functions of microtubules in the cytoskeleton, centrioles, spindles, and flagella, together with the observed tubulin sequence differences, have inspired the conclusion that the sequence differences between multiple tubulins within an organism have been positively selected during evolution (32). The murine β tubulin Mβ1 is only 78% identical to several other murine β tubulins and is expressed specifically in hematopoietic tissues, prompting the speculation that at least some of the sequence differences reflect functional specialization of individual β tubulins (34).

In the myxomycete *Physarum polycephalum*, three β-tubulin genes are known; one gene is expressed specifically in the amoebal cell type, another gene is expressed specifically in the plasmodial cell type, and the third gene, *betB*, is expressed in both amoebae and plasmodia (4, 28). The multinucleate plasmodium of *P. polycephalum* is unusual among eucaryotic cells in that it uses tubulins only in the mitotic-spindle microtubules; cytoskeletal, flagellar, and centriolar microtubules are absent from this cell type, even though they are present in amoebal or flagellate cell types (9, 23, 27). The β2-tubulin isotype expressed specifically in the plasmodium thus is used exclusively in the mitotic spindle (21). It is therefore of interest to determine the sequence of β2 tubulin and to analyze its structure in the context of the structures of other β tubulins. This work reports the identification of the gene encoding β2 tubulin and presents the sequence of a cDNA clone from this locus. The sequence of β2 tubulin is deduced from the cDNA sequence and compared with that of β tubulins from *Physarum* amoebae, yeasts, fungi, *C. reinhardtii*, trypanosome, fruit fly, chicken, and mouse.

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MATERIALS AND METHODS

Screening the cDNA library. The cDNA library was generously donated by V. Vogt and S. Kostelny, Cornell University, Ithaca, N.Y. cDNA was synthesized from plasmodial poly(A)$^+$ RNA (strain M_3CVIII) and treated with *Eco*RI methylase; *Eco*RI linkers were then added. After digestion with *Eco*RI, cDNAs were ligated into *Eco*RI-digested vector lambda gt11.

Approximately 10^6 plaques were screened for hybridization to a ^{32}P-labeled *Bgl*II-*Stu*I fragment of the chicken pT2 β-tubulin cDNA (5). Nitrocellulose filters were washed in 3× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) in 0.1% sodium dodecyl sulfate. DNA was extracted from bacteriophages containing β-tubulin-homologous inserts after plaque purification to yield 100% of plaques giving hybridization.

Subcloning cDNAs and sequencing strategy. Restriction enzymes were obtained from New England BioLabs, Beverly, Mass. *Eco*RI cDNA inserts were subcloned into the *Eco*RI site of plasmid pUC18 for nucleic acid hybridization studies and into phage M13mp18 (37) for sequencing by the dideoxynucleotide chain termination method (26). M13mp18 subclones were further subcloned by the Cyclone deletion system (International Biotechnologies, Inc., New Haven, Conn.) by the method of Dale et al. (6). To identify the locus of origin of the cDNAs, the 5′ *Eco*RI-*Rsa*I fragment of β102 was ligated into *Eco*RI-*Hin*cII-digested pUC18; this fragment was also subcloned into M13mp18 for sequencing. A detailed restriction map and the complete nucleic acid sequence can be obtained by contacting T. G. Burland.

For sequencing, the 1.3- and 0.3-kilobase-pair (kb) *Eco*RI inserts were initially subcloned separately, and a complete sequence was compiled from each. To confirm that the two *Eco*RI fragments were contiguous and in the correct orientation, a *Hin*dIII-*Nae*I fragment spanning the internal *Eco*RI site was ligated directly from the original lambda gt11 recombinant into *Hin*dIII-*Hin*cII-digested M13mp18 replicative-form DNA; single-stranded M13 recombinant DNA was obtained from this construct and sequenced.

For production of transcripts, fragments were subcloned into the *Eco*RI site of the pUC-derived plasmid pGEM4Blue (Promega Biotech, Madison, Wis.), which contains a multiple cloning site flanked by SP6 and T7 promoters. Restriction mapping was used to confirm that the 5′ end of the gene was adjacent to the SP6 promoter.

