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

EILEEN C. A. PAUL,[†] GARY L. BUCHSCHACHER, JR, DAVID B. CUNNINGHAM, WILLIAM F. DOVE and TIMOTHY G. BURLAND*

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, WI 53706, USA

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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 β 1A tubulin. We have also identified a partial cDNA for the unlinked *betB* β -tubulin gene, which encodes β 1B 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 fulfil 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.

* Author for correspondence. Tel. (608) 262 0982; fax (608) 262 2824; email TIM@WISCPSL.BITNET.

[†] Present address: Department of Physiology, 719 Veterinary Research Tower, Cornell University, Ithaca, NY 14853-6401, USA.

Abbreviations: MT, microtubule.

The nucleotide sequence of cDNA β 602 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 $4\text{--}10 \times 10^6$ 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 1.0% (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 *Eco*RI methylase. *Eco*RI linkers were then added, and after digestion with *Eco*RI, 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 λ gt10 digested with *Eco*RI. 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 4×10^5 phage plaques were obtained for ML5 and 1.4×10^6 for ML6. To screen the library, nitrocellulose filter replicas of phage plaques were probed with nick-translated β -tubulin cDNAs (Rigby *et al.*, 1977). ML5 filters were probed with a mixture of *Physarum betC* cDNA β 102 (Burland *et al.*, 1988) and chicken probe pT2 (Valenzuela *et al.*, 1979). ML6 filters were probed with *Physarum betC* cDNA β 102 (Burland *et al.*, 1988) and *betB* cDNA β 502 (from ML5). The filters were washed in $1 \times$ 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 *Eco*RI 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 λ into the *Eco*RI site of bacteriophage M13mp18 and were further subcloned using the Cyclone 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*, β 602 was subcloned into the plasmid pGEM4blue (Promega), with the translation start site adjacent to the SP6 promoter. This pGEM4blue β 602 plasmid was then linearized with *Xba*I and extracted with phenol and chloroform, precipitated with ethanol and resuspended in water. Linear pGEM4blue β 602 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 μ l 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 YL1/2 (Kilmartin *et al.*, 1982) and β -tubulin-specific monoclonal antibody KMX-1 (Birkett *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 radiolabelled 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 $\times$ MA275, was digested with the restriction enzyme *Stu*I, 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 μ g RNA was glyoxalated and electrophoresed on a 1.7% (w/v) agarose gel for 10 h at 50 V, then transferred to a Biotex A nylon membrane and prehybridized and hybridized as described by Schedl *et al.* (1984a). Membranes were washed six times for 20 min in $0.1 \times$ 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 4×10^5 clones from ML5 were screened; two clones hybridized to the β -tubulin probes. These were recloned to plaque purity and designated β 501 and β 502. Of 10^5 clones screened from ML6, 16 hybridized to the β -tubulin probes. One clone, β 602, was recloned and used for further analysis.

Locus assignments

(Schedl *et al.*, 1984*b*). Haploid strains like CLd and MA275 carry only one of each of these pairs of fragments, whereas diploids like CLd $\times$ MA275 carry both (Fig. 1). There are also two monomorphic *bet* bands, b and f, for which polymorphic restriction fragments have not been found (Schedl *et al.*, 1984*b*). For locus assignments, use of DNAs from polymorphic strains helps to make locus identification unequivocal between closely migrating bands.

The $\beta 501$ cDNA probe hybridizes preferentially to alleles d and e (Fig. 1), indicating that $\beta 501$ is derived from *betA*, and to the monomorphic band f, indicating that band f may also be derived from *betA*. $\beta 501$ hybridizes less strongly to alleles of other loci. $\beta 502$ cDNA preferentially hybridizes to *betB* alleles g and h (Fig. 1), indicating that $\beta 502$ is derived from *betB*, as well as to the monomorphic band b, indicating that band b may also be derived from the *betB* locus. Again, $\beta 502$ hybridizes only weakly to other tubulin loci. The $\beta 602$ cDNA was assigned to the *betA* locus based on its sequence identity with portions of $\beta 501$ (see below).

cDNAs for *betA* and *betB* contain a *StuI* restriction site in codon 206 (Fig. 1), indicating that *betA* and *betB* should each yield two genomic *StuI* fragments in haploid strains. For *betA*, subcloned cDNA probe β 501-33-7 (Fig. 1a), which contains only sequences 5' to the *StuI* site, hybridizes specifically to the polymorphic *betA* bands d and e, whereas probe β 501-3-106, containing only sequences 3' to this *StuI* site, hybridizes specifically to the monomorphic *betA* band f (not shown). For *betB*, the corresponding 5' cDNA probe β 502-1-10 (Fig. 1) hybridizes specifically to the polymorphic *betB* bands g and h, while the corresponding 3' probe β 502-8-4, which contains mostly sequence 3' to the *StuI* site, hybridizes preferentially to the *betB* monomorphic band b (not shown). Thus, the polymorphic bands from *betA* and *betB* 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.

