Cloning and characterization of the *altA* α -tubulin gene of *Physarum*

DAVID B. CUNNINGHAM,¹ GARY L. BUCHSCHACHER, JR.,¹ TIMOTHY G. BURLAND,¹
WILLIAM F. DOVE,^{1*} DIETRICH KESSLER² and EILEEN C. A. PAUL³

¹McArdle Laboratory for Cancer Research, University of Wisconsin–Madison, 1400 University Avenue, Madison, WI 53706, USA

²Department of Biology, Colgate University, Hamilton, NY 13346, USA

³Department of Physiology, 719 Veterinary Research Tower, Cornell University, Ithaca, NY 14853-6401, USA

(Received 20 May 1992; revised 28 August 1992; accepted 14 September 1992)

A cDNA clone derived from the *altA* locus, encoding one of several α -tubulins in *Physarum*, was sequenced and used to determine the developmental and cell cycle expression patterns of its corresponding gene. The predicted amino acid sequence of the *altA* gene product, α 1A-tubulin, is 92% identical to the other known *Physarum* α -tubulins, α 1B and α 2B, which are products of two tightly linked genes at the *altB* locus. The nucleotide sequence of the *altA* coding region is 82% identical to the two *altB* genes. Expression of the *altA* gene was found in all three cell types examined – amoeba, flagellate and plasmodium – but at substantially different levels in each. The peak level of *altA* message detected in flagellates was 14-fold higher than in amoebae, while the peak level in plasmodia was 5-fold lower than in amoebae. The expression pattern of *altA* and the predicted amino acid sequence of the α -tubulin it encodes suggest that α 1A is the substrate for post-translational acetylation, giving rise to the α 3-tubulin isoform found specifically in amoebae and flagellates. Northern blot analysis of plasmodial RNA samples from specific times in the cell cycle showed that the level of *altA* message varies over the cell cycle in a pattern similar to transcripts from other tubulin genes, with a peak at mitosis and little or no message detected during most of interphase.

Introduction

Microtubules are structural filaments found in virtually all eukaryotic cells. A diverse array of structures, including mitotic and meiotic spindles, centrioles, flagellar and ciliary axonemes, and the cytoskeleton, are composed of microtubules. The primary subunit of microtubules is a heterodimer of α - and β -tubulin. Most eukaryotes possess families of genes encoding α - and β -tubulin (reviewed by Cleveland & Sullivan, 1985; Little & Seehaus, 1988; Sullivan, 1988; Murphy, 1991). These multigene families range from isocoding to highly divergent. For example, the three known α -tubulin genes of the protist *Naegleria gruberi* encode identical proteins (Lai *et al.*, 1988), while the α 1 and α 4 genes of *Drosophila melanogaster* encode α -tubulins with only 67% amino acid sequence identity (Theurkauf *et al.*, 1986). The size of tubulin gene families is also variable. Thus, the genome of the budding yeast *Saccharomyces cerevisiae*

encodes two α -tubulins and one β -tubulin, the *Drosophila* genome includes four α -tubulin and four β -tubulin genes, and the chicken possesses six α -tubulin and seven β -tubulin genes, each giving rise to a different tubulin isoform (Sullivan, 1988). While some isoforms are found constitutively throughout the life cycle of an organism, others are expressed in spatially and temporally restricted patterns.

The variety of microtubular structures, tubulin isoforms and distinct patterns of isoform distribution have prompted speculation as to the functional significance of multiple tubulins within an organism. To encode tubulin in multigene families, as opposed to a single gene, may favour evolutionary survival in three ways: first, by allowing functional specialization of individual isoforms for particular microtubular structures (Fulton & Simpson, 1976); second, by facilitating complex patterns of tubulin gene expression over the cell cycle, in development and under varying environmental conditions (Raff, 1984); and third, by allowing for large amounts of tubulin to be synthesized rapidly through simultaneous expression from multiple genes. To what degree, if any, do each of these factors contribute to the ubiquity of tubulin multigene families among eukaryotes? We have

* Author for correspondence. Tel. (608) 262 0982; fax (608) 262 2824.

The nucleotide sequence of cDNA α 606 has been submitted to GenBank and has been assigned the accession number M98863.

explored this question by investigating the tubulin multigene family of the myxomycete *Physarum polycephalum*.

In *Physarum*, the tubulins are encoded by three β -tubulin genes and at least three, but perhaps as many as five, α -tubulin genes (Schedl *et al.*, 1984b). The sequence and developmental expression patterns of most of these genes have been analysed by genetic and molecular methods (reviewed in Sullivan, 1988).

The life cycle of *Physarum* includes several distinct cell-types, employing a number of microtubular structures. The amoeba is a haploid, uninucleate, proliferative cell that includes cytoplasmic microtubules, centrioles and an astral mitotic spindle. Amoebae can be induced to develop reversibly into flagellates – non-dividing cells with two anterior flagella. Amoebae of appropriate mating types may undergo irreversible, heterothallic development, giving rise to a diploid, syncytial plasmodium that is able to grow to indefinite size. Plasmodial nuclei undergo closed, or intranuclear, mitosis in natural synchrony. Several previous investigations were unable to detect cytoplasmic microtubules in the mature plasmodium, and it has been widely held that the mitotic spindle is the only microtubular structure in the plasmodium (Havercroft & Gull, 1983). However, a recent report has identified cytoplasmic microtubules in the plasmodium using immunofluorescence and immunogold electron microscopy (Salles-Pasador *et al.*, 1991). In response to specific environmental conditions, the plasmodium develops a fruiting body, undergoes meiosis and produces haploid spores, which are then able to hatch and give rise to amoebae.

Here we describe the coding sequence and expression pattern of the *altA* α -tubulin gene of *Physarum*. The *altA* gene product probably corresponds to the amoebal α 1-tubulin isoform that was previously characterized (Singhofer-Wowra *et al.*, 1986b; Walden *et al.*, 1989a). Relative to the other known *Physarum* tubulin genes, *altA* is unique in its broad and versatile pattern of expression. We show that, as with members of other multigene families of *Physarum*, the intra-specific and inter-specific divergence of the tubulins is correlated with the extent to which they are expressed throughout the life-cycle.

Methods

cDNA cloning. The construction and screening of two *Physarum* cDNA libraries, ML5 and ML6, are described by Paul *et al.* (1992). The source of RNA for ML6 cDNA synthesis was strain LU352 (Dee *et al.*, 1989), grown axenically (McCullough *et al.*, 1978). ML5 was derived from total myxamoebal RNA, while ML6 was generated using poly(A)⁺ RNA from a culture undergoing the amoeba–flagellate transition. Plaque lifts of the ML5 library were probed for α -tubulin sequences using the *Physarum* α -tubulin cDNA clone Ppc- α 125 (Krammer *et al.*, 1985) radiolabelled by nick-translation (Rigby *et al.*,

1977). Filters were washed in 1 × SSC (150 mM-sodium chloride, 15 mM-sodium citrate), 0.1% SDS at 55 °C. A single positive colony, designated λ gt10 α 501, was plaque-purified and grown up for DNA isolation. Digestion of the recombinant phage DNA with *Eco*RI revealed a 500 bp cDNA insert. Subsequently, this partial clone was used to screen the ML6 cDNA library. A recombinant phage carrying a 1450 bp *Eco*RI insert was identified and designated λ gt10 α 606. The insert was isolated and ligated into the *Eco*RI site of the plasmid vector pGEM-7Zf(+) (Promega) and the *Eco*RI site of the phage vector M13mp18.

Genomic DNA isolation. Nuclei from microplasmodia grown in shaking liquid culture (Turnock *et al.*, 1981) were isolated by the method of Mohberg & Rusch (1971), and further purified by centrifugation through 25% Percoll (Kunzler *et al.*, 1984). The nuclei were lysed and DNA was isolated according to Schedl & Dove (1982), except that dialysis and caesium chloride density-gradient centrifugation were omitted.

Southern blotting. *Physarum* genomic DNA was isolated from haploid plasmodia arising from apogamic development of amoebal strains CLd (Cooke & Dee, 1975) and MA275 (Schedl *et al.*, 1984b) and the diploid strain CLd × MA275. The DNA was digested with a twenty-fold excess of *Eco*RV restriction endonuclease (New England Biolabs) under conditions suggested by the supplier. About 4 μ g of diploid DNA or 2 μ g of the haploid DNAs was loaded per well on a 0.8% agarose gel and electrophoresed at 1 V cm⁻¹ for 40 h. The DNA was blotted and probed as described by Schedl *et al.* (1984b), except that dextran sulphate was omitted from the prehybridization and hybridization solutions and each step was incubated at 65 °C. The probes were radiolabelled with [α -³²P]dCTP by random hexamer priming (Prime-a-Gene, Promega) to a specific activity of about 1 × 10⁹ c.p.m. (μ g DNA)⁻¹. The α 606 probe was made using the full-length 1450 bp cloned cDNA *Eco*RI fragment, while the α 606-3' probe was derived from a 120 bp *Xho*I/*Eco*RI fragment at the 3' end of the α 606 clone. Filters were washed at 60 °C in either 1 × SSC, 0.1% SDS or 0.1 × SSC, 0.1% SDS as specified, with six changes of wash buffer over 2 h. After drying, the filters were exposed to X-ray film (XAR-5, Kodak) with intensifying screens (Lightning-Plus, DuPont).

