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Differential expression of an α-tubulin gene during the development of *Physarum polycephalum*

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The expression of an α-tubulin gene (*alt*B1 (NαTu) [(1987) J. Mol. Biol. 193, 427–438]) of *Physarum polycephalum* (strain CLdAXE) was found to be governed by a developmental switch since mRNA transcripts were detected, by S_1 nuclease analysis, in the plasmodial but not the amoebal phase of the life-cycle. The conclusion that the *alt*B1 (NαTu) allele codes for a plasmodial specific α-tubulin isotype is supported by recent amino acid sequence data.

α-Tubulin; Gene expression; Development; (*Physarum*, Myxamoebae, Plasmodia)

1. INTRODUCTION

Cellular assemblies such as the mitotic spindle, centrioles, cilia, flagella and elements of the cytoskeleton involve microtubules made up of α- and β-tubulin subunits, each with a molecular mass of approx. 55 kDa (reviews [1,2]). An advantageous model system for the study of microtubules is *Physarum polycephalum* which has not only a well-defined life-cycle suitable for the study of cellular differentiation [3,4] but also a macroplasmodial phase which has a naturally synchronous mitotic cycle suitable for the study of cell-cycle regulated events [5,6].

P. polycephalum has two proliferative vegetative phases. In the diploid plasmodial phase of the life-cycle mitosis is intranuclear and the mitotic spindle constitutes the only form of microtubules that are present. *Physarum* also grows as haploid, uninucleate, flagellated amoebae with nuclei undergoing an open mitosis [3,4].

At least two tubulin isotypes (α_1, β_1) were found to be present in myxamoebae. Paradoxically, at least four isotypes (α_1, α_2, β_1, β_2) were identified in plasmodia [3,4]. We have cloned and sequenced [7,8] one *Physarum* α-tubulin gene (phage λ NαTu) that is an allele [*alt*B1 (NαTu)] of one of 4 unlinked α-tubulin gene loci (*alt*A, *alt*B, *alt*C, *alt*D) [9]. Other tubulins that have been sequenced contain few introns and those that are present are located towards the 5′-end of the gene. α-Tubulins normally have a tyrosine residue at the carboxy-terminus which turns over as a result of cleavage and ligation [9,10]. The *Physarum* α-tubulin gene is unusual because it contains 7 introns and especially because it has a C-terminal methionine codon. This report provides evidence that the cloned gene (phage λ NαTu) is expressed in the plasmodial phase of the life-cycle and is not expressed in myxamoebae and so codes for a plasmodial specific α-tubulin isotype.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were bought from Bethesda

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Research Laboratories; acrylamide, AristaRurea and Analar formamide were obtained from BDH; Sigma supplied Pipes and nucleoside triphosphates; Klenow fragment was bought from Pharmacia; S_1 nuclease was supplied by Boehringer und Sohne; phage M13 primer (1.2.1.2) was purchased from New England Biolabs; Diagnostic Film X-Omat AR X-ray film was obtained from Kodak; radioisotopes were bought from Amersham International.

2.2. *Growth of cultures*

All cultures of *Physarum* were grown at 26°C [11]. Synchronous cultures were made by inoculating exponentially growing microplasmodia, concentrated by centrifugation, onto Whatman no.50 paper supported on a stainless-steel grid over growth medium. Alcohol-fixed smears of small pieces of the plasmodium were used to establish by phase-contrast microscopy the stage of the mitotic cycle [12]. The generation time was 12 h. CLdAXE amoebae were induced to form plasmodia by plating high concentrations of amoebae on water-agar medium [13].

2.3. *Isolation of poly(A)$^+$ RNA*

Poly(A)$^+$ RNA was isolated directly from cell lysates utilising poly(U)-Sepharose 4B [11].

