Preferential expression of one β-tubulin gene during flagellate development in *Physarum*

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The microbial eukaryote *Physarum polycephalum* displays several distinct cell types in its life cycle, including amoebae, flagellates and plasmodia. Despite its relative simplicity, *Physarum* has a tubulin gene family of complexity comparable to that of *Drosophila*. We have identified β-tubulin cDNAs from *Physarum* that are derived from the betA β-tubulin locus and encode β1A tubulin. We have also identified a partial cDNA for the unlinked betB β-tubulin gene, which encodes β1B tubulin. The polypeptide sequences encoded by betA and betB show 99% identity, but the nucleotide sequences show only 85% identity, consistent with an ancient duplication of these genes. The betB gene is expressed in amoebae, flagellates and plasmodia, whereas betA is expressed only in amoebae and flagellates. During the amoeba–flagellate transition the level of betA transcript increases over 100-fold, while the level of betB transcript changes very little. Thus *Physarum* has a mechanism for regulating the level of discrete β-tubulin transcripts differentially during flagellate development. A need for this differential regulation could account for the maintenance of the virtually isocoding betA and betB β-tubulin genes.

Introduction

Most eukaryotes have multiple genes for both α- and β-tubulins, the principal components of microtubules (MTs). Two hypotheses have been proposed to explain the multiplicity of tubulin genes. One suggests that different tubulin genes encode polypeptides with different functional properties; these in turn would compose MTs with distinct functions (Fulton & Simpson, 1976). The other hypothesis states that multiple tubulin genes fulfill a eukaryote’s need for differential regulation, especially in different cell types (Raff, 1984). Neither of these hypotheses excludes the other.

The myxomycete *Physarum polycephalum*, like most eukaryotes, has several functionally distinct sets of MTs. In *Physarum*, the distinct sets of MTs are differentially utilized in different cell types. The uninucleate amoeba utilizes microtubules in the cytoskeleton, centrioles and the open mitotic spindle. During the development of the amoeba into the flagellate, the cytoplasmic MTs change their central stellate arrangement to form a cone radiating from the anterior of the cell. The centrioles migrate to this region of the cell, where they form the basal bodies, nucleating two flagellar axonemes. The multinucleate, syncytial plasmodium, by contrast, utilizes MTs only in its intranuclear mitotic apparatus (Burland et al., 1983).

*Physarum* has at least five α-tubulin (alt) genes and three β-tubulin (bet) genes (Schedl et al., 1984b). Thus, this relatively simple eukaryote has a tubulin gene family comparable in complexity to that of *Drosophila* (Sanchez et al., 1980). We are studying the β-tubulin gene family of *Physarum* to try to understand how multiple tubulin genes may be of benefit to a simple microbial eukaryote. We show here that the multiple genes for β-tubulin show different patterns of expression in different cell types. Even two virtually isocoding β-tubulins are expressed differentially during flagellate development, consistent with the idea that multiple tubulins provide a mechanism for differential regulation.

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Abbreviations: MT, microtubule.

The nucleotide sequence of cDNA β602 has been submitted to GenBank and has been assigned the accession number M58521.
Methods

Cells and culture conditions. Amoebae of P. polycephalum strain LU352 (Dee et al., 1989) were grown in 50 ml SDM (Cooke & Dee, 1975) in 500 ml flasks at 26 °C in a rotary shaker at 50 r.p.m. To induce amoebae to form flagellates efficiently from axenic medium, we found it essential to use Oxoid bacteriological peptone in SDM as originally described (Cooke & Dee, 1975), rather than Difco Bacto Soytone, which is commonly used instead. Strains CLd (Dee et al., 1989) and MA275 (Schedl et al., 1984b) have been described previously.