In vitro transcription and translation. The 1450 bp α 606 cDNA clone was subcloned into the *Eco*RI site of the plasmid vector pGEM-7Zf(+) (Promega). The plasmid was linearized with *Hind*III and transcripts were synthesized *in vitro* from the T7 promoter (Studier & Moffatt, 1986). About 300 ng of α 606 transcript was translated *in vitro* as described (Paul *et al.*, 1992). A mixture of the radiolabelled product and unlabelled plasmodial lysate was resolved by two-dimensional PAGE and blotted onto a nitrocellulose membrane as described (Burland *et al.*, 1988). The blot was probed with a mixture of the anti- α -tubulin monoclonal antibody YL1/2 (Kilmartin *et al.*, 1982) and the anti- β -tubulin antibody KMX-1 (Birkett *et al.*, 1985b). Antibody binding was visualized using horseradish-peroxidase-conjugated secondary antibodies. The blot was then exposed to X-ray film to determine the position of the radiolabelled α 606 translation product relative to the immunostained plasmodial tubulins. An additional two-dimensional Western blot was generated from ³⁵S-labelled *in vitro* translation products of poly(A)⁺ RNA isolated from plasmodial G2 phase. An immunoblot and autoradiogram of this blot were used as additional references in identifying the translation product of α 606.

Sequencing. The 1450 bp α 606 cDNA fragment was ligated in both orientations into the *Eco*RI site of the bacteriophage vector M13mp18 (New England Biolabs) and a series of deletion subclones of the cDNA insert in each orientation was generated as described by Dale *et al.* (1985) using the Cyclone deletion system (International Biotechnologies). The nucleotide sequences of the subclones were determined by the dideoxynucleotide chain termination method (Sanger *et al.*,

1977) using Sequenase enzyme (United States Biochemical). Overlapping sequences were identified and combined to generate the full-length sequence of $\alpha 606$ using the computer program ALIGN (DNASTAR, Madison, Wisconsin). The predicted amino acid sequence encoded by the $\alpha 606$ cDNA clone was then determined using the TRANSLATE program. Pairwise comparisons of the nucleotide and amino acid sequences of $\alpha 606$ to other known *Physarum* α -tubulin sequences were made using the ALIGN and AALIGN programs.

Northern blotting. Total RNA was isolated from microplasmodia and flagellates as described by Burland *et al.* (1983). Amoebal RNA was isolated by the single-step acid guanidinium thiocyanate/phenol/chloroform (AGPC) extraction method (Chomczynski & Sacchi, 1987) from exponentially growing cells cultured axenically in liquid medium. RNA samples were isolated from a single plasmodium at various times in the cell cycle by the AGPC method, with an additional lithium chloride precipitation step, as described by Puissant & Houdebine (1990). In some samples (see figure legends) poly(A) tails were removed from mRNAs by the oligo-dT/RNAase H method (Mercer & Wake, 1985). Glyoxylated RNA was electrophoresed, blotted and hybridized to probes labelled with [α - 32 P]dCTP according to Schedl *et al.* (1984a). Probes for *altB* transcripts were either the 930 bp *SacI/BglII* fragment or the 250 bp *SacI/AluI* fragment of the *Physarum* cDNA clone Ppc- $\alpha 125$ (Schedl *et al.*, 1984a). Transcripts from the *altA* locus were detected using either the complete 1450 bp $\alpha 606$ cDNA clone or the 120 bp 3' *XhoI/EcoRI* fragment of $\alpha 606$. The 670 bp *Physarum* cDNA clone, Ppc-16, was radiolabelled and added to the hybridization reactions to verify RNA loading and integrity (Schedl *et al.*, 1984a). All blots were washed for 2 h at 65 °C in 0.1 $\times$ SSC with six changes of wash buffer.

Induction of flagellate formation. Myxamoebae of the strain LU352 (Dee *et al.*, 1989) were induced to form flagellates as described by Paul *et al.* (1992). Briefly, cells were grown axenically to a density of 0.4–1.0 $\times 10^7$ cells ml $^{-1}$ in a shaking culture of SDM (Cooke & Dee, 1975). At thirty-minute intervals, the cells were pelleted and re-suspended in increasingly dilute SDM. The percentage of flagellates within the population was monitored by phase contrast microscopy.

Results

Isolation of the $\alpha 606$ cDNA clone

Screening the amoebal cDNA library ML5 with the *Physarum* tubulin cDNA clone Ppc- $\alpha 125$ (Schedl *et al.*, 1984a) identified a single positive plaque. This clone included a 500 bp insert, designated $\alpha 501$, that hybridized preferentially to polymorphic restriction fragments characteristic of the *altA* locus on Southern blots of *Physarum* genomic DNA (data not shown). In order to obtain a full-length cDNA from this locus, the $\alpha 501$ fragment was used to probe the amoebal/flagellate cDNA library ML6 (Paul *et al.*, 1992), which includes size-selected cDNA inserts of greater than 1 kb. From among the positive plaques detected, one containing a 1450 bp cDNA insert, designated $\alpha 606$, was characterized further.

The $\alpha 606$ cDNA clone is derived from the *altA* locus

The locus encoding the $\alpha 606$ cDNA sequence was identified by Southern blotting. Previous analysis using restriction fragment length polymorphisms (RFLP)

indicated that the *Physarum* genome includes four unlinked α -tubulin loci, designated *altA* through *altD* (Schedl *et al.*, 1984b). The hybridization pattern of the $\alpha 606$ clone to polymorphic *EcoRV* restriction fragments of genomic DNA from *Physarum* is shown in Fig. 1. The blot shown in panel (a) was hybridized to the full-length $\alpha 606$ cDNA clone and washed at moderate stringency (1.0 $\times$ SSC, 60 °C). All fifteen polymorphic bands that define the four α -tubulin loci are visible (Schedl *et al.*, 1984b). By contrast, the blot in panel (b) was washed at high stringency (0.1 $\times$ SSC, 60 °C) and showed strong, preferential hybridization to the *altA* bands (b, l and o). Cross-hybridization to several of the *altB* bands was visible, though no hybridization to the *altC* or *altD* loci was detected. Finally, a 120 bp fragment from the 3' end of the $\alpha 606$ clone was used to probe the blot in panel (c). This probe is nearly locus-specific for *altA*, displaying only a trace of hybridization to the *altB* bands h and i.

The $\alpha 606$ cDNA clone encodes an $\alpha 1$ -tubulin

Evidence from sequencing of purified amoebal α -tubulin points to only one major α -tubulin isotype in this cell type (Singhofer-Wowra, 1986b). However, since there may be as many as five α -tubulin genes in *Physarum*, it is possible that minor α -tubulins may be expressed. The particular tubulin isotype encoded by $\alpha 606$ was identified by two-dimensional PAGE analysis of *in vitro* translation products of transcripts generated from an expression vector carrying the $\alpha 606$ clone. Unlabelled plasmodial cell lysate was mixed with the translation products so that both α - and β -tubulin could be detected by immunostaining in order to orient the autoradiogram (Paul *et al.*, 1992). A portion of an autoradiogram of 35 S-labelled *in vitro* translation products from plasmodial poly(A) $^{+}$ RNA is shown in Fig. 2(a). Two α -tubulin and two β -tubulin electrophoretic isotypes are visible in well-characterized positions (Burland *et al.*, 1983). Their identity was verified by probing with the anti- α -tubulin monoclonal antibody YL1/2 (Kilmartin *et al.*, 1982) and the anti- β -tubulin antibody KMX-1 (Birkett *et al.*, 1985b) (data not shown). The position of the radio-labelled translation product of $\alpha 606$ transcripts is shown by the autoradiogram of the immunoblot in Fig. 2(b). Arrows at corresponding positions of the tubulins detected by immunostaining indicate that $\alpha 606$ encodes an $\alpha 1$ -tubulin.