To determine the product of *betA*, RNA was transcribed from pGEM4Blue β 602 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 α 1, β 1 and β 2 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* α 1, β 1 and β 2-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* α 2-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 autoradiogram of the two-dimensional immunoblot of *in vitro* translation products 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.*, 1984a). The right panel shows the corresponding autoradiogram of the immunoblot for the *in vitro* translation product of cDNA β 602. Isoelectric focussing is from left (basic) to right (acidic); denaturing polyacrylamide gel electrophoresis is from top to bottom. The major radioactive translation product of β 602 is clearly β 1-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.

(Fig. 2) identified the position of the *in vitro* translation product relative to the immunologically detected marker tubulins in the gel sample mixture. The results show that β 602 encodes a β 1-tubulin polypeptide. β 1-Tubulin is composed of two gene products, β 1A- and β 1B-tubulin (Burland *et al.*, 1984). Since β 602 is derived from *betA*, and since *betB* encodes β 1B tubulin (Burland *et al.*, 1984), we can conclude that the β 1-tubulin encoded by *betA* is β 1A-tubulin.

Sequences of β -tubulin cDNAs

The *betA* cDNA β 501 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, β 501 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. β 501-33-3 (Fig. 1) is a subclone of β 501 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 β 602 has 1512 nucleotides, consisting of 53 nucleotides of 5' untranslated sequence, an open reading frame of 445 codons for β 1A-tubulin, 124 nucleotides of 3' untranslated sequence and 17 nucleotides of poly(A) tail. Sequence analysis indicated that β 602 is derived from the same gene as β 501, i.e. *betA*.

β 502 is approximately 600 nucleotides long (not shown). Comparison of preliminary nucleotide sequence for β 502 with that for *betB* genomic clone p β 40-21 (Werenskiold *et al.*, 1988) confirms that β 502 is derived from *betB* and starts at codon 103 of this gene. Since most of the *betB* sequence covered by β 502 is already published (Werenskiold *et al.*, 1988), we did not complete rigorous sequencing of β 502.

Comparisons of β -tubulin sequences

Singhofer-Wowra *et al.* (1986) determined 90% of the amino acid sequence of β 1 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 β 1A and β 1B 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 β 602 (Fig. 3) illustrates that serine-283 originates from this gene, which implies that alanine-283 comes from *betB*. Translation of the β 602 sequence fills in the gaps of the β 1A tubulin sequence not determined by the protein sequencing (Singhofer-Wowra *et al.*, 1986). We found four more differences in amino acids between the β 1 sequence derived from direct protein sequence of amoebal β -tubulin and the deduced amino acid sequence of β 602, at residues 39, 165, 196 and 238 (Fig. 3).

In Fig. 4, the nucleotide sequence of β 602 (*betA*) from codons 4 to 204 is compared to the partial nucleotide sequence of *betB* obtained from genomic clone p β 40-21 (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 β 501-33-3 (Fig. 1) and cDNA probe β 502, 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 1A	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGSYHGESDLQLERINVYNEATGGKYV	60
β 1 protein		
β 1B	...	
β 2	V V V V E S A T K D T V A S	
*39		
β 1A	PRAVLVDLEPGTMDSVRAGPFGQIFRPDNFVFGQTGAGNNWAKGHYTEGAELIDSVLDVV	120
β 1 protein		
β 1B		
β 2	V L I S S I S M T H A S V E V	
*165		
β 1A	RKEAESCDCLQGFQIAHSLGGGTGSGMGILLISKIREEYPDRMMCTFSVVPSPKVSDTVV	180
β 1 protein		
β 1B		
β 2	N C L F M	
*196		
β 1A	EPYNATLSVHQLVENADEVMCIDNEALYDISFRTLKLTPTTYGDLNHLVSAVMSGITCCL	240
β 1 protein	. T S .	
β 1B		
β 2	I C G A	
*238		
*283		
β 1A	RFPGQLNSDLRKLAVNLIPFRLHFFLVGFAPLTSRGSVGYRSLTVPCLTQOMFDAKNMM	300
β 1 protein	 A	
β 1B		
β 2	I Y A S A M F A I S R	
*360		
β 1A	AASDPRHGRYLTAAMFRGRMSTKEVDEQMLNVQKNSSYFVEWIPNNIKSSVCDIPPKG	360
β 1 protein	.	
β 1B		
β 2	T K H A T F	
*420		
β 1A	LKMAVTFIGNSTAIQELFKRVSEQFTAMFRRKAFHLHWYTGEGMDEMEFTEAESNMNDLVS	420
β 1 protein		
β 1B		
β 2	M S A N C I G L S	
*445		
β 1A	EYQQYQDATIDDEEGGEEEEGGAAE	445
β 1 protein		
β 1B		
β 2	E S V A M E D D A A E G G A	