Induction of flagellate development. To induce flagellate development, LU352 amoebae at 4-10 x 10^6 cells ml^-1 were centrifuged at 200 g for 2.5 min. Growth medium was decanted and cells were resuspended in 1 vol. SDM diluted fourfold in water; this point was taken as time zero. The culture was then shaken at 50 r.p.m., 26 °C for 30 min, then centrifuged at 50 g for 2.5 min and the supernatant decanted. Cells were resuspended in one volume of SDM diluted 16-fold in water and shaken at 50 r.p.m., 26 °C. Appearance of flagellates was monitored by phase-contrast microscopy of cells that were fixed by placing them on top of a layer of 1% (w/v) agarose, 3.7% (v/v) formaldehyde on a microscope slide.

Construction and screening of cDNA libraries. Total RNA was isolated from cells after lysis in guanidinium isothiocyanate (Schedl et al., 1984a). Poly(A)^+ RNA was prepared by oligo(dT) chromatography (Aviv & Leder, 1972).

Two cDNA libraries, designated ML5 and ML6, were prepared. ML5 was prepared from total RNA extracted from amoebae. ML6 was prepared from poly(A)^+ RNA extracted from a culture early in flagellate development, and was expected to include cDNAs of transcripts from both flagellates and amoebae. cDNA was synthesized using standard procedures for ML5 (Huynh et al., 1984) or, for ML6, using a kit (Amersham, cat. no. RPN.1257). cDNA was treated with EcoRI methylase. EcoRI linkers were then added, and after digestion with EcoRI, the cDNA was size-fractionated using a 1-0% (w/v) low-melting temperature agarose gel (for ML5), or on a BioGel A50 column (Huynh et al., 1984) for ML6). In ML5, cDNA of 0.5-3 kb was ligated into the vector pgt10 digested with EcoRI. In ML6, cDNA greater than 1 kb was ligated into the same vector. The phage DNA was packaged in vitro using extracts prepared by the method of Zehnbauer & Blattner (1982) using modifications of V. Burland (University of Wisconsin, Madison, personal communication). A total of 4 x 10^9 phage plaques were obtained for ML5 and 1 x 10^8 for ML6. To screen the library, nitrocellulose filter replicas of phage plaques were probed with a mixture of Physarum betC cDNA β102 (Burland et al., 1988) and chicken probe pT2 (Valenzuela et al., 1979). ML6 filters were probed with Physarum betC cDNA β102 (Burland et al., 1988) and betB cDNA β502 (from ML5). The filters were washed in 1 x SSC (150 mM-NaCl, 15 mM-sodium citrate) in 0.1% sodium dodecyl sulphate. After purifying the β-tubulin-positive recombinant plaques, DNA was extracted and the size of the cDNA insert determined by EcoRI digestion and agarose gel electrophoresis. Clones from ML5 were numbered in the 500s and from ML6 in the 600s.

Subcloning cDNA and sequencing strategy. The cDNAs were subcloned from β into the EcoRI site of bacteriophage M13mp18 and were further subcloned using the Cylon deletion system (International Biotechnologies) by the method of Dale et al. (1985) for sequencing by dideoxynucleotide chain termination (Sanger et al., 1977), using Sequenase (USB).

DNA sequences were entered into computer files manually and manipulated using DNASTAR software. DNA sequences were translated with the program TRANSLATE and protein sequence comparisons were made pairwise with the program AALIGN, which uses the Lipman method to identify homologies and introduces gaps and makes the final alignment by the protocol of Needleman & Wunsch (1970). All percentage identities were rounded to the nearest integer.