In vitro transcription and translation. The vector carrying β105 cDNA was linearized with *Xba*I and then extracted with phenol and chloroform, precipitated with ethanol, and suspended in 10 mM Tris (pH 8)–1 mM EDTA; capping of transcripts was not necessary for translation. Linear β105 cDNA was then transcribed in vitro by using SP6 polymerase (16). About 300 ng of the transcripts was translated in a wheat germ in vitro translation system (RPN.1; Amersham Corp., Arlington Heights, Ill.) in 30-μl reactions containing [^{35}S]methionine (SJ.204; Amersham) and 19 unlabeled amino acids. To identify the product of the transcripts, 1/15 volume of the translation products was coelectrophoresed with unlabeled plasmodial extracts as described previously (3), except that gels were first blotted to nitrocellulose and probed with β-tubulin-specific monoclonal antibody KMX-1 (2) before autoradiography. Reaction with primary antibody was detected by using horseradish peroxidase-conjugated secondary antibody.

Sequence comparisons. We chose only a subset of the available β-tubulin sequences for comparisons. Other vertebrate β tubulins were omitted because they are so similar to the mouse β5 and chicken β1 tubulins. For mouse and chicken sequences, we used only one canonical sequence plus the sequence most diverged from the canonical one. The *Drosophila* β1-tubulin sequence was not yet available.

Trypanosome (12), *C. reinhardtii* (38), *Saccharomyces cerevisiae* (19), *Schizosaccharomyces pombe* (10), chicken β1-tubulin (33), and *Neurospora crassa* (20) DNA sequences were obtained from GenBank, using the search software of DNASTAR, Inc. (Madison, Wis.). DNA sequences for *Drosophila* β2 and β3 tubulins (24), chicken β4 tubulin (31), and *Aspergillus* β1/2 (*benA*) and β3 (*tubC*) tubulins (15) were entered manually into the computer, using DNASTAR software, and the *Physarum* amoebal β1-tubulin (30) and mouse β1- and β5-tubulin sequences (34) were entered as polypeptides. For the *Physarum* β1 tubulin, we used the Ser-283 rather than the Ala-283 sequence (30). DNA sequences were translated with the DNASTAR program TRANSLATE, and comparisons were made pairwise with program AALIGN, which uses a Lipmann method to identify homologies and introduces gaps and makes the final alignment by the method of Needleman and Wunsch (18). Percent identities were calculated for the overlapping regions only of the β-tubulin polypeptides. Thus, any additional residues at the carboxyl terminus in one member of the pair were ignored for the purposes of calculating identities; this accounts for occasional slight variations in percent identities in Table 1 compared with published data. All percentages were rounded to the nearest integer. Figure 3 was produced with the aid of the GAP and PRETTY programs of the University of Wisconsin Genetics Computer Group.

RESULTS

Identification of β2-tubulin cDNA clones. Of 10^6 cDNA clones screened, 74 hybridized to the chicken β-tubulin probe, and three (β102, β105, and β106) were studied in detail. All three showed a similar restriction map. β102 contained a 1.3-kb *Eco*RI fragment that hybridized to the chicken probe, whereas β105 and β106 contained this fragment and a 0.3-kb *Eco*RI fragment that did not hybridize to the probe. The smaller fragment would not be expected to hybridize if it corresponded to the 3′ end of the transcript, since this end was truncated in the chicken DNA probe. The 1.3- and the 0.3-kb fragments comprise a nearly full-length cDNA for β tubulin, since they contain a complete coding region (see below) and the size of β-tubulin mRNA in plasmodia is approximately 1.6 kb (27). A 237-base-pair fragment from the 5′ *Eco*RI site to the first *Rsa*I site hybridized specifically to the *betC* β-tubulin locus in Southern blots (Fig. 1), indicating that the cDNA was derived from this rather than from either of the other two β-tubulin loci.

DNA from β105 was partially digested with *Eco*RI, and a 1.6-kb fragment corresponding to the 1.3- and 0.3-kb fragments was subcloned into plasmid pGEM4Blue to produce plasmid pβ105/SP6. RNA was transcribed from pβ105/SP6 by using SP6 RNA polymerase, and the resulting transcripts were translated in vitro. Translation products were coelectrophoresed on two-dimensional gels with unlabeled *Physarum* plasmodial homogenates. Western immunoblotting, using the β-tubulin-specific monoclonal antibody KMX-1 (2), served to locate both β1 and β2 tubulins on the gels, and autoradiography of the blot identified the position of the in vitro translation product relative to the authentic β tubulins of the plasmodium. The results (Fig. 2) demonstrate that β105 encodes plasmodium-specific β2-tubulin. RNA blotting has shown that the β2-tubulin transcript is also plasmodium specific (L. Solnica-Krezel, W. F. Dove, and T. G. Burland, J. Gen. Microbiol., in press).