Fig. 3. Alignment of the amino acid sequences of *Physarum* β -tubulins. β 1A is the sequence deduced for the *betA* product from cDNA β 602; β 1 protein is the sequence derived from purified amoebal protein (Singhofer-Wowra *et al.*, 1986); β 1B is the sequence deduced for the *betB* product from partial genomic clone p β 40-21 (Werenskiold *et al.*, 1988); and β 2 is the sequence deduced for the *betC* product (Burland *et al.*, 1988). All sequences were aligned with that deduced for *Physarum* β 1A tubulin. Amino acid residues, numbered starting at the amino termini, are represented by the standard one-letter codes. Only residues that differ from the β 1A 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 plasmodia. 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 β 502 appears to detect two incompletely resolved transcripts. In the amoebal and plasmodial RNAs, β 502

<i>betA</i>	ATGAGAGAGATTGTACACATCCAAGCCGGCAGTGTGGTAATCAAATTGGAGCCAAGTTCTGGGAGGTTATCTCTGATGAGCATGGCATTGACCCCACTG	100
<i>betB</i>	 C T G A C C C C G T	C
<i>betA</i>	GATCTACCACGGAGAGTCTGACCTGCAACTTGAGAGAATCAACGTCTACTACAATGAGGCCACCGCGGGAAGTATGTCCCCGTGCCGTCTTGTGA	200
<i>betB</i>	C A T T T A T C T A T A T T A C T T T G G	
<i>betA</i>	TCTTGAGCCTGGCACAATGGACTCTGTTCGTGCTGGTCCCTTGGTCAGATCTTCCGCCAGACAACCTTTGTATTGGACAGACTGGTGCCGGTAACAAC	300
<i>betB</i>	CT G C C C C A A A A A A A G	A A
<i>betA</i>	TGGGCCAAGGGTCACTACACAGAGGGTGCAGAACTGATCGATTCTGTCTTGGATGTCGTCAGAAAGGAAGCAGAGAGCTGTGACTGCCTACAGGGTTTCC	400
<i>betB</i>	A A T G C C G T C C C G T C	
<i>betA</i>	AAATTGCACACTCACTGGGAGGAGGCACAGGGTCTGGTATGGGAACACTTCTCATCTCCAAGATTCTGTGAAGAGTACCCCGACCGTATGATGTGCACATT	500
<i>betB</i>	G T T T T A G G T C	
<i>betA</i>	CTCCGTCGTGCCATCCCTAAGGTGTCAGACACAGTCGTCGAGCCATACAACGCCACCTCTCCGTCCACCAACTTGTAGAGAACGCTGATGAGGTGATG	600
<i>betB</i>	C A A G G C A T A A C	
<i>betA</i>	TGCATTGACAATGAGGCCT	619
<i>betB</i>	T C	

Fig. 4. Alignment of the nucleotide sequence of $\beta 602$ (*betA*) with the partial genomic sequence of p $\beta 40$ -21 (Werenskiold *et al.*, 1988) from *betB*. Only residues that differ from the *betA* sequence are shown; dots indicate undetermined nucleotides, and spaces indicate identical nucleotides.

Fig. 5. Northern blot analysis of total RNA isolated from amoebae (A), flagellates (F) and plasmodia (P). The hybridization probes (Fig. 1) were $\beta 501$ -33-3 for *betA* (left), and $\beta 502$, which detects both *betA* and *betB* (right). Exposures shown were at -70°C for 18 h. Longer exposures (not shown) reveal the low level of *betA* transcript in amoebae more clearly.

detects one transcript (Fig. 5), which we interpret to be from *betB*; we already know that *betB* is expressed in amoebae and plasmodia (Burland *et al.*, 1984). In flagellate RNA, $\beta 502$ detects not only the *betB* transcript, but also another transcript of slightly faster mobility (Fig. 5). We interpret the faster-mobility species as cross-hybridization to *betA* transcript, especially since this species corresponds to the position of the *betA* transcript detected by probe $\beta 501$ -33-3 (Fig. 5, left panel). These results suggest that *betB* is expressed in flagellates as well as in amoebae and plasmodia.