In vitro transcription and translation. For the production of transcripts in vitro, β602 was subcloned into the plasmid pGEM4blue (Promega), with the translation start site adjacent to the SP6 promoter. This pGEM4blueβ602 plasmid was then linearized with XbaI and extracted with phenol and chloroform, precipitated with ethanol and resuspended in water. Linear pGEM4blueβ602 DNA was then transcribed in vitro using SP6 polymerase (Melton et al., 1984). About 300 ng of transcript was translated in a wheatgerm translation system (RPN-1; Amersham) in 30 μl reactions containing [35S]methionine (SJ.204; Amersham) and 19 unlabelled amino acids. To identify the products of the transcripts, 0.06 vol. of the translation mixture was co-electrophoresed with unlabelled lysates of Physarum plasmodia and blotted onto nitrocellulose filters as described previously (Burland et al., 1988). Blots were probed with a mixture of α-tubulin-specific monoclonal antibody YLI/2 (Kilmartin et al., 1982) and β-tubulin-specific monoclonal antibody KMX-1 (Birckett et al., 1985). Reaction with the antibodies was detected using horseradish-peroxidase-conjugated secondary antibodies. The same filters to which electrophoretically separated proteins had been blotted and immunostained were then exposed to X-ray film to detect radio labelled translation products. Translation products were distinguished using the immunologically detected unlabelled marker tubulins from the cell lysates.

Agarose gel electrophoresis and filter hybridization. For Southern blotting, Physarum DNA from haploid strains CLd and MA275, and diploid CLd × MA275, was digested with the restriction enzyme SstI, separated by electrophoresis on a 0.7% agarose gel, partially hydrolysed by acid depurination, and transferred to nitrocellulose filters using standard procedures (Southern, 1975). Nitrocellulose filters were prehybridized, hybridized and washed under the stringent conditions described by Schedl et al. (1984b).

For Northern blotting, 3 μg RNA was glyoxalated and electrophoresed on a 1-7.5% (w/v) agarose gel for 10 h at 50 V, then transferred to a Biodyne A nylon membrane and prehybridized and hybridized as described by Schedl et al. (1984a). Membranes were washed six times for 20 min in 0.1 x SSC at 55 °C and exposed to pre-flashed Kodak X-ray film (Laskey & Mills, 1977). RNA was quantified by scanning autoradiographs using a Biomed Instruments soft laser scanning densitometer model SL-504-XL. Linearity was assessed by densitometry of a series of different exposures of the blots.

Results

Identification of β-tubulin cDNA clones

We made two separate cDNA libraries, the first from amoebae (ML5), the second from an early stage of flagellate development (ML6), which should represent both amoebal and flagellate transcripts. All 4 x 10^5 clones from ML5 were screened; two clones hybridized to the β-tubulin probes. These were recloned to plaque purity and designated β501 and β502. Of 10^5 clones screened from ML6, 16 hybridized to the β-tubulin probes. One clone, β602, was recloned and used for further analysis.
Tubulin gene expression in Physarum flagellates

Locus assignments

On Southern blots of StuI-digested Physarum DNA hybridized with β-tubulin DNA probes, three pairs of β (β-tubulin) alleles show restriction fragment length differences between strains CLd and MA275 (Fig. 1; Schedl et al., 1984b). Bands d and e are alleles of βA; g and h are alleles of βB; a and c are alleles of βC (Schedl et al., 1984b). Haploid strains like CLd and MA275 carry only one of each of these pairs of fragments, whereas diplods like CLd × MA275 carry both (Fig. 1). There are also two monomorphic β bands, b and f, for which polymorphic restriction fragments have not been found (Schedl et al., 1984b). For locus assignments, use of DNAs from polymorphic strains helps to make locus identification unequivocal between closely migrating bands.

The β501 cDNA probe hybridizes preferentially to alleles d and e (Fig. 1), indicating that β501 is derived from βA, and to the monomorphic band f, indicating that band f may also be derived from βA. β501 hybridizes less strongly to alleles of other loci. β502 cDNA preferentially hybridizes to βB alleles g and h (Fig. 1), indicating that β502 is derived from βB, as well as to the monomorphic band b, indicating that band b may also be derived from the βB locus. Again, β502 hybridizes only weakly to other tubulin loci. The β602 cDNA was assigned to the βA locus based on its sequence identity with portions of β501 (see below).