Sequence of β105. The β105 cDNA includes 89 nucleotides of 5′ untranslated sequence, an open reading frame of 454 codons for β tubulin, 103 nucleotides of 3′ untranslated sequence, and 37 nucleotides of poly(A) tail. A putative poly(A)-addition signal AAUAUA occurs 15 base pairs upstream from the poly(A) tail. Codon usage shows no strong bias; indeed, every amino acid codon except CGG (arginine) is used at least once. The CGG codon is also not used in a *Physarum altB* α-tubulin gene (17).

In view of the marked variation in level and stability of tubulin transcripts at certain times during the plasmodial cell cycle (27; L. L. Green, Ph.D. thesis, University of Wisconsin, Madison, 1987), it was of interest to search for the sequence AUUU in the 3′ untranslated region, since the presence of multiple copies of this sequence has been implicated in mRNA instability (29). However, only one copy of this sequence was found in the 3′ untranslated region of β105 (and of the plasmodium-specific α-tubulin gene *altB* [17]), suggesting that a distinct mechanism of regulation may be responsible for the variation in tubulin transcript level during the cell cycle.

Birkett et al. (1) determined the reaction of *Physarum* tubulins with several monoclonal antibodies and noted an anomaly in that β2 tubulin reacted with the anti-α-tubulin antibody YL1/2 and the plasmodium-specific α2 tubulin did not. To account for this observation, they proposed that the gene for β2 tubulin arose as a result of recombination between the genes for *Physarum* α1 and β1 tubulins. The epitope usually recognized by YL1/2 on α tubulins is Glu-

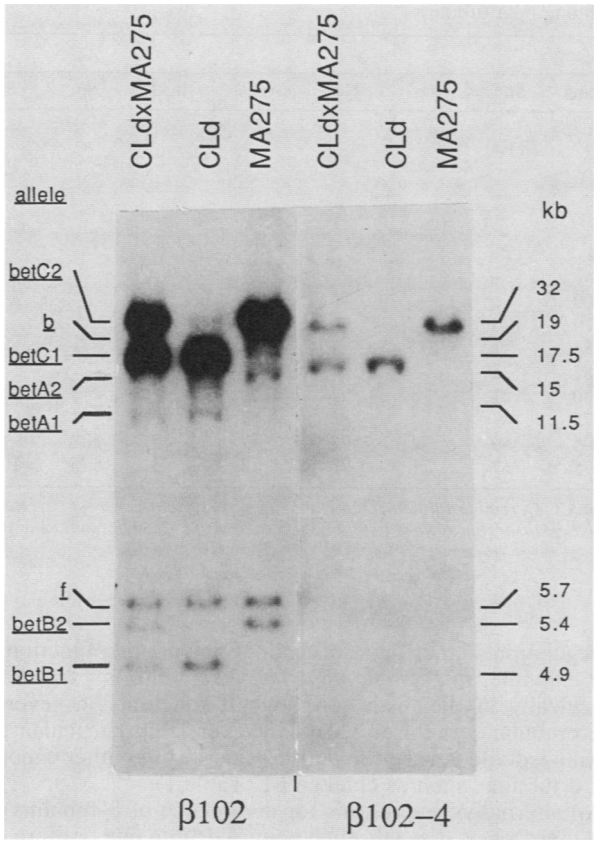

FIG. 1. Identification of the locus for β105. DNAs from haploid strains CLd and MA275 and from the diploid CLd×MA275 were digested with *StuI*, electrophoresed, and blotted to nitrocellulose as described previously (28) and then were probed with ^{32}P-labeled DNA as indicated. Three pairs of *bet* (β-tubulin) alleles show restriction-fragment-length polymorphism between the two strains (28); their positions are indicated on the left, and molecular sizes (in kilobases [kb]) are shown on the right. Use of these strains helps to make locus identification unequivocal for closely migrating bands. There are also two monomorphic *bet* bands, *b* and *f* (28). β102 is the 1.3-kb *Eco*RI fragment, and β102-4 is the 5′ 238-base-pair *Eco*RI-*Rsa*I fragment. The β102 blot was washed at low stringency (3× SSC; 55°C), yet β102 still hybridized preferentially to *betC* and only weakly to *betA*, *betB* and monomorphic bands *b* and *f*. The β102-4 blot was washed stringently (0.1× SSC; 55°C) and showed β102-4 hybridizing specifically to *betC*.

