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Developmental Regulation and Identification of an Isotype Encoded by *altB*, an Alpha-Tubulin Locus in *Physarum polycephalum*

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A subcloned portion of the 5′ nontranslated sequence from a *Physarum* α-tubulin cDNA is specific for a single α-tubulin locus, *altB*, of *Physarum polycephalum*. We find that this locus is expressed only in the plasmodium and encodes at least an α1-tubulin isotype, which we have designated α1B. Hybridization patterns of other subclones of this cDNA reveal two sequences for α-tubulin at the *altB* locus.

Physarum polycephalum possesses different microtubule structures and expresses different tubulin isotypes during the distinct phases of its life cycle (2; for reviews, see references 6 and 16). The syncytial plasmodium uses only spindle microtubules, whereas the uninucleate amoeba uses both cytoplasmic and spindle microtubules (9) and the nonproliferative flagellate possessess axonemal and cytoskeletal microtubules (28). Two-dimensional gel electrophoresis resolves α1-, α2-, β1-, and β2-tubulin isotypes in the plasmodium (2). Each tubulin isotype is part of the mitotic spindle (14, 17). Immunoblots with KMP-1, a monoclonal antibody specific for a subset of *Physarum* α-tubulins, reveal that the α1 isotype in the plasmodium is composed of at least three different subspecies (1). α1, α3, and β1 constitute the tubulins of the amoeba and flagellate (2, 5, 18).

Tubulin isotype complexity in *Physarum polycephalum* originates both from posttranslational modification and from transcription of different genes. α3-tubulin is made by posttranslational acetylation of an α-tubulin, almost surely α1-tubulin (5, 8, 18). The other tubulins arise from individual mRNAs, probably transcribed from different loci (2). Extensive genetic analysis has associated the β-tubulin isotypes with the loci encoding them (3, 20). Four α-tubulin loci, *altA*, *altB*, *altC*, and *altD*, have been identified by the segregation of restriction-fragment-length polymorphisms via Southern blotting. The *altB* locus is complex; it contains at least two sequences for α-tubulin (20).

Here we continue the study of differential expression of the *alt* loci with a series of subclones from Ppc-α125, an α-tubulin cDNA clone from a plasmodium of strain WisI (14). The α125 cDNA lacks approximately 80 base pairs (bp) of 3′ coding and 140 bp of 5′ or 3′ nontranslated sequence; it contain 70 bp corresponding to 5′ nontranslated sequence (11). Figure 1 displays a partial restriction map of the α125 cDNA and demarcates the subclones we constructed. Insertion of these DNA fragments into pIC-20R vector DNA (13) and transformation of recombinant DNAs into *Escherichia coli* JM101 or JM109 (29) were accomplished by standard techniques (12, 21, 24).

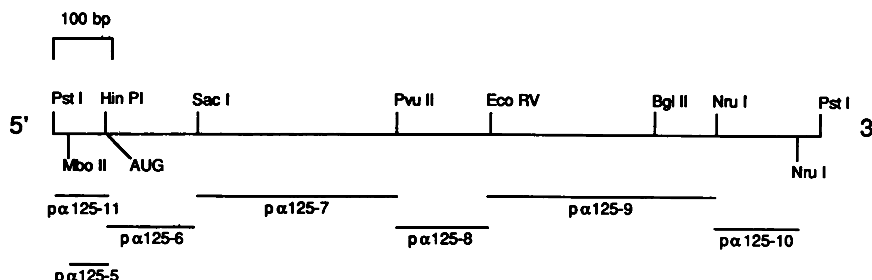

FIG. 1. Partial restriction map of the cDNA insert of Ppc-α125. The fragments used to make the pα125 series of clones are marked with bars, and the name of each subclone is given below the bar. The AUG for translation initiation is marked. The locations of the *Sac*I and *Bgl*II sites used to isolate the 930-bp fragment for the general α-tubulin probe are indicated. Sequences from the *Pst*I to *Mbo*II sites and from the 3′ *Nru*I to *Pst*I sites are composed of GC homopolymers used for tailing the cDNA in its original cloning (11). Consequently, pα125-11 was used as an intermediate subclone from which the insert for pα125-5 was isolated. Because pα125-11 carries GC tails, which hybridize to repetitive sequences in the *Physarum* genome (T. Schedl, Ph.D. thesis, University of Wisconsin, Madison, 1984), it was not used as a hybridization probe.

pα125-5, the subclone containing 70 bp of 5′ nontranslated sequence, detected only a subset of the *alt* DNA fragments, compared with those detected by the 930-bp *Sac*I-*Bgl*II fragment of Ppc-α125, a general α-tubulin probe (compare Fig. 2A versus Fig. 2B, lane 5). In these Southern blots (20) of 2 μg of *Eco*RV-digested genomic DNA from the haploid *Physarum* strain CLd (WisI background) (4), pα125-5 detected only two bands, f and j; these correspond to part of the *altB* locus (Table 1) (20). Thus, pα125-5 is specific for the