cDNAs for βA and βB contain a StuI restriction site in codon 206 (Fig. 1), indicating that βA and βB should each yield two genomic StuI fragments in haploid strains. For βA, subcloned cDNA probe β501-3-7 (Fig. 1a), which contains only sequences 5’ to the StuI site, hybridizes specifically to the polymorphic βA bands d and e, whereas probe β501-3-106, containing only sequences 3’ to this StuI site, hybridizes specifically to the monomorphic βA band f (not shown). For βB, the corresponding 5’ cDNA probe β502-1-10 (Fig. 1) hybridizes specifically to the polymorphic βB bands g and h, while the corresponding 3’ probe β502-8-4, which contains mostly sequence 3’ to the StuI site, hybridizes preferentially to the βB monomorphic band b (not shown). Thus, the polymorphic bands from βA and βB are derived from the 5’ ends of these genes, and the monomorphic bands are derived from the 3’ ends of these genes. This also confirms that the monomorphic bands are not fragments of unlinked β-tubulin genes.

Gene product assignment

To determine the product of βA, RNA was transcribed from pGEM4Blueβ602 using SP6 RNA polymerase, and the resulting transcripts were translated in vitro. Unlabelled plasmodial lysates were mixed with the translation products and the mixtures were then resolved by two-dimensional gel electrophoresis. Immunoblotting using the tubulin-specific monoclonal antibodies stained the α1, β1 and β2 tubulins present in the unlabelled plasmodial lysates (not shown). There was too little protein among the translation products to detect immunologically, but autoradiography of the immunoblot
Fig. 2. Identification of the product of betA. The mixture of anti-α-tubulin and anti-β-tubulin antibodies detected *Physarum* α1, β1 and β2-tubulins (arrows; see also Burland et al., 1988) in the mixture of cell lysates and *in vitro* translation products co-electrophoresed on two-dimensional gels. *Physarum* α2-tubulin, detected on the left autoradiogram, does not have an epitope that YL1/2 recognizes (Walden et al., 1989). The blots (not shown) served to determine the precise orientation for comparison of blot and autoradiogram. The left panel shows the tubulins (arrows; see also Burland et al., 1984) in this mixture of plasmodial poly(A)+ RNA; this RNA was isolated from the G2 phase of the cell cycle, when tubulin mRNAs are abundant (Schedl et al., 1984). The right panel shows the corresponding autoradiogram of the immunoblot for the *in vitro* translation product of cDNA β602. Isoelectric focusing is from left (basic) to right (acidic); denaturing polyacrylamide gel electrophoresis is from top to bottom. The major radioactive translation product of β602 is clearly β1-tubulin. No signal in the vicinity of the other tubulins was detected after longer exposures of the autoradiogram. Other radioactive species detected were minor and of lower apparent molecular mass than tubulins; they may result from translation of prematurely terminated *in vitro* transcripts.

Sequences of β-tubulin cDNAs

The betA cDNA β501 has approximately 1610 nucleotides (not shown), including an extensive open reading frame for β-tubulin. However, despite the presence of poly(A) 3' to the coding region, β501 contains an intron-like sequence between nucleotides 1210 and 1318, and we experienced difficulties in accurately sequencing this region of the cDNA. We therefore do not report the sequence for this cDNA. β501-33-3 (Fig. 1) is a subclone of β501 which contains 730 base pairs from the 5' coding region of the betA gene; this subclone hybridizes specifically to betA on Southern blots (not shown), so it was chosen as a DNA probe for Northern blotting.

cDNA β602 has 1512 nucleotides, consisting of 53 nucleotides of 5' untranslated sequence, an open reading frame of 445 codons for β1A-tubulin, 124 nucleotides of 3' untranslated sequence and 17 nucleotides of poly(A) tail. Sequence analysis indicated that β602 is derived from the same gene as β501, i.e. betA.

β502 is approximately 600 nucleotides long (not shown). Comparison of preliminary nucleotide sequence for β502 with that for betB genomic clone pβ40-21 (Werenskiold et al., 1988) confirms that β502 is derived from betB and starts at codon 103 of this gene. Since most of the betB sequence covered by β502 is already published (Werenskiold et al., 1988), we did not complete rigorous sequencing of β502.