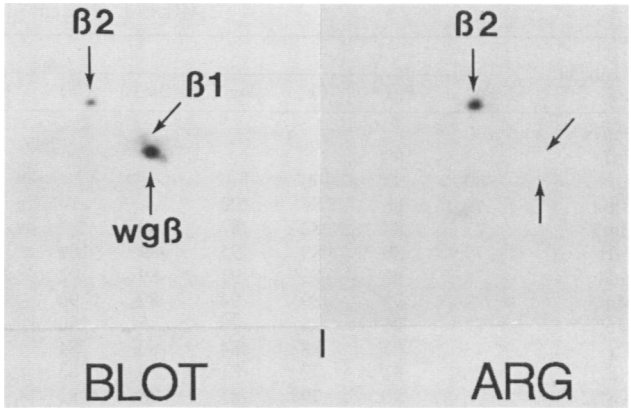

FIG. 2. Identification of the product of the *betC* β-tubulin locus. Both panels are from the same gel. Isoelectric focusing is from left (basic) to right (acidic); denaturing polyacrylamide gel electrophoresis is from top to bottom. The *Physarum* β2 tubulin (β2) is well resolved from β1 tubulin (β1), although β1 tubulin is almost occluded by an endogenous β tubulin (wgβ) from the wheat germ system. Parallel gels of the translation products of plasmodial poly(A) RNA confirm that β1 tubulin migrates almost to the same position as wgβ (not shown). Nevertheless, the blot functions to determine the precise orientation for comparison of blot and autoradiogram (ARG). The major radioactive translation product of β105 is clearly β2 tubulin. Other radioactive species are minor and are of lower apparent molecular weight than β tubulins; they may result from translation of prematurely terminated β105 transcripts.

Glu-Tyr at the carboxyl terminus, but the epitope Glu-Glu-Phe, which we have shown occurs at the β2-tubulin carboxy terminus, is recognized even more strongly (36), and this sequence thus accounts for the reactivity of β2 tubulin with YL1/2. There is no evidence that a recombination event occurred between the genes for α1 and β1 tubulins to produce the β2-tubulin gene.

Intraspecies comparison of *Physarum* β tubulins. Three β-tubulin polypeptides are known in *P. polycephalum*. Amoebae express two β tubulins, β1A and β1B, that are the products of different genes but that are indistinguishable on two-dimensional gels; plasmodia express β1B and β2 tubulins, which are easily resolved (3, 4). A large portion of the sequence of amoebal β tubulins was determined by directly sequencing purified amoebal polypeptide (30), and evidence was obtained for only one amino acid residue difference between the two amoebal β tubulins. Comparison of the β2-tubulin sequence with the available β1A-β1B sequence indicates only 83% identity (Table 1). Thus, β2 tubulin differs from the other two *Physarum* β tubulins almost as much as the mammalian hematopoietic β tubulin differs from other mammalian β tubulins. Indeed, *Physarum* β2 tubulin is essentially as different from *Physarum* β1 tubulin as it is from β tubulins from several other organisms (Table 1). Even when the sequences of *Physarum* β1 and β2 tubulins are compared with one another at the level of individual residues, there are no special regions of similarity that distinguish these *Physarum* β tubulins from other β tubulins.

Interspecies comparisons of β tubulins. Figure 3 presents comparisons of *Physarum* β2 tubulin with the polypeptide sequences corresponding to those of the β-tubulin genes from yeasts, *C. reinhardtii*, fungi, trypanosome, fruit fly, chicken, and mouse, along with the polypeptide sequence from *Physarum* amoebal β tubulin. As expected, certain regions of all β-tubulin sequences are highly conserved, including the putative nucleotide-binding site (residues 58 through 64) and the putative phosphate-binding site (residues 137 through 151), along with other notably conserved regions around residues 1 through 5, 9 through 16, 101 through 114, 176 through 185, 240 through 267, 340 through 349, and 394 through 429. The carboxy terminus is notably the most heterogeneous region.