2.4. *Construction of the ^{32}P-labelled actin probe*

A *Hin*dIII-*Sst*I fragment of the ardC2 (NAc) allele [14] spanning four exons near the 5′-end of the gene was cloned into phage M13mp19, and a single-strand template was isolated. M13 primer was hybridized with the template which was copied using the Klenow fragment of DNA polymerase I and [α-^{32}P]dTTP (spec. act. 3000 Ci/mmol). After treatment with *Hin*dIII the DNA was denatured, the products separated electrophoretically on a sequencing gel [8], and the radioactive fraction corresponding to a full-length copy was recovered [15].

2.5. *Construction of the ^{32}P-labelled tubulin probe*

The *Eco*RI-*Xba*I fragment of phage λ NαTu containing the 3′-terminus of the tubulin gene [8] was subcloned into phage M13mp18. A radioactive copy was synthesized as described for the actin probe. After treatment with *Eco*RI the DNA was denatured, the products were separated on a sequencing gel and the radioactive component corresponding to a full-length copy of the *Eco*RI-*Xba*I fragment was recovered.

2.6. *RNA-DNA hybridization and S_1 nuclease treatment of hybrids*

The procedure of Berk and Sharp [16] was followed. Poly(A)$^+$ RNA (10 μg) and ^{32}P-labelled probe (100000 cpm) were mixed, precipitated with ethanol and the precipitate was redissolved in 10 μl hybridization buffer [80% (v/v) formamide, 0.4 M NaCl, 1 mM EDTA, 0.04 M Pipes (pH 6.4)], boiled for 5 min and then placed in a water bath at 52°C for 18 h. Ethanol (1 ml) was added and the solution cooled to −70°C, the precipitate recovered and redissolved in 100 μl S_1 buffer (0.25 M NaCl, 1 mM $ZnSO_4$, 0.03 M Na acetate, pH 4.6) containing native calf thymus DNA (5 μg) and S_1 nuclease (300 U). The reaction mixture was kept at 32°C for 1 h, extracted first with phenol and then with ether and RNA·DNA hybrids were precipitated with ethanol. The precipitate was dissolved in water (20 μl), heated to 95°C for 5 min, cooled on ice and analyzed by electrophoresis on 6% (w/v) polyacrylamide/8 M urea sequencing gel [8]. The gel was then soaked in 10% (v/v) methanol, 10% (v/v) acetic acid solution, dried, and subjected to autoradiography.

3. RESULTS

The *alt*B1 (NαTu) allele was isolated from the strain M_3CVIII and was shown to be expressed in the plasmodial phase of the *Physarum* life-cycle [8]. However, this strain is little used for the study of myxamoebae. It was more convenient to use the strain CLdAXE currently used in studies of *Physarum* tubulins [17], in order to establish whether the expression of the *alt*B1 (NαTu) allele was confined to the plasmodial phase of the *Physarum* life-cycle. CLdAXE amoebae were grown in axenic liquid culture and samples were induced to form plasmodia. The poly(A)$^+$ mRNA fraction was isolated from both phases, hybridized with a probe specific for the *alt*B1 (NαTu) allele and the reaction mixtures subjected to S_1 nuclease analysis. The principal result is presented in fig.1 which shows that transcripts of the *alt*B1 (NαTu) allele were detected in the plasmodial phase and