Comparisons of β-tubulin sequences

Singhofer-Wowra et al. (1986) determined 90% of the amino acid sequence of β1 tubulins purified from *Physarum* amoebae. Evidence was obtained for heterogeneity at only one amino acid residue (Singhofer-Wowra et al., 1986) in this mixture of β1A and β1B polypeptides (Burland et al., 1984), at position 283, where serine and alanine both occurred. The amino acid sequence deduced from the sequence of betA cDNA β602 (Fig. 3) illustrates that serine-283 originates from this gene, which implies that alanine-283 comes from betB. Translation of the β602 sequence fills in the gaps of the β1A tubulin sequence not determined by the protein sequencing (Singhofer-Wowra et al., 1986). We found four more differences in amino acids between the β1 sequence derived from direct protein sequence of amoebal β-tubulin and the deduced amino acid sequence of β602, at residues 39, 165, 196 and 238 (Fig 3).

In Fig. 4, the nucleotide sequence of β602 (betA) from codons 4 to 204 is compared to the partial nucleotide sequence of betB obtained from genomic clone pβ40-21 (Werenskiold et al., 1988). While the amino acids show 99.5% identity, the nucleotide sequence shows only 85% identity. Thus although betA and betB genes encode virtually identical proteins, there has been considerable drift in their nucleotide sequence and codon usage.

Expression of betA and betB

In order to examine the expression patterns of the betA and betB genes in *Physarum*, RNA was isolated from amoebae, flagellates and plasmodia, and analysed by Northern blotting using the betA-specific cDNA probe β501-33-3 (Fig. 1) and cDNA probe β502, which hybridizes to both betA and betB on Southern blots.

The betA gene is expressed at only a low level in amoebae but the level of the betA transcript increases...
**β-Tubulin gene expression in Physarum flagellates**

**Fig. 3.** Alignment of the amino acid sequences of *Physarum* β-tubulins. β1A is the sequence deduced for the betA product from cDNA β02; β1 protein is the sequence derived from purified amoebal protein (Singhofer-Wowra et al., 1986); β1B is the sequence deduced for the betB product from partial genomic clone pβ40-21 (Werenskiold et al., 1988); and β2 is the sequence deduced for the betC product (Burland et al., 1988). All sequences were aligned with that deduced for *Physarum* β1A tubulin. Amino acid residues, numbered starting at the amino termini, are represented by the standard one-letter codes. Only residues that differ from the β1A sequence are shown; dots indicate undetermined residues, and spaces indicate identical residues. Asterisks indicate positions (numbered) specifically discussed in the text.

Dramatically in the flagellate (Fig. 5). No betA expression was detected in plasmodium. Since we do not have a DNA probe specific for the betB gene, it is more difficult to assess betB expression. However, betB cDNA probe β502 appears to detect two incompletely resolved transcripts. In the amoebal and plasmodial RNAs, β502...
In order to determine if the two bet transcripts detected by Northern blotting differed only in the length of poly(A) tail, the RNA from flagellates was treated with RNAase H in the presence of oligo(dT) to remove poly(A) tails (Mercer & Wake, 1985). We also hoped to improve resolution of the two transcripts with this technique. However, both transcripts appeared to be shortened to the same extent (not shown), indicating that the difference between these two transcripts is not simply the length of their poly(A) tails.