Percent identities between the different β tubulins are listed in Table 1. All β tubulins studied, including *Physarum* β1 tubulin, have 70 to 83% identity with *Physarum* β2 tubulin. However, *Physarum* β1 tubulin shows considerably more identity to the β tubulins of other organisms, particularly those of *C. reinhardtii*, *D. melanogaster*, chicken, mouse, and trypanosome, than it does to *Physarum* β2 tubulin.

DISCUSSION

Since Fulton and Simpson (8) proposed the multitubulin hypothesis, much enthusiasm has been expressed for the

TABLE 1. Percent identities among beta tubulins

β Tubulin[a]	% Identity														
	Pp2	Pp1	Cr	Dm2	Dm3	Gd1	Gd4	Mm5	Mm1	Tb	Nc	An1/2	An3	Sp	Sc
Pp2	100														
Pp1	83	100													
Cr	80	90	100												
Dm2	79	88	87	100											
Dm3	77	84	82	87	100										
Gd1	79	89	89	95	89	100									
Gd4	78	85	85	91	89	92	100								
Mm5	79	90	89	94	89	96	92	100							
Mm1	72	76	75	78	75	80	78	79	100						
Tb	78	87	88	84	80	84	81	86	71	100					
Nc	76	81	79	81	80	83	81	82	72	78	100				
An1/2	76	80	79	81	78	81	80	80	71	78	93	100			
An3	72	75	75	76	73	76	74	75	67	74	84	83	100		
Sp	71	74	73	75	73	76	75	75	69	72	78	77	75	100	
Sc	70	74	71	74	73	74	75	75	67	71	78	78	73	75	100

[a] Species are indicated by the following two-letter abbreviations. Pp, *P. polycephalum*; Cr, *C. reinhardtii*; Dm, *D. melanogaster*; Gd, *Gallus domesticus*; Mm, *Mus musculus*; Tb, *Trypanosoma brucei*; Nc, *N. crassa*; An, *Aspergillus nidulans*; Sp, *Schizosaccharomyces pombe*; Sc, *S. cerevisiae*. Numbers indicate the multiple β-tubulin polypeptides within the species that are being compared.

idea that the presence of multiple, distinct isotypes for α and β tubulins in a single organism reflects functional specificity for the different isotypes. Support for this view has been drawn from demonstrations that the distinct isotypes usually differ in primary sequence. Despite its attractiveness, this idea has faced some opposition based on experimental data. The compositions of the amoebal cytoskeleton and the plasmodial mitotic spindle in *P. polycephalum* have been analyzed, and both were found to contain the *betB* β-tubulin gene product (E. C. A. Paul, T. G. Burland, and K. Gull, submitted for publication). Lewis et al. (13) studied the distribution of different mammalian β-tubulin isotypes; they expressed the murine Mβ1 gene in HeLa cells and found that even this highly diverged isotype was incorporated into both cytoskeletal and mitotic-spindle microtubules despite its apparent specific in vivo location in the marginal band in hematopoietic tissues. Clearly, this kind of evidence against functional specificity needs to be assessed carefully. For example, coassembly of different β-tubulin isotypes into a microtubular organelle does not rule out a superior functional capacity of one of the isotypes for that organelle, nor does it prove that one particular β-tubulin isotype could assemble into a specific microtubular organelle in the absence of the other β-tubulin isotypes. Nevertheless, these experiments are not encouraging for the notion of functional specificity. Neither is the situation in yeasts, in which one β-tubulin gene is adequate for all of the functions that are required in these organisms (10, 19).

The clearest experimental evidence supporting the idea that distinct tubulin isotypes are used for specific functions comes from studies of flagellated organisms such as *C. reinhardtii*, *Crithidia* sp., and *P. polycephalum* (7, 14, 25), in which the use of an α-tubulin isotype in the flagellum correlates with its posttranslational modification. However, this modification may involve the same gene product that is used in other microtubular organelles, rather than the utilization of a novel gene product as a substrate. There remains serious doubt whether functional specialization can account for differences in the primary structure of tubulins.