Nucleotide sequence of the $\alpha 606$ cDNA clone

Both strands of the cloned 1450 bp *EcoRI* cDNA insert were sequenced from a series of deletion subclones in an M13 bacteriophage vector (Dale *et al.*, 1985). An open

Fig. 1. Southern blots of *Physarum* genomic DNA probed with the $\alpha 606$ cDNA clone. DNA from the diploid plasmodial strain CLdxMA275 includes 15 polymorphic *EcoRV* fragments (a–o) that hybridize to α -tubulin sequences (Schedl *et al.*, 1984*b*). These bands are more clearly distinguished in the haploid amoebal strains CLd and MA275, which were crossed to generate the heterozygous, diploid plasmodial strain. Each band can be assigned to one of four unlinked α -tubulin loci, *altA* to *altD*, as follows: *altA* (b, l, o), *altB* (f, g, h, i, j, k, m, n), *altC* (a, c), *altD* (d, e). (a) Autoradiogram of a blot probed with the full-length $\alpha 606$ cDNA clone and washed at moderate stringency ($1.0 \times \text{SSC}$). (b) Autoradiogram of a replicate blot probed with $\alpha 606$, but washed at high stringency ($0.1 \times \text{SSC}$). Here the probe shows a strong preferential hybridization to the *altA* bands. (c) Autoradiogram of a replicate blot probed with the 120 bp *EcoRI/XhoI* restriction fragment from the 3' end of $\alpha 606$, and washed at high stringency. Since band o represents the 5' region of the *altA1* allele, it cannot be detected by this probe. Relatively weak hybridization to the *altB* bands, h and i, is visible.

reading frame of 1353 bp, including the start and stop codons, was found to share about 82% nucleotide sequence identity with two other α -tubulin genes of *Physarum*. Fig. 3 displays the complete coding sequence of $\alpha 606$, compared to the coding regions of two closely related α -tubulin genes from the *altB* locus, the E α -tubulin gene and the N α -tubulin gene (Monteiro & Cox, 1987; Walden *et al.*, 1989*b*), here designated *altB(E)* and *altB(N)*. Only positions of nonidentity to *altA* are given for the *altB* genes. Nucleotide substitutions are scattered throughout the 450 codons of sequence, with roughly 200 out of 240 being silent substitutions in each case. The $\alpha 606$ clone includes a short tract of 5'

untranslated sequence. Since the transcriptional start site of *altA* has not been mapped, it is not known if this region corresponds to the full-length transcript. Previous Northern blot analysis of deadenylated amoebal α -tubulin transcripts found a single size species of approximately 1490 bases (Green, 1987), suggesting that the 1450 bp $\alpha 606$ clone is not complete in the non-coding regions. The 3' untranslated region consists of 71 nucleotides, including a short poly(A) tract.

A putative polyadenylation signal, similar to known functional sequences in other organisms (Manley, 1988), is the underlined TATAAA sequence found 13 nucleotides upstream from the beginning of the poly(A) tract.

Fig. 2. (a) Autoradiogram of ^{35}S -labelled *in vitro* translation products of poly(A)⁺ RNA from *Physarum* plasmodial G2 phase resolved by two-dimensional PAGE and blotted onto nitrocellulose. The identity of the α - and β -tubulin spots was verified by immunostaining the blot with anti- α - and anti- β -tubulin monoclonal antibodies (data not shown). (b) Autoradiogram of the ^{35}S -labelled *in vitro* translation product of transcripts synthesized from the $\alpha 606$ cDNA clone. The two upper arrows indicate the position of unlabelled *Physarum* β -tubulins detected by immunostaining the blot with an anti- β -tubulin antibody (data not shown). The radiolabelled translation product comigrates to the $\alpha 1$ -tubulin position.

The same sequence is found at this distance from the poly(A) tract in several other *Physarum* genes, most notably the two profilin genes, *proA* and *proP* (Binette *et al.*, 1990). However, it is found in neither *altB(E)* nor *altB(N)* (Monteiro & Cox, 1987; Walden *et al.*, 1989b), both of which are known to generate polyadenylated transcripts (Green & Dove, 1988), nor in a number of other cDNA clones from *Physarum* (D. Pallotta, personal communication).

Amino acid sequence of $\alpha 1A$ -tubulin

The amino acid sequence of the *altA* gene product, designated $\alpha 1A$ in reference to its electrophoretic isotype ($\alpha 1$) and the locus from which it arises (*altA*), was inferred from the nucleotide sequence of $\alpha 606$. The *altA* sequence is consistent with the published partial amino acid sequence of amoebal α -tubulin, and identifies the 20 amino acid residues that complete the sequence (Singhofer-Wowra *et al.*, 1986b). A comparison of $\alpha 1A$ and

the two other known *Physarum* α -tubulins, $\alpha 1B$ and $\alpha 2B$, is shown in Fig. 4. The $\alpha 1A$ sequence is approximately 92% identical to both $\alpha 1B$ and $\alpha 2B$. The greatest divergence is evident among the 12 amino acids at the carboxyl terminus, as is typical of both α - and β -tubulins in general (Sullivan, 1988). Listed below the *Physarum* sequences are α -tubulin sequences from a variety of other organisms, highlighting the divergent carboxy-terminal domain juxtaposed to a region of extraordinary evolutionary conservation (residues 391–435).

The percent identity among α -tubulins within *Physarum* is similar to that seen between *Physarum* and several other protists (*Naegleria gruberi*, *Chlamydomonas reinhardtii* and *Stylonicchia lemnae*) (Table 1). *Physarum* $\alpha 1A$ shows a similar level of identity ($\sim 86\%$) to α -tubulins from multicellular eukaryotes as diverse as mammals (*Mus musculus*), insects (*Drosophila melanogaster*) and plants (*Arabidopsis thaliana*). The most divergent α -tubulins are found in the two yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, which show only about 70% identity to the α -tubulins of *Physarum* and most of the other organisms examined.

Transcripts from the *altA* gene are detected in amoebal, flagellate and plasmodial RNA

Northern blot analysis of total RNA from three *Physarum* cell types – amoeba, flagellate and plasmodium – indicated that *altA* transcripts are present at different levels in each (Fig. 5). Prior to electrophoresis, all of the RNA samples were incubated with oligo d(T) and subsequently digested with RNAase H to remove the poly(A) tails from mRNA (Mercer & Wake, 1985). This treatment reduces size heterogeneity due to variable poly(A) tail length that may exist within a single species of tubulin transcript in *Physarum* plasmodia (Green & Dove, 1988).

The blot shown in panel (a) was probed with the 120 bp 3' fragment of the $\alpha 606$ cDNA clone and washed at high stringency to detect *altA* transcripts. Both the amoebal lane (am) and the flagellate lane (fl) showed relatively strong hybridization with this probe. These bands do not represent cross-hybridization to *altB(E)* or *altB(N)* transcripts, since the expression of these genes is plasmodium-specific (Green *et al.*, 1987; Walden *et al.*, 1989a). Furthermore, it is unlikely that the signal is attributable to *altC* or *altD* transcripts, since the $\alpha 606$ cDNA probe failed to cross-hybridize with the *altC* and *altD* loci at this stringency of washing on Southern blots. Thus, while we cannot absolutely rule out the possibility of cross-hybridization, the signal is likely to represent only *altA* transcripts.