**Kinetics of betA RNA accumulation**

We analysed how the changes in betA RNA level occurred with respect to the timing of flagellate development. Amoebae were induced to develop, and RNA was isolated at subsequent times. Northern blots of RNA were then probed with radiolabelled betA-specific cDNA subclone 8501-33-3 (Fig. 6), and the levels of the betA transcripts were quantified. The peak level of betA transcript during flagellate development is 110-fold higher than the level of betA transcript present in the amoebae. Prior to the first cytological detection of flagellates (40 min) the level of betA transcript was already 18% of its peak value (Fig. 6b). The peak level of betA transcript occurred when 21% of the cells were flagellates. By the time the proportion of flagellates was at a maximum (240 min), the level of the betA transcript was decreasing (Fig. 6b). Control RNA detected by the cDNA probe Ppc16, which is expressed constitutively during flagellate development (Green & Dove, 1984), remained at approximately constant levels, while the level of actin RNA decreased slightly (not shown) when
p-Tubulin gene expression in Physarum flagellates

Fig. 6. (a) Northern blot analysis of total RNA isolated at different times after amoebae were induced to form flagellates. The numbers above each lane indicate the time in minutes after induction of flagellate development. Upper panel, probed with 8501-33-3, specific for \( \beta A \); lower panel, probed with 8502, which reacts with both \( \beta A \) and \( \beta B \) transcripts. The low level of \( \beta A \) transcript present in amoebae (0 min) is evident only on longer autoradiographic exposures. (b) Quantification of RNA (□, percentage of maximum) and percentage of flagellates (△) after induction of development.

normalized to \( \text{OD}_{260} \) units of RNA loaded for each sample.

Discussion

Comparisons of Physarum \( \beta \)-tubulins

One amino acid difference between the two \( \text{Physarum} \) \( \beta \)-tubulins had already been identified at position 283 by sequencing protein purified from amoebae (Singhofer-Wowra et al., 1986). In view of our observations of only a low level of \( \beta A \) transcript in amoebae, it is remarkable that readily detectable amounts of both alanine and serine could be found at position 283 by protein sequencing (Singhofer-Wowra et al., 1986). We suspect that manipulations used to harvest amoebae from culture induce elements of flagellate development, leading to increased levels of \( \beta A \) gene product in cell extracts.

Our cDNA sequencing indicated four other differences among \( \text{Physarum} \) \( \beta 1 \)-tubulins, at residues 39, 165, 196 and 238 (Fig. 3). These differences might be expected simply to reflect differences between \( \beta 1 \)A and \( \beta 1 \)B tubulins, but only the differences at residues 39 and 238 seem to fit this interpretation. Each of these differences could have arisen by a change in a single nucleotide between \( \beta A \) and \( \beta B \). At position 165, however, cysteine is indicated in both \( \beta 602 \) (\( \beta A \)) and \( \beta 40-21 \) (\( \beta B \)), but aspartate was found in the \( \beta 1 \) protein sequence (Singhofer-Wowra et al., 1986). Similarly, position 196 is alanine in \( \beta 602 \) and \( \beta 40-21 \), but threonine in the \( \beta 1 \) protein sequence. The Ala–Thr discrepancy at codon 196 could be accounted for by a single nucleotide change, whereas the Cys–Asp discrepancy would need two nucleotide changes. These discrepancies in the protein sequence where \( \beta A \) and \( \beta B \) DNA sequences appear isocoding could be due to errors in sequencing, strain differences, or post-transcriptional modifications. We discount the possibility of a third \( \beta 1 \)-tubulin gene as this would surely have been detected by Southern blotting. The complete \( \beta 1 \)A polypeptide remains 88-90\% identical to \( \beta \)-tubulins from a broad range of organisms, including \( \text{Chlamydomonas} \), \( \text{Drosophila} \) and vertebrates, but only 83\% identical to \( \text{Physarum} \) \( \beta 2 \) tubulin, which is expressed primarily in plasmodia (Burland et al., 1988).