Raff (22) suggested that multiple β tubulins in the same organism may serve regulatory requirements, i.e., multiple genes may arise through positive selection for distinct forms of regulation, rather than for distinct polypeptide functions. For example, in *D. melanogaster*, β2 tubulin is expressed specifically in the testis, although it functions in several microtubular structures (11). However, this particular β-tubulin isotype is very similar (95% identity) to other canonical β tubulins such as chicken β1 (Table 1).

An alternative hypothesis for divergence of β tubulins is that divergence arises through neutral drift in one or more of the multiple genes. Such drift would be more feasible for cases in which a particular isotype is required to perform fewer functions, allowing more amino acid substitutions to be tolerated. Thus, functional constraints would be the driving force for conservation of amino acid sequence rather than for generation of distinct sequences. Plausible candidates for this scenario would be the use of *Physarum* β2 tubulin exclusively in the mitotic spindle of the plasmodium, the use of mouse Mβ1 tubulin exclusively in hematopoietic tissues, predominantly in the marginal band, and the use of *Aspergillus* β3 tubulin specifically in conidiation, a developmental process that involves many mitoses in the generation of asexual spores (35). Each of these three tubulins shows no more than 83% identity with other β tubulins within the same organism. The restricted expression of *Drosophila* β2 tubulin to the testis would not be expected to allow as much divergence as, say, that of *Physarum* β2 tubulin, since *Drosophila* β2 tubulin performs multiple functions.

But if β tubulins tend to diverge more when they perform fewer functions, why is *Drosophila* β3 tubulin, which is expressed in more tissues than *Drosophila* β2, more diverged from the canonical β tubulins than *Drosophila* β2? *Drosophila* β3 tubulin is apparently expressed only transiently during certain developmental stages (24) and probably is only a minor expressed β tubulin at these stages. A similar situation seems to apply for chicken β4 tubulin, a minor isotype that shows 92% identity to the canonical chicken β1 (31). Perhaps low levels of expression could allow more divergence in a β-tubulin isotype, if selection pressure for conservation of sequence was relaxed on the less abundant β tubulins. Here, one could argue that a minor isotype would simply need to avoid poisoning microtubular structures when coassembled along with more major isotypes. Even widely diverged β tubulins can copolymerize