While the signal in the flagellate lane was approx-

```

altA   ATGCGTGAAGTGATTTCACATCCACATTGGACAAGCCGGAACCCAGGTCCGTAACGCTTGCTGGGAGCTCTACTGCTTGGAGCATGGCATCCAACCTGATG 100
altB(N) C  GA T C T T TG T A T C T G T C A A T C
altB(E) GA AC C T T TG T T C T G T C T A A G C

altA   GCCAGATGCCCTCCGACAAGTCCGTAGGCTATGGAGATGACGCCTTCAACACCTTCTCTCTGAGACCGGTGCTGGCAAGCACGTGCCCCGTGCCGTCTT 200
altB(N) A A A T T AGGG T A C GA CT A A AA G AA A
altB(E) A A G T T AGGA C T A C GA CT A A AA A AA

altA   CTTGGACTTGAGAGCCCACTGTGATCGATGAAGTCAGGACCGGAACCTACCGCCAGTTGTTCCACCCCGAACAACCTCATCACTGGCAAGGAGGATGCCGCG 300
altB(N) A T T GA A A C C T G T A C A
altB(E) A T GA A A C C T T A C A

altA   AACAACTATGCCCGTGGTCATTACACCATTGGAAAGGAAATCGTGGACCTCTGCCTCGACAGAGTGAGGAAGCTCGCCGACCAGTGCACCTGGTCTCCAAG 400
altB(N) T G C A T G T G CT G
altB(E) T G C A C T G G CT G

altA   GTTTCCTCGTCTTCCACTCTGTTGGTGGTGGTACAGGATCTGGTTTCGGCTCTCTTCTCCTTGAGAGACTCTCCGTTGACTACGGAAAGAAGAGCAAGCT 500
altB(N) G T C G A C CT GT G G T A
altB(E) G T C G A C CT GT G G T A

altA   CGACTTCTGCGTCTACCCATCTCCCCAAGTGTCCACTGCCGTGCTGGAGCCCTACAACCTCCGTGCTCTCCACCCACTCTTTGTTGGAGCACACCGATGTG 600
altB(N) T T C G A G A A T C T GGGC C C
altB(E) T T C G A G A A T C T GGGC C C

altA   GCCTTCATGCTCGACAATGAGGCTATCTACGACATCTGCAAGAGAGCCCTCGACATCGACCGCCCACTACACCAACTTGAACAGGCTGGTCCGCCAGG 700
altB(N) A G C A C T C AGT G TT G G C C TC C T T T
altB(E) A G C A C C C GT A A TT G G C C C T T T T

altA   TCATCTCCTCCTTGACCACCTCTCTTCGTTTCGATGGTGCCCTCAACGTCGATATCACTGAGTTCCAAACCAACTTGGTGCCCTACCTCGCATCCATTT 800
altB(N) G G TC C G C T C GT G T AC G C
altB(E) G TC C T G T G G G T G G

altA   CATGCTCTGCTCGTACGCCCCCGTGATCTCCGCCGAGAAGGCCCTACCACGAGCAACTTACCGTTGCTGAGATCACCAACTCTGTGTTTCGAGCCCGCCAAC 900
altB(N) GGCG T T T T G CT T G AC T A A T T G G
altB(E) TGCG C T G T T T CT T G C A G T C T G G

altA   ATGATGGCGAAGTGCAGCCCCCGTCACGGCAAGTACATGGCTTGCTGCTTGATGTATCGTGGTATGTTGTTCCCAAGGATGTTAACGCGTCCGTGGCCG 1000
altB(N) C T C A T T C A A C C G C CG A G
altB(E) C T C A A CA A A C C T C G AG G T G

altA   TCATCAAGACCAAGCGCACCATCCAGTTCGTCGACTGGTGTCCACCGGATTCAAGTGCGGTATCAACTACCAACCCCCACCGTCGTGCCTGGAGGAGA 1100
altB(N) A G C A A G G T G C
altB(E) A G C A A A A AT G C

altA   CCTCGCCAAAGTCCAGCGCGCCGTGTGTATGCTTTCCAACACCACCGCCATTGCTGAGGTGTTCTCTCGCATTGACCACAAGTTCGATCTCATGTACGCC 1200
altB(N) T G G T C A G G C C A AGC C T
altB(E) T G A G T C A G T G A AGC C T C T G

altA   AAGCGTGCCTTCGTCCACTGGTACGTCGGAGAGGGTATGGAGGAGGGTGAAGTTCAGCGAGGCCCGTGAGGATCTCGCCGCCCTCGAGAAGGACTACGAGG 1300
altB(N) G T G A A TCG A T C C T G G A
altB(E) C T T G T A TCG A T C T T G A

altA   AGGTCGGAGCCGAGAGTTCGAGGCTGGTGGCGACGAGGAAGGAGAGATTAA 1353
altB(N) A A CCGT G AC A AA CG A AGAT A...
altB(E) A G C G CCG A A A T CA T AGAT A...

```

altA 5' Untranslated TTTTTTTTTTTTTTATTCTTCAATT

altA 3' Untranslated ACTGCGTAAACTCCTGTTGTATTTTGTGTCCATATAAACTGTGTACCTCATAAAAAAAAAAAAAAAAAAAAA

imately twice as strong as the amoebal signal, the flagellate lane was loaded with only one third the amount of RNA of the amoebal lane. From this, we conclude that the steady state level of *altA* message is about six times higher in flagellate RNA than in amoebal RNA. In contrast, the signal seen in the plasmodial lane (p1) was about five-fold less intense than that in the amoebal sample.

Does the signal seen in the plasmodial lane in panel (a) represent cross-hybridization to *altB* transcripts? Indeed, the *altA* probe showed a small amount of cross-hybridization to the *altB* locus on Southern blots under similar conditions of washing (Fig. 1c), and it is known that *altB* transcripts are relatively abundant in plasmodial RNA. Fig. 5(b) shows a duplicate blot generated from aliquots of the RNA samples used in panel (a), but probed with α 125, a partial cDNA clone encoded by the *altB* locus (Schedl *et al.*, 1984a). Consistent with previous data, no signal was detected in the amoebal or flagellate lanes, even on long exposures of the blot (not shown). As expected, the plasmodial lane showed a relatively strong signal. However, this band had a slightly lower electrophoretic mobility than the bands detected by the *altA* probe. This difference is demonstrated more clearly in (c), where a third blot was probed with both α 606-3' and α 125. Here, the prominent band detected in the plasmodial lane had lower electrophoretic mobility than the bands seen in the amoebal and flagellate lanes. This pattern is distinct from that seen in panel (a), where the RNA detected in all three lanes exhibits the same mobility. We conclude that the small amount of signal detected in plasmodial RNA by the *altA* probe is not due to cross-hybridization to *altB* transcripts, but rather to the presence of *altA* transcripts at relatively low abundance.

The level of altA message changes during flagellate induction

Northern blot analysis of total RNA from cells in transition from amoebae to flagellates revealed a substantial increase in the level of *altA* message over the first two hours of flagellate induction, during which approximately 20% of the cells had become flagellates (Fig. 6). The level of *altA* message then dropped over the following two hours of induction, even as the percentage of flagellates continued to rise to a maximum of about 90% of the population. The peak level of message

detected at 120 min of induction was approximately 14-fold greater than that seen in the 0 min sample, and about 3-fold higher than seen at 240 min. Duplicate blots were probed with an actin cDNA and the cDNA clone Ppc-16 (Schedl *et al.*, 1984a), to verify the integrity of the RNA and the uniform loading of the six samples (data not shown). The signal obtained with the Ppc-16 probe varied by less than two-fold among the six timepoint samples, while the actin signal showed a slight decrease over the time course of induction, in agreement with previous experiments (Green & Dove, 1984).

The level of altA message changes over the plasmodial cell cycle

A series of RNA samples isolated from a single plasmodium over a portion of the naturally synchronous cell cycle was analysed by Northern blotting (Fig. 7). Duplicate blots were prepared in order to compare the expression pattern of *altA* with the two *altB* genes. Both blots were probed with Ppc-16, which detects an 850 bp transcript at a relatively constant level throughout the cell cycle and life cycle (Schedl *et al.*, 1984a). The autoradiogram shown in Fig. 7(a) is a two-day exposure of a blot that was also probed with a 250 bp *SacI/AluI* fragment of the *altB* cDNA α 125, presumably detecting transcripts from both *altB(E)* and *altB(N)*. The *altB* signal was relatively weak in mid-G2 phase (–240 min), but increased substantially over the last three hours of G2, peaking at metaphase (0 min). At 30 min post-metaphase, the signal had dropped to about half the peak level, and by 90 min after metaphase, no *altB* signal was detected. This pattern is in agreement with previous studies (Schedl *et al.*, 1984a; Green & Dove, 1988). The slight change in mobility of the *altB* transcripts between the metaphase sample and the +30 min sample is due to the shortening of the poly(A) tail of both α - and β -tubulin messages immediately following metaphase in the plasmodium (Green, 1987; Green & Dove, 1988). A flagellate RNA sample included on the blot showed no hybridization to the *altB* probe, as expected.

A duplicate blot was probed for *altA* expression, using the 120 bp *XhoI/EcoRI* fragment of α 606 (Fig. 7b). A pattern of cell cycle modulation similar to that of the *altB* genes was observed, with the highest level of message detected in late G2 and mitosis, and little or no signal observed in late S-phase and early G2. The overall level of detectable *altA* message was substantially lower than that of the *altB* genes, as indicated by the seven-fold

Fig. 3. Nucleotide sequence alignment of the coding regions from three α -tubulin genes in *Physarum*. Only positions of non-identity with *altA* (α 606) are shown for *altB(N)* (Walden *et al.*, 1989b) and *altB(E)* (Monteiro & Cox, 1987). The inferred 5' and 3' untranslated regions of *altA* are included at the bottom of the figure. A putative polyadenylation signal is underlined in the 3' untranslated sequence.

Table 1. Comparison of amino acid sequences of α -tubulin from *Physarum* and other organisms

Figures are percentage identity between the two sequences. For abbreviations and source references see Fig. 4 legend, except for Sc1 and Sc3, α 1- and α 3-tubulins of *S. cerevisiae* (Schatz *et al.*, 1986).