Differential expression of \( \beta \)-tubulins

During the transition of an amoeba into a flagellate, the level of \( \beta A \) transcript increases over 100-fold, while the level of the \( \beta B \) transcript changes very little. Based on the levels of \( \beta A \) and \( \beta B \) transcripts, we would expect that in amoebae, \( \beta 1 \)A-tubulin is of minor abundance and \( \beta 1 \)B-tubulin is the major \( \beta \)-tubulin; immunoblotting of axenic amoebal lysates supports this conclusion (Paul et al., 1989). In cultures radiolabelled as amoebae and induced to undergo the amoeba–flagellate transition, the \( \beta 1 \)A polypeptide is radiolabelled more heavily than \( \beta 1 \)B (Burland et al., 1984), indicating that the level of \( \beta 1 \)A tubulin increases with the increase in its mRNA level. Some synthesis of \( \beta 1 \)B tubulin continues, however, so both polypeptides may be abundant in flagellates. Since most cells seem not to have a mechanism for excluding expressed tubulins from MTs (e.g. Lewis et al., 1987), we would expect that both \( \beta 1 \)A-and \( \beta 1 \)B-tubulin are assembled into the flagellum.
The prediction that \( \beta \)-tubulin is of minor abundance in amoebae would contradict an earlier proposal that the two \( \beta \)-tubulins are of similar abundance in this cell type (Singhofer-Wowra et al., 1986), but would explain why mutation of \( \beta B \) alone is sufficient to confer resistance to antitubulin benzimidazoles on amoebae (Burland et al., 1984). However, if \( \beta B \)-tubulin is indeed the principal \( \beta \)-tubulin of the amoeba, our earlier hypothesis that mutation of \( \beta A \) alone can also lead to benzimidazole resistance (Burland et al., 1984) would warrant further investigation.

Strong differential expression of \( Physarum \) \( \beta \)-tubulins is further illustrated during the developmental transition of an amoeba into a plasmodium. At some point during this transition \( \beta A \) transcripts disappear, and can no longer be detected in the plasmodium, while \( \beta C \) transcripts (encoding \( \beta 2 \)-tubulin), which are not detected in the amoeba, are first diagnosed when cells become committed to plasmodium development (Solnica-Krezel et al., 1988). Our results show that the \( \beta B \) gene does not undergo such dramatic regulation as is seen for the \( \beta A \) and \( \beta C \) tubulin genes during these developmental transitions. However, in plasmodia, \( \beta B \) transcript and \( \beta B \)-tubulin levels are significantly lower than \( \beta C \) transcript and \( \beta 2 \)-tubulin levels (Burland et al., 1983; Solnica-Krezel et al., 1988). Thus, each of the three cell types we have studied preferentially expresses one of the three \( \beta \)-tubulin genes: \( \beta A \) in the flagellate, \( \beta B \) in the amoeba, and \( \beta C \) in the plasmodium.

**Regulation of \( \beta \)-tubulin expression**

Several examples of a rise in tubulin RNA levels during flagellar development have been noted. In \( Physarum \), \( \alpha \)-tubulin transcript levels increase five- to sevenfold over the same time frame that \( \beta A \) rises 110-fold (Green & Dove, 1984). In \( Naegleria \) (Lai et al., 1988; Lee & Walsh, 1988), \( \alpha \)-tubulin RNA is first detected 15 min after flagellate development is induced, and during flagellate development the level of \( \alpha \)-tubulin RNA increases dramatically. Similar increases in tubulin transcript levels during flagellar development have been extensively documented for \( Chlamydomonas \) (Lefebvre & Rosenbaum, 1986), where increases in both transcription and stability of tubulin RNA have been noted (Baker et al., 1984). In contrast to \( Physarum \), it appears that expression of the two isocoding \( \beta \)-tubulin genes of \( Chlamydomonas \) (Youngbloom et al., 1984), like that of the two \( \alpha \)-tubulin genes, is coordinate regulated during flagellar growth (Brunke et al., 1982). However, in the case of \( Chlamydomonas \), the appearance of flagella occurs at a specific cell cycle stage, and is not associated with a change in cell type.