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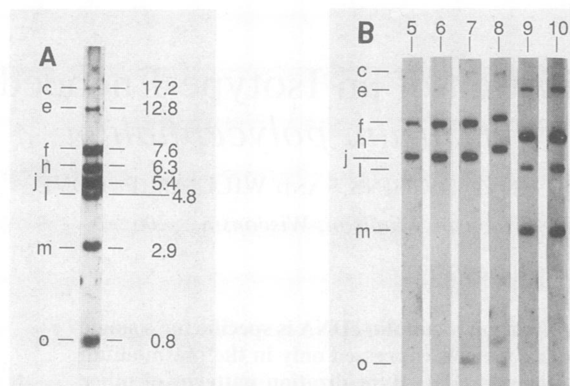

FIG. 2. Southern blots of 2 μg of *Eco*RV-digested *Physarum* DNA from the haploid CLd strain. (A) The DNA fragments were probed with the ^{32}P-labeled (15) 930-bp *Sac*I-*Bgl*II fragment of Ppc-α125, giving the complete pattern of *alt* fragments. Fragment sizes are given in kilobase pairs (20). (B) The DNA fragments were probed with the different ^{32}P-labeled (15) pα125 subclones. Probes used were pα125-5 (lane 5), pα125-6 (lane 6), pα125-7 (lane 7), pα125-8 (lane 8), pα125-9 (lane 9), and pα125-10 (lane 10). In this figure, the mobility differences of the *alt* bands in lane 8 versus those in lanes 5 through 7 arose because the DNA in lane 8 was electrophoresed on an agarose gel separate from that used for lanes 5 through 7.

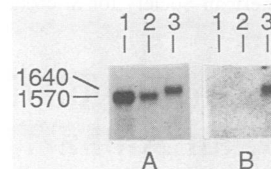

FIG. 3. Northern blots of total RNA (2 μg) isolated at different times in the *Physarum* life cycle. Total RNA, prepared by the guanidinium thiocyanate method (19) from amoebae of strain CLd, flagellates of strain CLd, or microplasmodia of strain CLd × LU215 (Wis1 background), was glyoxalated, electrophoresed for 10 h at 3.3 V/cm on 1.7% agarose gels, blotted to Biodyne A membranes (Pall Corp.) (19) and probed with either the general probe for α-tubulin mRNA, the 930-bp *Sac*I-*Bgl*II fragment of Ppc-α125 (A), or pα125-5, the *altB*-specific probe (B). Hybridization and washing in 1× SSC at 55°C was performed as outlined in Schedl et al. (19). Lanes: 1, flagellate RNA; 2, amoebal RNA; 3, microplasmodial RNA. Fragment sizes are given in nucleotide bases.

altB locus. Because the α125 cDNA was made from plasmodial RNA (11), *altB* is expressed at least in the plasmodium.

The other subclones, while not locus specific, allow us to infer the 5′ and 3′ order of the *altB* and *altA* bands. pα125-6, pα125-7, and pα125-8 detected the same two *altB* bands as did pα125-5; they also weakly detected other *alt* loci (Fig. 2B, lanes 6 to 8). Because these three subclones represent the 5′ portion of the α125 RNA, then bands f and j must be the 5′ portions of the sequences at *altB*, and band o must correspond to the 5′ coding region of *altA*. pα125-9 and pα125-10 detected a subset of *altB* and *altA* bands different from those seen with the 5′ subclones (Fig. 2B, lanes 9 and 10). As these subclones represent the 3′ sequences of the α125 cDNA, bands h and m must be the 3′ portions of *altB*, and band l must contain the 3′ coding region of *altA*.

These data are consistent with only two sequences for α-tubulin at *altB*. In all cases, a subclone hybridized to a pair of *altB* bands, in genomic DNA either from strain CLd (Fig. 2B) or from strain MA275 (data not shown), a strain polymorphic for *Eco*RV restriction fragment length at all the *alt* loci (20). Conceivably, three sequences for α-tubulin could lie in an *altB* allele, with two having identical sizes of *Eco*RV restriction fragments while the third has different fragment lengths.

TABLE 1. *Physarum alt* loci and corresponding *Eco*RV restriction fragments

Locus	*Eco*RV bands[a]
altA	o (5′), l (3′)
altB	f-j (5′), h-m (3′)[b]
altC	c
altD	e

[a] 5′ and 3′ designations for *altA* and *altB* fragments are given (see text).
[b] The designations f-j and h-m indicate the 5′ and 3′ *Eco*RV bands at *altB*, respectively. We cannot assign the fragments that are joined to make the two sequences for α-tubulin in *altB*.