	Pp1B	Pp2B	Ng1	Cr1	Sl1	Mm1	Dm1	At1	Sp2	Sc1	Sc3	Sp1
Pp1A	91.5	91.5	91.3	90.6	89.5	86.6	85.8	85.8	71.6	71.9	71.2	70.3
Pp1B		96.4	87.3	86.6	85.3	82.1	81.7	84.4	71	70.8	69.8	69.5
Pp2B			87.5	87.2	86	82.3	81.7	84.4	70.8	69.8	68.9	69.4
Ng1				93.1	92.2	85.7	85.5	86.6	71.7	69.3	69.3	70.4
Cr1					92.2	86.3	85.8	86.9	71.9	69.7	70	70.5
Sl1						85.3	84.5	85.7	69.6	68.6	68.2	69.4
Mm1							96.9	79.4	76.5	74.7	73.4	76.3
Dm1								78.9	76.3	74.9	74.3	75.8
At1									68.2	66.8	67.1	67.9
Sp2										75.5	73.9	85.7
Sc1											90.8	76.3
Sc3												74.5

Fig. 5. Northern blot analysis of *altA* transcripts in total RNA from amoebae, plasmodia and flagellates. Three replicate Northern blots were generated and probed differently as indicated in the three panels. Each 'am' lane was loaded with 3 μ g amoebal RNA, each 'pl' lane received 3 μ g plasmodial RNA and each 'fl' lane contained 1 μ g flagellate RNA. All of the RNA samples were treated to remove poly(A) tails and denatured by glyoxylation prior to electrophoresis on a single gel. (a) Autoradiogram of a blot probed with the 120 bp *Xho*I/*Eco*RI fragment from the 3' end of the α 606 cDNA clone. (b) Autoradiogram of a blot probed with the 930 bp *Sac*I/*Bgl*II fragment of the α 125 cDNA clone. (c) Autoradiogram of a blot double probed with both fragments. All of the blots were washed under stringent conditions (0.1 $\times$ SSC, 65 $^{\circ}$ C). The autoradiograms shown were exposed to the blots for 72 h at -80° C with an intensifying screen.

longer time of exposure required for the *altA* autoradiogram (compare the relative Ppc-16 signal between Figs 7a and b). It should be noted that the different levels of signal seen between the *altB* and *altA* blots can be attributed in part to the larger size of the *altB* probe relative to the *altA* probe. The strong *altA* signal seen in

Fig. 6. (a) Northern blot analysis of total RNA isolated at different times after amoebae were induced to form flagellates. Numbers over each lane indicate the time in minutes after induction of flagellate development. The blot was probed with α 501, a 500 bp partial cDNA from the 3' region of the *altA* mRNA. (b) Densitometric quantification of the Northern blot signal expressed as a percentage of the peak signal at 120 min (□) compared to the percentage of flagellates among the cells harvested at each timepoint (△).

the flagellate sample indicated that the α 606 probe was able to detect abundant message levels under the hybridization and wash conditions used.

Fig. 7. Northern blot analysis of total RNA isolated from a single plasmodium at various times in the cell-cycle. The numbers over the lanes indicate the time in min relative to metaphase (0) that each sample was collected. Approximately 6 μ g RNA was loaded onto each lane. Lane F1 on each blot was loaded with 3 μ g flagellate RNA. Both blots were probed with the cDNA clone Ppc-16, which recognizes an 850 bp constitutive transcript, to gauge the loading and integrity of each RNA sample. The blot in (a) was additionally probed with the 250 bp *SacI*/*AluI* fragment of α 125 to detect transcripts of the *altB* genes. A two-day exposure at -80°C is shown. The blot in (b) was probed with the 120 bp 3' *XhoI*/*EcoRI* fragment of α 606 in order to detect *altA* transcripts. A two-week exposure at -80°C is shown.

Discussion

Developmental expression pattern of *altA*

Analysis of the sequence and expression pattern of the *altA* locus using the α 606 cDNA clone confirmed several previous observations concerning the α -tubulin family of *Physarum*. First, the *altA* gene product, α 1A-tubulin, is expressed in both the amoeba and the plasmodium. Walden *et al.* (1989a) tested the epitope requirements of two anti- α -tubulin monoclonal antibodies, 6-11B-1 (Piperno & Fuller, 1985) and KMP-1 (Birkett *et al.*, 1985a) on *Physarum* proteins. It was found that both

antibodies recognize an epitope that includes lysine 40 (Lys⁴⁰). Recognition by 6-11B-1 requires acetylated Lys⁴⁰, while KMP-1 requires unacetylated Lys⁴⁰ as well as a tyrosine at position 44. Because KMP-1 detects α -tubulin in amoebae and plasmodia, and neither of the plasmodium-specific α -tubulin genes encodes Tyr⁴⁴ (Monteiro & Cox, 1987; Walden *et al.*, 1989a) it was proposed that the α 1-tubulin isotype detected in the amoeba, which is known to include both Lys⁴⁰ and Tyr⁴⁴ (Singhofer-Wowra *et al.*, 1986b), is also found at reduced levels in the plasmodium. It is not possible to exclude the possibility that the *altC* and/or *altD* loci give rise to gene products in the plasmodium that are recognized by KMP-1. However, the Northern blot data reported here (Fig. 5) indicate that the *altA* gene is transcribed in amoebae and plasmodia. We infer that the α 1-tubulin previously detected by the KMP-1 antibody in amoebae and plasmodia (Walden *et al.*, 1989a) is the product of the *altA* gene.

The detection of *altA* expression in the plasmodium corroborates previous studies that revealed multiple α -tubulin isotypes present in this cell type (Burland *et al.*, 1983; Birkett *et al.*, 1985b). Green *et al.* (1987) proposed that at least one plasmodium-specific α 1-tubulin, designated α 1B, arises from a pair of closely related and tightly linked genes constituting the *altB* locus. This is confirmed and extended by Walden *et al.* (1989a), using monoclonal antibodies specific for each of the α -tubulins encoded by the *altB(E)* and *altB(N)* genes. They demonstrate that one of these, E α -tubulin, is a plasmodium-specific α 2-tubulin, while N α -tubulin is a plasmodium-specific α 1-tubulin. We refer to these tubulins as α 2B and α 1B, respectively, in reference to the original descriptions of electrophoretic isotypes (Burland *et al.*, 1983) and genetic loci (Schedl *et al.*, 1984b). Hence, at least three α -tubulin genes are expressed in the plasmodium. In contrast, *altA* is the only known α -tubulin gene expressed in the amoeba.

From existing data, it is not clear whether α 1A participates in all plasmodial microtubules or a subpopulation. Plasmodial mitotic spindles are stained by the monoclonal antibody KMP-1 (Birkett *et al.*, 1985a; Solnica-Krezel *et al.*, 1991), which is probably specific for α 1A, as discussed above. That α 1A participates in the plasmodial mitotic spindle is also implied by the cell cycle modulation in *altA* transcript level, which was found to peak at mitosis in the plasmodium (Fig. 7b). However, the single description of plasmodial cytoplasmic microtubules to date (Salles-Pasador *et al.*, 1991) employed mainly YL1/2, an antibody which detects both α 1- and β 2-tubulin (Birkett *et al.*, 1985a). An integrated analysis of plasmodial microtubules, using the two specific anti- α -tubulin antibodies raised by Walden *et al.* (1989a) along with KMP-1 (Birkett *et al.*, 1985a, b) may reveal whether

the three known plasmodial α -tubulin isotypes are segregated among specific subpopulations of microtubules. No such segregation has been detected for the two β -tubulins, β 1B and β 2, that are coexpressed in the plasmodium (Diggins-Gilcinski *et al.*, 1989; Paul *et al.*, 1989).

Possible acetylation of α 1A-tubulin

Post-translational modification of amoebal α 1-tubulin produces the α 3-tubulin isotype found in both amoebae and flagellates (Green & Dove, 1984). Recognition of α 3-tubulin by the 6-11B-1 monoclonal antibody indicates that Lys⁴⁰ is acetylated (Piperno & Fuller, 1985). Two-dimensional gel electrophoresis of protein labelled *in vivo* reveals that the level of α 3-tubulin detected in flagellates is perhaps ten-fold higher than in amoebae. At the same time, α 1-tubulin shows a more modest increase of about two-fold (Green & Dove, 1984). This result is borne out by indirect immunofluorescence of fixed cells stained with the 6-11B-1 antibody. In amoebae, α 3-tubulin is found localized solely to the microtubule organizing centre (MTOC). Flagellates, by contrast, exhibit α 3-tubulin in all microtubular structures detected, including the flagellar axonemes, basal bodies and flagellar cone (Diggins & Dove, 1987; Sasse *et al.*, 1987). Since α 3-tubulin arises by modification of α 1-tubulin, a substantial increase in α 1-tubulin synthesis must accompany flagellate induction. The 14-fold increase in the level of *altA* message detected by Northern blots during flagellate induction (Fig. 6) leads us to conclude that the *altA* gene product, α 1A-tubulin, is probably the substrate for the acetylation that produces α 3-tubulin.

