\(\alpha\)-Tubulin marker gene of neural territory of sea urchin embryos detected by whole-mount in situ hybridization

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ABSTRACT We have used Northern blot and whole-mount in situ hybridization to analyze the temporal and spatial expression pattern of the \(P \alpha a2\) \(\alpha\)-tubulin gene in \(Paracentrotus lividus\) sea urchin embryos. The \(P \alpha a2\) transcript is first detectable at 14 h post-fertilization (blastula stage) and it is only expressed in the oral ectoderm. The amount of transcripts of this gene increases throughout development and accumulates up to the pluteus stage. In this stage the \(P \alpha a2\) transcript is localized in the neural structures of the embryo. We conclude that the \(P \alpha a2\) gene is an early neurogenic territory marker. Furthermore we have observed the same localization of the \(P \alpha a2\) transcript in the Zn\(^{++}\) or phenytoin-treated embryos, confirming the animal localization of the \(P \alpha a2\) transcript and its specific relation to neurogenic territory, whose differentiation starts from few founder cells present at blastula stage.

KEY WORDS: sea urchin, \(\alpha\)-tubulin expression, neural territory, in situ hybridization

Introduction

Vertebrate \(\alpha\) and \(\beta\) tubulins are encoded by small multigene families (for review see Cleveland, 1987; Little and Seehaus, 1988; Sullivan, 1988) but usually only five to seven genes are functional, encoding distinct polypeptide isotypes differing primarily in the carboxy-terminal domains. These specific variable domain sequences have been highly conserved among species, despite their divergence within the same species. Accordingly, \(\alpha\) and \(\beta\) tubulins have been classified into distinct isotypic classes (Sullivan and Cleveland, 1986; Lopata and Cleveland, 1987; Little and Seehaus, 1988; Luduena, 1993). Among these classes, one or more members are ubiquitously expressed, while others are expressed differentially with respect to developmental stage and/or tissue localization. Two hypotheses have been advanced to explain the functional significance of multiple tubulin isoforms. The multitubulin hypothesis, originally presented by Stephens (1978) and by Fulton and Simpson (1976) and subsequently developed by others, proposes that chemically distinct tubulins may possess different polymerization properties or may contribute to formation of microtubules with different functional characteristics. The alternative hypothesis proposed by Raff (1984), argues that tubulin isotypes are functionally equivalent and that their genes have evolved unique regulatory sequences to place them under alternative programs of expression during development and differentiation. It is likely that both possibilities apply to differing extents in different systems. Recent studies indicate that qualitative or quantitative differences in isotypic composition and/or subunit modification may confer unique functional properties to tubulins, to microtubules, or both. On the other hand, from protists to multicellular eukaryotes, the number of the \(\alpha\) and \(\beta\) isotypes increases, as shown by the identification of specific mRNAs and the cloning of the corresponding cDNAs (Alexandraki and Ruderman, 1983; Harlow and Nemer, 1987a; Di Bernardo et al., 1989; Gianguzza et al., 1989, 1990, 1992). The transcription of \(\alpha\) and \(\beta\) tubulin genes has been related to the differentiation of specific cell types at the gastrula stage, as well as to the formation of mitotic spindle and cilia during embryogenesis (Harkey and Whiteley, 1983; Gong and Brandhorst, 1987). In \(Strongylocentrotus purpuratus\), the expression of a specific \(\beta\) isotype (\(\beta 1\)) at blastula stage, has been shown to be temporally coordinated with ciliogenesis (Harlow and Nemer, 1987b). Evidence has also been accumulated for transcriptional and post-transcriptional regulation of \(\alpha\) and \(\beta\) tubulin genes during sea urchin embryogenesis (Alexandraki and Ruderman, 1985; Gong and Brandhorst, 1988a,b; Gianguzza et al., 1989, 1992). We have previously reported that in \(P. lividus\) at least five \(\alpha\) and \(\beta\) tubulin mRNAs are expressed during...
embryogenesis according to a regulative program that is both maternal and embryonic. Furthermore, both gene families contain members that are transcribed only during oogenesis and stored in the unfertilized eggs. We have also reported previously the isolation of two different α-tubulin cDNA clones encoding for different isotypes (Pdx1, Pdx10), and of three different β-tubulin cDNA clones encoding almost for two different isotypes (Pβ1, Pβ3, Pβ2) (Gianguzza et al., 1990, 1992). As an attempt to assign a specific function to the various tubulin genes we started to determine their spatial expression in the embryo. In this paper we report the isolation and characterization of a cDNA encoding for a third α-tubulin isotype of P. lividus (Pdx2). We show that this gene is transcriptionally activated at blastula stage, and that its expression is specific of the presumptive neural territory of the pluteus.