The relatively equal intensities of hybridization of pα125-5 to the 5′ nontranslated region of both sequences at *altB* (Fig. 2B, lane 5) suggest that a recent duplication event has occurred at this locus, as 5′ nontranslated sequences in most α-tubulin genes are highly divergent (7, 22, 27, but see references 25 and 26). The relative hybridization intensities of the *altB* bands were unaffected by high-stringency washing (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 65°C) of genomic DNA blots probed with the 930-bp *Sac*I-*Bgl*II fragment of Ppc-α125 (data not shown). The coding sequences are thus highly conserved, supporting the hypothesis that the duplication happened recently in *Physarum* evolution. Less plausibly, divergence of these sequences may be constrained for functional or regulatory reasons, even for the third positions in codons.

The *altB*-specific probe, pα125-5, detected α-tubulin mRNA only in the plasmodium, in contrast to the *Sac*I-*Bgl*II fragment of Ppc-α125, which detected α-tubulin mRNA throughout the life cycle (Fig. 3). Thus, the *altB* locus is expressed only in the plasmodium. However, we cannot discern whether each sequence at *altB* is expressed. The other subclones detected α-tubulin mRNA in all phases of the life cycle, although with various intensities (data not shown). Nevertheless, silence of *altB* in the amoeba and flagellate means that *altA*, *altC*, and/or *altD* encode(s) the amoeba and flagellate α1-tubulin(s).

To quantify the failure to detect *altB* transcripts in the amoeba and flagellate, pα125-5, ^{32}P-labeled by nick translation (15), was hybridized to spots of serially diluted, denatured microplasmodial RNA on nitrocellulose filters, and then the spots were cut out and counted by scintillation (19). Signal could not be detected in a quantity of RNA one-tenth of that used in the Northern blots (data not shown). Thus, an upper limit on the level of *altB* expression in the amoeba and flagellate is one-tenth of that in the microplasmodium.

Two data corroborate our claim that *altB* is silent in the amoeba and flagellate. First, size differences between α-tubulin mRNA of the plasmodium versus that of the amoeba and flagellate (Fig. 3, panel A) are in the length of the primary transcript, and are not due to heterogeneity in the length of the poly(A) tract (L. L. Green, Ph.D. thesis, University of Wisconsin, Madison, 1987). Second, the deduced sequence of the polypeptide encoded by the mRNA from Ppc-α125 (11) contains multiple amino acid differences compared with a sequenced α1-tubulin from the amoeba, suggesting that these two α-tubulins are not the same gene product (23).

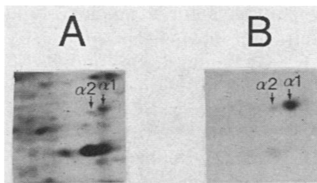

FIG. 4. Autoradiograms of [^{35}S]methionine-labeled proteins made by in vitro translation of RNA in a reticulocyte lysate (Promega Biotech). Shown are 4-day exposures of translation products of (A) 2 μg of poly(A)$^+$ RNA from CLd × LU215 microplasmodia and of (B) RNA hybrid selected by Ppc-α125 from 2 μg of poly(A)$^+$ RNA from CLd × LU215 microplasmodia. Isolation of poly(A)$^+$ RNA by oligo(dT) cellulose column chromatography (12) and hybrid selections (2) were performed as outlined.

We infer that *altB* encodes at least an α1-tubulin isotype. With Ppc-α125 bound to activated diazobenzyloxymethyl paper, we hybrid selected homologous RNA from 2 μg of poly(A)$^+$ plasmodial RNA as described by Burland et al. (2). The recovered RNA was translated in vitro in a reticulocyte extract with [^{35}S]methionine (SJ204; Amersham Corp.), and the polypeptides were electrophoresed on two-dimensional polyacrylamide gels (2) and electrotransferred to nitrocellulose filters. The polypeptides were confirmed to be α-tubulins by mixing the in vitro translation products with unlabeled plasmodial proteins before electrophoresis and probing the filters immunochemically (5) with the general anti-α-tubulin antibody YOL1/34 (10). Comparing the amounts of α1-tubulin versus α2-tubulin made from RNAs hybrid selected by Ppc-α125 versus the ratio of amounts of these α-tubulin isotypes made from total poly(A)$^+$ RNA (Fig. 4, panel B versus panel A), it is clear that Ppc-α125 preferentially selected mRNA encoding α1-tubulin. Since the Ppc-α125 cDNA corresponds to the *altB* locus (Fig. 2B), *altB* must encode at least an α1-tubulin, which we designate α1B. However, we cannot eliminate the possibility that the second α-tubulin sequence at *altB* encodes α2-tubulin, thereby enabling *altB* to encode two different α-tubulin isotypes.

The lack of any other detectable microtubules in the plasmodium requires that α1B-tubulin be used in the spindle apparatus, if it assembles into microtubules. Thus, the plasmodium has at least two α-tubulin isotypes which are used only in the mitotic spindle, α1B and α2. This confirms the observations of Birkett et al. (1). Whether α1B- and α2-tubulins are functionally interchangeable or instead have functional differences within the spindle apparatus remains to be seen.

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