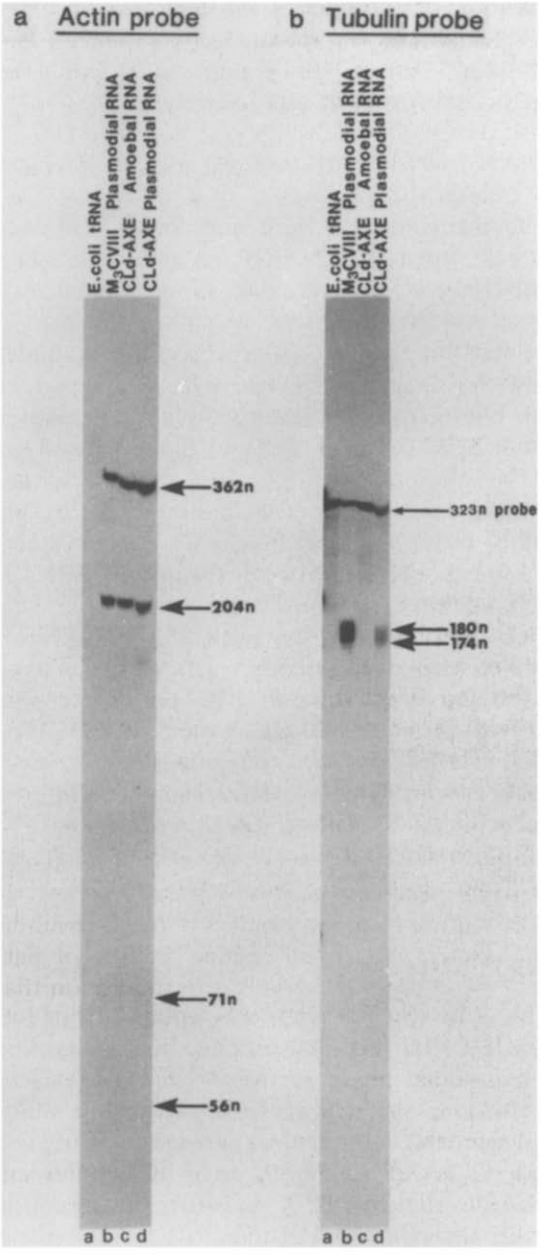

Fig.1. The tubulin *alt*B1 (NαTu) allele is expressed in plasmodia but not in amoebae. As described in section 2, a ^{32}P-labelled probe for actin mRNA (panel a, lanes a–d) or a ^{32}P-labelled probe for tubulin mRNA transcribed from the *alt*B1 (NαTu) allele (panel b, lanes a–d) was hybridized with the poly(A)$^+$ mRNA isolated either from microplasmodia or from amoebae. The reaction mixture was treated with S_1 nuclease and the size of radioactive fragments protected from enzymic hydrolysis was measured by electrophoresis through a 6% (w/v) polyacrylamide-urea sequencing gel which was dried and an autoradiogram was obtained. The gel was calibrated by using ^{32}P-labelled molecular size markers derived from an M13 clone of known sequence. Lanes: a, *E. coli* tRNA (10 μg); b, M_3CVIII–microplasmodial poly(A)$^+$ mRNA (10 μg); c, CLdAXE–amoebal poly(A)$^+$ mRNA (10 μg); d, CLdAXE–microplasmodial poly(A)$^+$ mRNA (10 μg).

not in the amoebal phase of the strain CLdAXE. We infer that the gene is expressed in plasmodia and not in amoebae.

An actin probe was used to check on both the amount and quality of poly(A)$^+$ mRNA used in each assay (see fig.1a). The probe was derived from a 1.1 kbp *Hin*dIII/*Xho*I fragment of the ardC2 allele [14] containing part of 2 exons, 2 complete exons, and 3 introns. Starting from the 5′-end the sizes of the exons or parts of exons are 56, 204, 362 and 71 bp (Gonzalez-y-Merchand, J.A. et al., unpublished). The probe would be expected to hybridize with transcripts of not only the ardC2 allele, but also of the ardC1, ardB1 and ardB2 alleles [14], yielding protected fragments of 56, 71, 204 and 362 bp. Radioactive bands were found at the appropriate positions for both amoebal and plasmodial poly(A)$^+$ mRNA samples (see fig.1a). The protected fragments were equally abundant in both plasmodial and amoebal poly(A)$^+$ mRNA fractions confirming that the amounts of poly(A)$^+$ mRNA loaded were similar in each case.

The radioactive probe complementary to transcripts of the *alt*B1 (NαTu) allele comprised the last 101 nucleotides of the coding region, 157 nucleotides of the 3′-flanking region of the gene and 65 nucleotides of M13 sequence. The size of fragment expected if transcripts of the *alt*B1 (NαTu) allele were present is approx. 180 nucleotides comprising 101 nucleotides of coding region and 79 nucleotides of the 3′-nontranslated sequences of mRNA [8].