No acetylated α -tubulin is detected in the plasmodium (Diggins & Dove, 1987; Sasse *et al.*, 1987), despite the fact that all three α -tubulins known to be present in the plasmodium possess a Lys⁴⁰ residue that can be acetylated *in vitro* (Walden *et al.*, 1989a). As expected, none of the microtubular structures that include acetylated α -tubulin in amoebae and flagellates is present in the plasmodium. The cellular and subcellular localization of acetylated α -tubulin in *Physarum* tempts one to speculate that this isoform has a specific function. However, the cellular distribution of acetylated α -tubulin among various microtubular arrays exhibits such diversity among organisms that its possible functional significance remains unclear (Sullivan, 1988).

*Induction of *altA* expression during flagellate development*

The sharp increase in *altA* message seen during the amoeba-flagellate transition (Fig. 6) is accompanied by a coordinate induction of a specific β -tubulin message

(Paul *et al.*, 1992). Two β -tubulin genes, *betA* and *betB*, are expressed in amoebae (Burland *et al.*, 1984). During flagellate induction, however, only *betA* message shows an increased abundance (Paul *et al.*, 1992). Remarkably, the magnitude of *betA* induction is much greater than *altA* induction over the same time course: while *altA* message increased by about 14-fold from its steady-state level in amoebae (Fig. 6), *betA* message increases at least 100-fold (Paul *et al.*, 1992). Since α - and β -tubulin bind to each other with 1:1 stoichiometry to form heterodimers, one might expect parallel induction of these genes during flagellate formation. However, the steady-state level of *betA* message in the amoeba appears to be much lower than that of *altA* message. Prior to flagellate induction, *betA* message is virtually undetectable in amoebal RNA, while *altA* and *betB* messages are relatively abundant. Since the basal levels of *altA* and *betA* message are unequal, the different magnitudes of induction may result in an equivalent amount of each message at their peak levels.

*Cell cycle modulation of *altA* message levels*

Changes in the levels of both α - and β -tubulin mRNA over the plasmodial cell cycle are well-characterized (Schedl *et al.*, 1984a; Green & Dove, 1988). We have found that while *altA* message constitutes only a small fraction of the total α -tubulin message in the plasmodium, its level rises to a peak at metaphase and subsequently falls below the level of detection roughly in parallel to *altB* mRNA (Fig. 7). In addition to its lower level of expression, *altA* appears to differ from the *altB* genes in two ways. First, there appears not to be as great a change in size of the *altA* transcripts as there is in the *altB* transcripts between the metaphase sample (0 min) and the +30 min sample. Second, the decrease in *altA* transcript level between the 0 min and 30 min samples is not as great as the decrease in *altB* level. These differences may be related, as it has been shown that the change in poly(A) tail length of the *altB* messages before and after metaphase is correlated with a change in their half-life (Green & Dove, 1988). Short-term labelling of RNA *in vivo* indicates that changes in both the transcription rate and the half-life of *altB* messages contribute to their observed modulation in level over the cell cycle (Green, 1987; Green & Dove, 1988).

*Divergence of *Physarum* tubulins*

A comparison of nucleotide and amino acid sequence identity among several *Physarum* gene families is shown in Table 2. Among the three α -tubulin genes, the *altB(N)* and *altB(E)* genes are more similar to each other than to *altA*. Since the two *altB* genes are expressed exclusively

Table 2. Sequence divergence within *Physarum* gene families

Comparison of nucleotide sequence identity and amino acid sequence identity among members of *Physarum* gene families. Percentage identity was determined using previously published sequences of *altB(E)* (Monteiro & Cox, 1987), *altB(N)* (Walden *et al.*, 1989b), *betA* and *betB* (Paul *et al.*, 1992), *betC* (Burland *et al.*, 1988), *ardA*, *ardB* and *ardC* (Hamelin *et al.*, 1988), *ardD* (Adam *et al.*, 1991), *proA* and *proP* (Binette *et al.*, 1990).

Gene family	Loci	Sequence identity (%)	
		Nucleotide	Amino acid
α -Tubulin	<i>altA</i> vs <i>altB1</i>	82	92
	<i>altA</i> vs <i>altB2</i>	82	92
	<i>altB1</i> vs <i>altB2</i>	94	96
β -Tubulin	<i>betA</i> vs <i>betB*</i>	85	99.5
	<i>betA</i> vs <i>betC</i>	74	83
Actin	<i>ardA</i> vs <i>ardB</i>	93	100
	<i>ardA</i> vs <i>ardC</i>	93	100
	<i>ardB</i> vs <i>ardC</i>	99	100
	<i>ardC</i> vs <i>ardD</i>	84	84
Profilin	<i>proA</i> vs <i>proP</i>	60	66

* Codons 4 to 204.

in the plasmodium, one might ask whether divergence of their sequences is constrained by a plasmodium-specific function. In fact, the percentage of non-synonymous substitutions among the three α -tubulin genes appears to be essentially the same. The *altB(E)* and *altB(N)* genes differ from each other by 81 nucleotide substitutions out of a 1347 bp coding region. These result in 15 amino acid substitutions out of 449 residues in each polypeptide (Walden *et al.*, 1989b). Thus, about 19% of the nucleotide substitutions are non-synonymous. The *altB(E)* and *altB(N)* genes each differ from *altA* by about 240 nucleotide substitutions. From these arise about 40 amino acid substitutions, giving a non-synonymous substitution rate of 16%. We hypothesize that the greater similarity between the *altB(E)* and *altB(N)* genes is due to their emergence from a more recent gene duplication than that which gave rise to *altA* and the progenitor *altB* gene. If, instead, the two *altB* gene products were being constrained by selection for a plasmodium-specific function, we might expect to observe a lower percentage of non-synonymous substitutions relative to each other than to *altA*. Since all three genes are apparently diverging from each other at about the same rate, we have no evidence that the plasmodium-specific *altB* genes are functionally specialized or limited.

The β -tubulin genes of *Physarum* present a clear example of differential conservation of sequence within a tubulin gene family. A single amino acid substitution arising from among ninety nucleotide substitutions

between the *betA* gene and the known *betB* sequence (Paul *et al.*, 1992) indicates a dramatically lower tolerance of non-synonymous substitutions than that seen among the α -tubulin genes. Interestingly, the percentage of non-synonymous substitutions between the plasmodium-specific *betC* gene and the *betA* and *betB* genes is similar to that of the plasmodium-specific *altB(E)* and *altB(N)* α -tubulin genes. About 20% of the nucleotide substitutions between *betA* and *betC* result in amino acid substitutions. It has been proposed that the greater divergence of the plasmodium-specific *betC* gene product, β 2-tubulin, may be a consequence of increased neutral drift permitted by the relatively limited set of microtubular structures in the plasmodium (Singhofer-Wowra *et al.*, 1986a; Burland *et al.*, 1988). Likewise, the *betA* and *betB* gene products, β 1A- and β 1B-tubulin, are highly conserved relative to each other and to β -tubulins of other species because they both apparently participate in a number of microtubular structures. Similar relationships are seen among the α -tubulins, as previously described by Singhofer-Wowra *et al.* (1986b). The more widely expressed α 1A-tubulin shows greater interspecies conservation than the two plasmodium-specific α -tubulins, α 1B and α 2B (Table 1).

Divergence of other *Physarum* multigene families

The *Physarum* actin and profilin gene families show a broader range of non-synonymous substitution rates than the tubulins. While the nucleotide sequences among the *ardA*, *ardB* and *ardC* genes have drifted by as much as 77 nucleotide substitutions, the actins they encode are identical (Hamelin *et al.*, 1988). However, the *ardD* gene seems not to be subject to the same conservative selective pressure as acts on *ardA*, *ardB* and *ardC*, as its amino acid sequence has diverged substantially. The *ardD* and *ardC* genes differ by 182 nucleotide substitutions, resulting in 59 amino acid changes (Adam *et al.*, 1991). Expression of *ardD* transcripts is limited to plasmodia and developing spherules, a dormant phase of plasmodia, with no expression detected in amoebae (Adam *et al.*, 1991). The profilin genes, *proA* and *proP*, are the most highly diverged *Physarum* gene family, with only 60% nucleotide sequence identity and 66% amino acid sequence identity (Binette *et al.*, 1990). The 151 nucleotide differences between them result in 42 amino acid substitutions. The expression of both genes is cell-type-specific, with *proA* expressed in the amoeba and *proP* expressed in the plasmodium (Binette *et al.*, 1990).