How do cells adjust mRNA levels when major physiological changes occur? For \( \beta \)-tubulin, there is evidence for negative feedback regulation of mRNA level by \( \beta \)-tubulin protein, in organisms as diverse as cultured mammalian cells (Yen et al., 1988) and sea urchin (Gong & Brandhorst, 1987; Harlow & Nemer, 1987); high levels of free tubulin lead, directly or indirectly, to decreased tubulin RNA levels. In mammalian cells, the first four codons are necessary for this negative regulation of \( \beta \)-tubulin transcript levels (Yen et al., 1988), and it is remarkable that the sequence of the first four amino acid residues is very highly conserved, including for all three \( Physarum \) \( \beta \)-tubulins (Burland et al., 1988). This mechanism could thus be important for tubulin expression in many organisms. Indeed, the timing of changes in tubulin transcript level with respect to flagellar assembly fits well with a negative regulatory mechanism. In flagellates of \( Physarum \), \( Chlamydomonas \) and \( Naegleria \), the peak level of tubulin transcripts occurs prior to the completion of flagellar development (Baker et al., 1984; Lai et al., 1988; Lee & Walsh, 1988). By the time construction of the flagellar axoneme is completed, the level of tubulin RNA is on the decline. If these cells synthesize tubulin protein beyond the level required for the construction of axonemes, the increase in the level of free tubulin subunits could then homeostatically depress tubulin transcript level. It remains to be determined what mechanisms might turn on tubulin expression in advance of flagellar assembly.

**Functions of \( \beta \)-tubulins**

The \( \beta A \) and \( \beta B \) genes of \( Physarum \) have drifted from one another in their nucleotide sequence but their products nevertheless remain virtually identical in amino acid sequence. This implies that the duplication that gave rise to these two genes is ancient, but that there is a strong constraint on the \( \beta \) tubulin polypeptides acting to conserve their sequences. The close similarity of the \( \beta A \) and \( \beta B \) gene products does not preclude a functional distinction between these two molecules, but we have no evidence to support such a distinction with respect to microtubular function.

Some evidence for preferential assembly of specific \( \beta \)-tubulins into MTs has been obtained, for example in differentiating neurites (Joshi & Cleveland, 1989). However, most investigations have examined only participation in MTs by distinct \( \beta \)-tubulin isotypes of minor abundance, rather than active function by isotypes of major abundance. We previously noted that isotypes of minor abundance might be carried as "passengers" in MTs, and predicted that genetic drift would occur to the extent that such passengers did not
poison microtubular function (Burland et al., 1988). The demonstration that the Drosophila minor divergent β3-tubulin isotype impairs functional axoneome assembly when it exceeds 20% of cellular β-tubulin provides experimental confirmation of this idea (Hoyle & Raff, 1990). We would not expect to observe this particular distinction for Physarum β1A and β1B tubulins, since both are probably abundant during flagellate development. But perhaps high levels of expression of Physarum β2-tubulin would impair the function of flagella.

Even where substantial polypeptide sequence differences are observed between multiple β-tubulins within a single cell, it has been difficult to find compelling evidence for distinct functional roles (e.g. Sullivan, 1988). For example, the divergent β2 tubulin (the product of the betC gene) of Physarum has been detected in all the MT types observed during the amoebal-plasmodial developmental transition, including cytoplasmic, open and closed mitotic-spindle, flagellar, and centriolar MTs (Diggins-Gilicinski et al., 1989; Solnica-Krezel et al., 1990).

The 17% difference in amino acid sequence between β1 and β2 tubulins is remarkable in view of the similarity between the β1A and β1B tubulins. Singhoefer-Wowra et al. (1986) predicted that Physarum β2-tubulin would be more diverged than β1 tubulins since it usually participates only in mitosis and meiosis; they suggested that participation of the β1 tubulins not only in the mitotic spindle but also in more complex organelles like the flagellum may constrain sequence divergence. This interpretation is consistent with the idea that both β1A and β1B-tubulins are utilized in the flagellum, and would argue against the maintenance of the two β1-tubulin genes for distinct microtubular functions. Our results indicate that the existence of the betA–betB gene pair provides a mechanism for strong differential regulation of tubulin genes in different cell types, and the selective advantage of this mechanism may be the evolutionary force that maintains both genes.

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