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 $\beta 501$ -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

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 β 501-33-3, specific for *betA*; lower panel, probed with β 502, which reacts with both *betA* and *betB* transcripts. The low level of *betA* 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 OD₂₆₀ units of RNA loaded for each sample.

Discussion

Comparisons of *Physarum* β -tubulins

One amino acid difference between the two *Physarum* β 1-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* 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* gene product in cell extracts.

Our cDNA sequencing indicated four other differences among *Physarum* β 1-tubulins, at residues 39, 165, 196 and 238 (Fig. 3). These differences might be expected simply to reflect differences between β 1A and β 1B 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* and *betB*. At position 165, however, cysteine is indicated in both β 602 (*betA*) and β 40-21 (*betB*), but aspartate was found in the β 1 protein sequence (Singhofer-Wowra *et al.*, 1986). Similarly, position 196 is alanine in β 602 and β 40-21, but threonine in the β 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* and *betB* DNA sequences appear isocoding could be due to errors in sequencing, strain differences, or post-transcriptional modifications. We discount the possibility of a third β 1-tubulin gene as this would surely have been detected by Southern blotting. The complete β 1A polypeptide remains 88-90% identical to β -tubulins from a broad range of organisms, including *Chlamydomonas*, *Drosophila* and vertebrates, but only 83% identical to *Physarum* β 2 tubulin, which is expressed primarily in plasmodia (Burland *et al.*, 1988).

Differential expression of β -tubulins

During the transition of an amoeba into a flagellate, the level of *betA* transcript increases over 100-fold, while the level of the *betB* transcript changes very little. Based on the levels of *betA* and *betB* transcripts, we would expect that in amoebae, β 1A-tubulin is of minor abundance and β 1B-tubulin is the major β -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 β 1A polypeptide is radiolabelled more heavily than β 1B (Burland *et al.*, 1984), indicating that the level of β 1A tubulin increases with the increase in its mRNA level. Some synthesis of β 1B 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 β 1A- and β 1B-tubulin are assembled into the flagellum.

The prediction that β 1A-tubulin is of minor abundance in amoebae would contradict an earlier proposal that the two β 1-tubulins are of similar abundance in this cell type (Singhofer-Wowra *et al.*, 1986), but would explain why mutation of *betB* alone is sufficient to confer resistance to antitubulin benzimidazoles on amoebae (Burland *et al.*, 1984). However, if β 1B-tubulin is indeed the principal β -tubulin of the amoeba, our earlier hypothesis that mutation of *betA* alone can also lead to benzimidazole resistance (Burland *et al.*, 1984) would warrant further investigation.

Strong differential expression of *Physarum* β -tubulins is further illustrated during the developmental transition of an amoeba into a plasmodium. At some point during this transition *betA* transcripts disappear, and can no longer be detected in the plasmodium, while *betC* transcripts (encoding β 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 *betB* gene does not undergo such dramatic regulation as is seen for the *betA* and *betC* tubulin genes during these developmental transitions. However, in plasmodia, *betB* transcript and β 1B-tubulin levels are significantly lower than *betC* transcript and β 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 β -tubulin genes: *betA* in the flagellate, *betB* in the amoeba, and *betC* in the plasmodium.

Regulation of β -tubulin expression

Several examples of a rise in tubulin RNA levels during flagellar development have been noted. In *Physarum*, α -tubulin transcript levels increase five- to sevenfold over the same time frame that *betA* rises 110-fold (Green & Dove, 1984). In *Naegleria* (Lai *et al.*, 1988; Lee & Walsh, 1988), α -tubulin RNA is first detected 15 min after flagellate development is induced, and during flagellate development the level of α -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 β -tubulin genes of *Chlamydomonas* (Youngbloom *et al.*, 1984), like that of the two α -tubulin genes, is coordinately 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 β -tubulin, there is evidence for negative feedback regulation of mRNA level by β -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 β -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* β -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 β -tubulins

The *betA* and *betB* 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 β 1 tubulin polypeptides acting to conserve their sequences. The close similarity of the *betA* and *betB* 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 β -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 β -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 β 3-tubulin isotype impairs functional axoneme 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* β 1A and β 1B tubulins, since both are probably abundant during flagellate development. But perhaps high levels of expression of *Physarum* β 2-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 β 2 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 β 1 and β 2 tubulins is remarkable in view of the similarity between the β 1A and β 1B tubulins. Singhofer-Wowra *et al.* (1986) predicted that *Physarum* β 2-tubulin would be more diverged than β 1 tubulins since it usually participates only in mitosis and meiosis; they suggested that participation of the β 1 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 β 1A and β 1B-tubulins are utilized in the flagellum, and would argue against the maintenance of the two β 1-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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