This survey of several *Physarum* gene families reveals a correlation between the relative divergence of an individual protein within a family and its developmental expression pattern. Those proteins with the most restricted expression pattern also show the greatest

intraspecific and interspecific rate of amino acid sequence divergence (for further discussion see Little, 1985; Burland *et al.*, 1988; Silflow, 1991). A similar trend is seen among tubulins from a variety of organisms, including the α -tubulins of the fruit fly (Theurkauf *et al.*, 1986), chicken (Pratt *et al.*, 1987) and mouse (Hecht *et al.*, 1988).

Discussions of tubulin multigene families have focused primarily on the relative contributions of functional specificity and/or regulatory diversity in the evolutionary maintenance of these genes (Little & Seehaus, 1988; Sullivan, 1988; Murphy, 1991). Indeed, evidence consistent with both views continues to accumulate. Here, we would like to emphasize the third selective advantage offered by multigene families. That is, the functional significance of some members of multigene families may be primarily quantitative. In *Naegleria*, the three known α -tubulin genes encode identical proteins and are coordinately induced during flagellate formation (Lai *et al.*, 1988). Similarly, the two isocoding β -tubulin genes of *Chlamydomonas* are regulated coordinately during flagellar regeneration (Youngblom *et al.*, 1984). The mouse testis-specific α -tubulin genes, $M\alpha 3$ and $M\alpha 7$, are isocoding, with 43 silent nucleotide substitutions between their coding sequences and extensive divergence in their noncoding regions (Villasante *et al.*, 1986). In *Physarum*, the isocoding actin genes, *ardB* and *ardC*, show parallel modulation of expression throughout the life-cycle (Hamelin *et al.*, 1988).

How can genes that appear to be fully redundant with regard to amino acid sequence and expression pattern be maintained evolutionarily? Multiple gene copies may allow for more rapid production of abundant structural proteins such as tubulin and actin when they are required during proliferation, development and in response to stress. Given that some tubulin gene families are able to maintain isocoding genes with indistinguishable patterns of expression, it is plausible that a gene whose coding sequence has in fact drifted from its sibling genes is not functionally specialized, yet its presence in the genome provides a quantitative advantage to the organism. That tubulin gene copy number may be biologically significant is illustrated by elegant genetic analysis of the testis-specific $\beta 2$ -tubulin gene of *Drosophila*. Male flies that carry only a single wild-type copy of this gene ($\beta 2^+/\beta 2^{\text{null}}$) exhibit decreased fecundity relative to males carrying two normal gene copies (Hoyle & Raff, 1990). By contrast, reducing the level of β -tubulin in *S. cerevisiae* by more than 50% has no discernible effect on the growth and viability of these cells under laboratory conditions (Katz *et al.*, 1990).

Bailey and Lilianna Solnica-Krezel for gifts of RNA samples. This research was supported by program project grant CA23076 and by core grant CA07175 from the National Cancer Institute. D. K. was supported by research grant DCB-8710791 from the National Science Foundation. E. C. A. P. was supported by postdoctoral training grant CA09230 from the National Cancer Institute. D. B. C. was supported by predoctoral training grant GM07133 from the National Institutes of Health.

References

- ADAM, L., LAROCHE, A., BARDEN, A., LEMIEUX, G. & PALLOTTA, D. (1991). An unusual actin-encoding gene in *Physarum polycephalum*. *Gene* **106**, 79–86.
- BINETTE, F., BENARD, M., LAROCHE, A., PIERRON, G., LEMIEUX, G. & PALLOTTA, D. (1990). Cell-specific expression of a profilin gene family. *DNA Cell Biology* **9**, 323–334.
- BIRKETT, C. R., FOSTER, K. E. & GULL, K. (1985a). Evolution and patterns of expression of the *Physarum* multi-tubulin family analysed by the use of monoclonal antibodies. In *Molecular Genetics of Filamentous Fungi*, pp. 265–275. Alan R. Liss.
- BIRKETT, C. R., FOSTER, K. E., JOHNSON, L. & GULL, K. (1985b). Use of monoclonal antibodies to analyse the expression of a multi-tubulin gene family. *FEBS Letters* **187**, 211–218.
- BURLAND, T. G., GULL, K., SCHEDL, T., BOSTON, R. S. & DOVE, W. F. (1983). Cell type-dependent expression of tubulins in *Physarum*. *Journal of Cell Biology* **97**, 1852–1859.
- BURLAND, T. G., SCHEDL, T., GULL, K. & DOVE, W. F. (1984). Genetic analysis of resistance to benzimidazoles in *Physarum*: differential expression of β -tubulin genes. *Genetics* **108**, 123–144.
- BURLAND, T. G., PAUL, E. C. A., OETLIKER, M. & DOVE, W. F. (1988). A gene encoding the major beta-tubulin of the mitotic spindle in *Physarum polycephalum* plasmodia. *Molecular and Cellular Biology* **8**, 1275–1281.
- CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.
- CLEVELAND, D. W. & SULLIVAN, K. F. (1985). Molecular biology and genetics of tubulin. *Annual Review of Biochemistry* **54**, 331–365.
- COOKE, D. J. & DEE, J. (1975). Methods for the isolation and analysis of plasmodial mutants in *Physarum polycephalum*. *Genetical Research* **24**, 175–187.
- DALE, R. M. K., MCCLURE, B. A. & HOCHINS, J. P. (1985). A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18S rDNA. *Plasmid* **13**, 31–40.
- DEE, J., FOXON, J. L. & ANDERSON, R. W. (1989). Growth, development and genetic characteristics of *Physarum polycephalum* amoebae able to grow in liquid, axenic medium. *Journal of General Microbiology* **135**, 1567–1588.
- DIGGINS, M. A. & DOVE, W. F. (1987). Distribution of acetylated alpha-tubulin in *Physarum polycephalum*. *Journal of Cell Biology* **104**, 303–309.
- DIGGINS-GILCINSKI, M. A., SOLNICA-KREZEL, L., BURLAND, T. G., PAUL, E. C. A. & DOVE, W. F. (1989). The localization of the divergent $\beta 2$ -tubulin isotype in the microtubular arrays of *Physarum polycephalum*. *Journal of Cell Science* **94**, 217–226.
- FULTON, C. & SIMPSON, P. A. (1976). Selective synthesis and utilization of flagellar tubulin: the multi-tubulin hypothesis. In *Cell Motility*, pp. 987–1005. Edited by R. Goldman, T. Pollard and J. Rosenbaum. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- GREEN, L. L. (1987). *Regulation of tubulin gene expression through the life and cell cycle of Physarum polycephalum*. PhD thesis, University of Wisconsin.
- GREEN, L. L. & DOVE, W. F. (1984). Tubulin proteins and RNA during the myxamoeba-flagellate transformation of *Physarum polycephalum*. *Molecular and Cellular Biology* **4**, 1706–1711.
- GREEN, L. L. & DOVE, W. F. (1988). Correlation between tubulin mRNA stability and poly(A) length over the cell cycle of *Physarum polycephalum*. *Journal of Molecular Biology* **200**, 321–328.
- GREEN, L. L., SCHROEDER, M. M., DIGGINS, M. A. & DOVE, W. F.

We thank Laurie Larson and Mike Nuveen for technical assistance, Linda Clipson for preparation of figures, and Larry Green, Juliet