Results and Discussion

The transcription of α-tubulin genes in Paracentrotus lividus produces several major different molecular forms during development (Fig. 1A): maternal transcripts of 2 Kb are present up to the morula stage and disappear at later stages. The other two transcripts, namely the 2.4 Kb (Pdx1) and 1.8 Kb (Pdx10) RNAs, whose corresponding genes we already described (Gianguzza et al., 1989, 1990), belong to a different regulative program. Both mRNAs are in fact expressed in the unfertilized eggs and in all embryonic stages examined. Finally a 1.5 Kb transcript, undetectable in unfertilized eggs and in stages preceding blastulation, appears at blastula stage and increases thereafter.

In order to shed some light on the regulation of tubulin gene expression during sea urchin development, and to understand whether these different transcripts correlate with different tubulin isotypes and functions, we analyzed a cDNA clone (Pdx2) which corresponds to the 1.5 Kb mRNA. This clone (Fig. 2) contains an insert of 1079 bases, 84 of which (plus polyA tail) correspond to the 3’UTR, and lacks the coding information for the first 121 N-terminal amino acids. Comparison of the nucleotide coding sequences (data not shown) of the Pdx2 with the same regions of the Pdx10 and Pdx1 clones previously described (Gianguzza et al., 1989, 1990) reveals a high sequence homology and also that
Fig. 3. Comparison of the 3'UTR nucleotide sequences of Pla1, Pla10 and Pla2 cDNA clones. The alignment starts from termination triplets (###). The comparison shows a very low sequence homology (α1 versus α10: 31.6%; α1 versus α2: 30.8% and α10 versus α2: 27.6%). This allows us to identify three different genes.

As shown in Fig. 1B, the PA10 oligonucleotide (corresponding to 3'UTR) specifically hybridizes to the 1.5 Kb RNA band, demonstrating that Pla2 is the corresponding clone. The temporal expression of Pla2 indicates that this gene is activated at blastula stage and that its transcript accumulates at pluteus stage.

In order to investigate the spatial distribution of Pla2 during embryogenesis we performed whole-mount in situ hybridization experiments, as described in Materials and Methods. Figure 5 shows the results of in situ hybridization of blastula (5A), gastrula (5B) prism (5C) and pluteus (5D-E) embryos, with specific antisense 3' untranslated probe of Pla2 (see Materials and Methods). In situ hybridizations of morula (5F), gastrula (5G) and pluteus (5H) stages were also performed using as probe an antisense RNA of 200 nt in length transcribed in vitro from Pla2 coding sequences (see Materials and Methods). With the latter probe we noticed a uniform distribution of α-tubulin mRNAs throughout the whole embryos as expected due to the high homology intra- and inter-species of coding tubulin sequences. On the contrary, using the specific antisense 3' untranslated probe of Pla2 we were able to observe a very specific territorial localization of the corresponding transcript. At blastula stage, in fact, the 1.5 Kb transcript is localized only in a few cells, corresponding to the thickened epithelium of the apical tuft. At gastrula, this localization is more evident and enhanced throughout the oral hood; at plume stage, it extends in the ciliated band. Finally, in the pluteus, hybridization is also evident in the oral cavity at the level of the esophageal muscles. The cell lineage determined for S. purpuratus by Cameron and Davidson (1991) implies that the oral ectoderm consists of two cell types: the squamous epithelial and the columnar epithelial cells. The latter encircle the squamous cells to form the ciliated band which marks the interface between the two ectodermal territories and constitutes the neurogenic territory in the late pluteus. In early stages of sea urchin embryo, the apical tuft, which is an indistinct region of elongated cilia arising from the animal pole soon after hatching, has been suggested to perform a sensory function. In the pluteus larva, neurons and tracts of axons within the ciliated bands outline the oral field and form the rim of the larval mouth. The few neuroblasts, already present in the thickened epithelium of the apical tuft or oral hood in the late gastrula, increase in number in the pluteus, extending axonal processes along the length of the ciliated band, and forming the apical ganglion. This comprises neurons and an extensive neuropile, and lies between the anterolateral arms on the top of the oral hood. A second nerve center, the oral ganglion, is located in the lower lip of the larval mouth (Bisgrove and Burke, 1966; Nakajima, 1986). The authors suggest that the founder cells, identified by antibodies against neurotransmitters or by histochemical methods at gastrula stage, can be committed before, possibly at the end of, the cleavage phase (Cameron and Davidson, 1991).