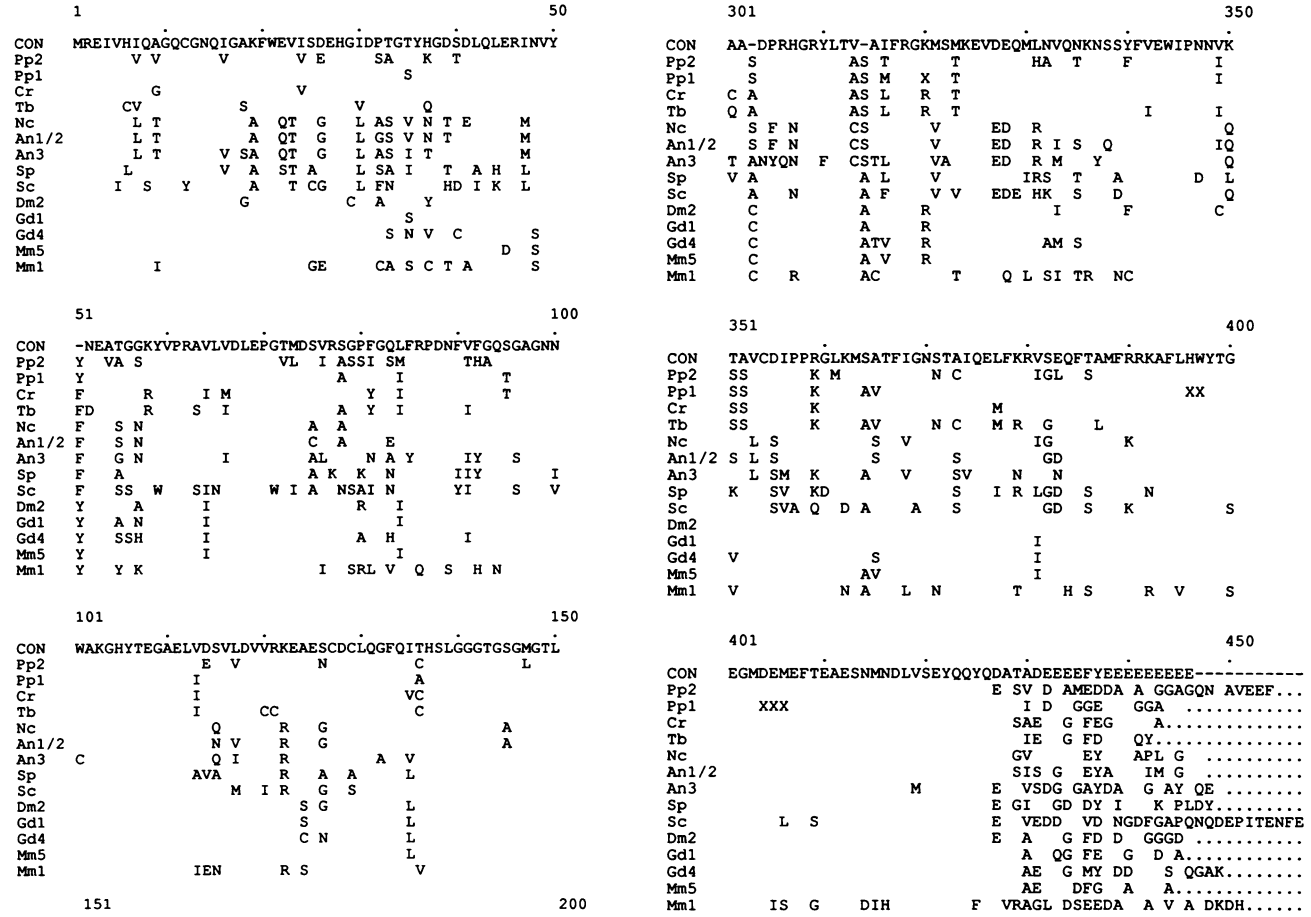

FIG. 3. Alignment of 14 β-tubulin sequences. The two-letter species abbreviations are described in Table 1, footnote *a*. All sequences were aligned with that for *Physarum* β2 tubulin; gaps were not needed to optimize alignments. Amino acid residues, numbered starting at the amino termini, are represented by the standard one-letter codes. The consensus sequence (CON) at the top indicates the majority residue at each position. It is used simply to highlight divergence and is not intended to imply any specific significance. Only residues that differ from the consensus are shown; a blank space under the consensus indicates identity. A hyphen in the consensus sequence indicates no consensus at that position. Dots at the carboxyl termini indicate that no residue is present. X, Not determined.

into microtubular organelles in which they are not normally found when other, less diverged β tubulins are also present in the cell (13).

An interesting observation for the vertebrates chicken, mouse, and human is that the β tubulins can be classified into four distinct isotype classes on the basis of interspecies conservation of intraspecies variation (32) (this classification excludes the divergent hematopoietic class). Although none of the four isotypes differs at more than 8 to 9% of the residues compared with the other three isotypes in the same organism, variation in a 25-residue region near the amino terminus and a in 15- to-20-residue region near the carboxyl terminus has been tightly conserved across species lines. This has led to the speculation that positive selection for specific sequence divergence has occurred during vertebrate evolution (32).

The temptation to ascribe functional significance to tubulins with distinct sequences is great. This is especially so

when tubulins are compared with, for example, actins, which are much more highly conserved. For example, *P. polycephalum* contains three actin genes that encode the same polypeptide, and this polypeptide is 96% identical to human cytoplasmic actin (M. Hamelin, L. Adam, G. Lemieux, and D. Pallotta, submitted for publication). However, interpreting the structures of tubulin gene families in the context of evolution remains a difficult problem. As with any multigene family, such interpretations require firm knowledge of whether the different gene products are functionally equivalent or functionally distinct, an issue in considerable contention at present for tubulins.

We conclude that neutral drift can account for the occurrence of variant β tubulins within the same organism, particularly where the variant β tubulin performs fewer functions than usual. The alternative hypothesis that variant β tubulins arise through positive selection for specific function should not be ruled out, however. The idea that multiple β tubulins arise through requirements for distinct modes of regulation (36) can link these two hypotheses, for one can postulate that once expression and function of a β tubulin are sufficiently restricted, positive selection may act more freely upon the variant gene to refine the function or mode of regulation of the product.

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