- (1987). Developmental regulation and identification of an isotype encoded by *altB*, an alpha-tubulin locus in *Physarum polycephalum*. *Molecular and Cellular Biology* **7**, 3337–3340.
- HAMELIN, M., ADAM, L., LEMIEUX, G. & PALLOTTA, D. (1988). Expression of the three unlinked isocoding actin genes of *Physarum polycephalum*. *DNA* **7**, 317–328.
- HAVERCROFT, J. C. & GULL, K. (1983). Demonstration of different patterns of microtubule organization in *Physarum myxamoebae* and plasmodia using immunofluorescence microscopy. *European Journal of Cell Biology* **32**, 67–74.
- HECHT, N. B., DISTEL, R. J., YELICK, P. C., TANHAUSER, S. M., DRISCOLL, C. E., GOLDBERG, E. & TUNG, K. S. K. (1988). Localization of a highly divergent mammalian testicular α -tubulin that is not detectable in brain. *Molecular and Cellular Biology* **8**, 996–1000.
- HELFTENBEIN, E. (1985). Nucleotide sequence of a macronuclear DNA molecule coding for α -tubulin from the ciliate *Stylonychia lemnae*. Special codon usage: TAA is not a translation termination codon. *Nucleic Acids Research* **13**, 415–433.
- HOYLE, H. D. & RAFF, E. C. (1990). Two *Drosophila* beta-tubulin isoforms are not functionally equivalent. *Journal of Cell Biology* **111**, 1009–1026.
- KATZ, W., WEINSTEIN, B. & SOLOMON, F. (1990). Regulation of tubulin levels and microtubule assembly in *Saccharomyces cerevisiae*: consequences of altered copy number. *Molecular and Cellular Biology* **10**, 5286–5294.
- KILMARTIN, J. V., WRIGHT, B. & MILSTEIN, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new non-secreting rat cell line. *Journal of Cell Biology* **93**, 576–582.
- KRAMMER, G., SINGHOFFER-WOWRA, M., SEEDORF, K., LITTLE, M. & SCHEDL, T. (1985). A plasmodial α -tubulin cDNA from *Physarum polycephalum*. Nucleotide sequence and comparative analysis. *Journal of Molecular Biology* **183**, 633–638.
- KUNZLER, P., PAULI, U. & BRAUN, R. (1984). Regions in the ribosomal minichromosome of *Physarum polycephalum* are protected from restriction nucleases; protein is insensitive to high salt in the G phase and sensitive in the M phase of the cell cycle. *Journal of Molecular Biology* **179**, 651–666.
- LAI, E. Y., REMILLARD, S. P. & FULTON, C. (1988). The α -tubulin gene family expressed during cell differentiation in *Naegleria gruberi*. *Journal of Cell Biology* **106**, 2035–2046.
- LITTLE, M. (1985). An evaluation of tubulin as a molecular clock. *Biosystems* **18**, 241–247.
- LITTLE, M. & SEEHAUS, T. (1988). Comparative analysis of tubulin sequences. *Comparative Biochemistry and Physiology* **90B**, 655–670.
- LUDWIG, S. R., OPPENHEIMER, D. G., SILFLOW, C. D. & SNUSTAD, D. P. (1987). Characterization of the alpha-tubulin gene family of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 5833–5837.
- MCCULLOUGH, C. H. R., DEE, J. & FOXON, J. L. (1978). Genetic factors determining the growth of *Physarum polycephalum* amoebae in axenic medium. *Journal of General Microbiology* **106**, 297–306.
- MANLEY, J. L. (1988). Polyadenylation of mRNA precursors. *Biochimica et Biophysica Acta* **950**, 1–12.
- MERCER, J. F. B. & WAKE, S. A. (1985). An analysis of the rate of metallothionein mRNA poly(A) shortening using RNA blot hybridization. *Nucleic Acids Research* **13**, 7929–7943.
- MOHBERG, J. & RUSCH, H. P. (1971). Isolation and DNA content of nuclei of *Physarum polycephalum*. *Experimental Cell Research* **66**, 305–316.
- MONTEIRO, M. J. & COX, R. A. (1987). Primary structure of an α -tubulin gene of *Physarum polycephalum*. *Journal of Molecular Biology* **193**, 427–438.
- MURPHY, D. B. (1991). Functions of tubulin isoforms. *Current Opinion in Cell Biology* **3**, 43–51.
- PAUL, E. C. A., BURLAND, T. G. & GULL, K. (1989). Location of a single β -tubulin gene product in both the cytoskeletal and mitotic spindle microtubules in *Physarum polycephalum*. *Journal of General Microbiology* **135**, 623–628.
- PAUL, E. C. A., BUCHSCHACHER, G. L., JR., CUNNINGHAM, D. B., DOVE, W. F. & BURLAND, T. G. (1992). Preferential expression of one β -tubulin gene during flagellate development in *Physarum*. *Journal of General Microbiology* **138**, 229–238.
- PIPERNO, G. & FULLER, M. (1985). Monoclonal antibodies specific for an acetylated form of α -tubulin recognize the antigen in cilia and flagella from a variety of organisms. *Journal of Cell Biology* **101**, 2085–2094.
- PRATT, L. S., OKAMURA, S. & CLEVELAND, D. W. (1987). A divergent testis-specific α -tubulin isotype that does not contain a coded C-terminal tyrosine. *Molecular and Cellular Biology* **7**, 552–555.
- PUISSANT, C. & HOUDEBINE, L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biotechniques* **8**, 148–149.
- RAFF, E. C. (1984). Genetics of microtubule systems. *Journal of Cell Biology* **99**, 1–10.
- RIGBY, P. W., DIECKMAN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–332.
- SALLES-PASADOR, I., MOISAND, A., PLANQUES, V. & WRIGHT, M. (1991). *Physarum* plasmodia do contain cytoplasmic microtubules! *Journal of Cell Science* **100**, 509–520.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SASSE, R., GLYN, M. C. P., BIRKETT, C. R. & GULL, K. (1987). Acetylated α -tubulin in *Physarum*: immunological characterization of the isotype and its usage in particular microtubular organelles. *Journal of Cell Biology* **104**, 41–49.
- SCHATZ, P. J., PILLUS, L., GRISAFI, P., SOLOMON, F. & BOTSTEIN, D. (1986). Two functional α -tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. *Molecular and Cellular Biology* **6**, 3711–3721.
- SCHEDL, T. & DOVE, W. F. (1982). Mendelian analysis of the organization of actin sequences in *Physarum polycephalum*. *Journal of Molecular Biology* **160**, 41–57.
- SCHEDL, T., BURLAND, T. G., GULL, K. & DOVE, W. F. (1984a). Cell cycle regulation of tubulin RNA level, tubulin protein synthesis, and assembly of microtubules in *Physarum*. *Journal of Cell Biology* **99**, 155–165.
- SCHEDL, T., OWENS, J., DOVE, W. F. & BURLAND, T. G. (1984b). Genetics of the tubulin gene families of *Physarum*. *Genetics* **108**, 143–164.
- SILFLOW, C. D. (1991). Why do tubulin gene families lack diversity in flagellate/ciliate protists? *Protoplasma* **164**, 9–11.
- SILFLOW, C. D., CHISHOLM, R. L., CONNER, T. W. & RANUM, L. P. W. (1985). The two alpha-tubulin genes of *Chlamydomonas reinhardtii* code for slightly different proteins. *Molecular and Cellular Biology* **5**, 2389–2398.
- SINGHOFFER-WOWRA, M., CLAYTON, L., DAWSON, P., GULL, K. & LITTLE, M. (1986a). Amino-acid sequence data of β -tubulin from *Physarum polycephalum* myxamoebae. *European Journal of Biochemistry* **161**, 669–679.
- SINGHOFFER-WOWRA, M., LITTLE, M., CLAYTON, L., DAWSON, P. & GULL, K. (1986b). Amino acid sequence data of α -tubulin from myxamoebae of *Physarum polycephalum*. *Journal of Molecular Biology* **192**, 919–923.
- SOLNICA-KREZEL, L., BURLAND, T. G. & DOVE, W. F. (1991). Variable pathways for developmental changes of mitosis and cytokinesis in *Physarum polycephalum*. *Journal of Cell Biology* **113**, 591–604.
- STUDIER, F. & MOFFATT, B. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* **189**, 113–130.
- SULLIVAN, K. F. (1988). Structure and utilization of tubulin isotypes. *Annual Review of Cell Biology* **4**, 687–716.
- THEURKAUF, W. E., BAUM, H., BO, J. & WENSINK, P. C. (1986). Tissue-specific and constitutive α -tubulin genes of *Drosophila melanogaster* code for structurally distinct proteins. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8477–8481.
- TODA, T., ADACHI, Y., HIRAOKA, Y. & YANAGIDA, M. (1984). Identification of the pleiotropic cell division cycle gene *NDA2* as one of two different α -tubulin genes in *Schizosaccharomyces pombe*. *Cell* **37**, 233–242.
- TURNOCK, G., MORRIS, S. R. & DEE, J. (1981). A comparison of the proteins of the amoebal and plasmodial phases of the slime mould,

- Physarum polycephalum*. *European Journal of Biochemistry* **115**, 533–538.
- VILLASANTE, A., WANG, D., DOBNER, P., DOLPH, P., LEWIS, S. A. & COWAN, N. J. (1986). Six mouse α -tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. *Molecular and Cellular Biology* **6**, 2409–2419.
- WALDEN, P. D., BLINDT, A. B., BIRKETT, C. R., COX, R. A. & GULL, K. (1989*a*). Recognition of specific *Physarum* α -tubulin isotypes by a monoclonal antibody. *European Journal of Biochemistry* **185**, 383–389.
- WALDEN, P. D., MONTEIRO, M. J., GULL, K. & COX, R. A. (1989*b*). Structure and expression of an α -tubulin gene of *Physarum polycephalum*. *European Journal of Biochemistry* **18**, 583–592.
- YOUNGBLOM, J., SCHLOSS, J. A. & SILFLOW, C. D. (1984). The two β -tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. *Molecular and Cellular Biology* **4**, 2686–2696.