Nuclease S_1-resistant fragments 174–180 nucleotides were recovered after the ^{32}P-labelled probe was hybridized with poly(A)$^+$ mRNA isolated from M_3CVIII plasmodia [8]. A similar result was obtained when poly(A)$^+$ mRNA isolated from CLdAXE plasmodia was analysed (see fig.1b). In contrast, no protected fragments of this

size range were found when poly(A)$^+$ mRNA isolated from CLdAXE amoebae was examined (see fig.1b). We conclude that the *alt*B1 (NαTu) allele is present in both strains of *Physarum*, viz. M_3CVIII and CLdAXE and that it codes for an α-tubulin isotype that is expressed in plasmodia but not in amoebae.

4. DISCUSSION

The results of S_1 nuclease analysis show that the *alt*B1 (NαTu) allele of the α-tubulin gene family of *Physarum* is controlled by a developmental switch because the allele is expressed in CLdAXE plasmodia but not in CLdAXE amoebae. This conclusion is reinforced by recently available amino acid sequence data. α-Tubulin isolated from CLdAXE amoebae was shown to have a tyrosine residue at the C-terminus, in common with most α-tubulins for which sequence data are available [18]. Comparison of the amoebal α-tubulin sequence [18] with the amino acid sequence inferred from the nucleotide sequence of the *alt*B1 (NαTu) allele [8] reveals 39 differences. The C-terminal regions of the two isotypes differ in 10 out of 12 amino acid residues (see table 1). Antibodies raised to the oligopeptide NH_2-Ala-Glu-Thr-Val-Gly-Asp-Asp-Glu-Ala-COOH are specific for the gene product of the *alt*B1 (NαTu) isotype and reacted with plasmodial α-tubulin at the position of the α_1 isotype in two-dimensional gel electrophoresis (Cox, R.A. et al., unpublished) and did not react with amoebal α-tubulin.

The developmental regulation of the *alt*B1 (NαTu) allele of the α-tubulin multigene family of *Physarum* may be an example of a more general phenomenon. Thus, α-tubulin genes of *Drosophila melanogaster* are also regulated during development [19]. Of the four α-tubulin genes, two (α_1, α_3) were found to be constitutively expressed and to code for α-tubulins that are very similar to previously sequenced α-tubulins, including a C-terminal tyrosine residue. In contrast, genes α_2 and α_4 are tissue specific. In particular, α_4 mRNA was found only in ovarian nurse cells, eggs and early embryos. It was inferred that the α_1 and α_4 isotypes differ in 149 positions including the C-terminus where phenylalanine was found in the place of tyrosine.

In mouse, the expression of α-tubulin genes is also tissue specific. For example, transcripts of genes Mα_1 and Mα_2 are abundant in brain whereas transcripts of two sister genes (Mα_3, Mα_7) are found exclusively in testis [20]. Most of the isotype-specific amino acids are clustered at the carboxy-terminus. Four of the five isotypes that have been identified have a C-terminal tyrosine residue. One gene (Mα_4), which was found to be expressed in the somatic tissues examined but especially in striated muscle, codes for an isotype that has a C-terminal glutamic acid residue instead of the more common tyrosine [20].

The C-terminal regions of both α- and β-tubulins are implicated in the binding of regulatory microtubule-associated proteins [21] and also the polymerization between tubulins [22]. The amino acid sequence data (see table 1 and [19,20]) indicate that the ten or so residues at the C-terminus including the C-terminal residue may be of particular importance because it is in this region that the developmentally regulated isotype differs most radically from constitutively expressed isotypes.

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Table 1

The amino acid sequence at the C-terminus of myxamoebal and plasmodial α-tubulins of *Physarum*

	440										450
Amoebal [18]	Ser	Ser	Glu	Ala	Gly	Gly	Asp	Glu	Glu	Gly	Glu	Tyr	COOH
	440										
Plasmodial [8] [*alt*B1 (NαTu) gene product]	Thr	Val	Gly	Asp	Asp	Glu	Ala	Glu	Glu	Glu	Met	COOH	

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