In order to demonstrate that the localization of the Pla2 transcript is related to the animal pole, we performed whole-mount in situ hybridization experiments on embryos cultured in the presence of Zn++ with a specific antisense probe corresponding to 3'UTR, as described in Materials and Methods. The results (Fig. 6) show that Zn++ is able to induce a significant degree of localization of Pla2 transcript in the oral region.
Fig. 5. Whole-mount in situ hybridization of various stage *Paracentrotus lividus* embryos illustrating the developmental pattern of expression of the α-tubulin transcripts (blue staining region). Embryos were photographed with an Axioskop 20 (Zeiss) photomicroscope equipped with an automatic MC80 exposure system at a magnification of x40. Scale bar, 20 μm. The DIG-probes used are: for stage from A to E the 3' UTR antisense of the Pxl2 clone at a concentration of 5 ng/ml, or for the stage from F to H the 200 bp coding antisense transcript of the same clone at a concentration of 10 ng/ml. (A) Blastula, only few epithelial cells are marked in the animal pole in the region thickened by apical tuft; (B) gastrula, the same region corresponding to the oral ectoderm show an enhanced localization of Pxl2 transcript; (C) prism, the localization is enhanced in the region that will correspond to the apical ganglion, and starts to appear in the ciliated band; (D-E) early pluteus, it is clearly visible the localization of the Pxl2 transcript in the apical ganglion (AG, arrow), in the ciliated band and in the oral ganglion (OG, arrow). Morula (F), gastrula (G) and pluteus (H) stages hybridized with a Pxl2 coding antisense α-tubulin probe. The results show the spreading distribution of all α-tubulin mRNA.
clearly show that the expression of the mRNA coding for Pk2 is restricted to and enhanced in the animal pole of the zinc-animalized embryo. Since the animalization leads to the development of a hyperciliated embryo (with enlarged apical tuft), these results confirm the localization of Pk2 transcript in the thickened epithelium of apical tuft. Furthermore, to exclude the possibility that the initial localization of Pk2 transcript was related to events of gastrulation, we analyzed embryos cultured in the presence of Phenyltoin which induces exogastrula or the deviation of the axis of the invagination of archenteron (Sconzo, personal communication). The results clearly show that the localization of Pk2 transcript in the thickened epithelium and ciliated band is not correlated with the invagination of the archenteron and/or the formation of the mouth (Fig. 6B-C).

In our opinion this localization appears to be of some interest because while many genes whose expression is specifically restricted to other territories and cell types have been identified (for review see Giudice, 1993), this, to our knowledge, is the first gene identified in sea urchin embryos that marks ectoderm-derived neurons and their founder cells. Moreover, our results in Paracentrotus lividus, are in agreement with the cell lineage described by Cameron and Davidson (1991) for Strongylocentrotus purpuratus, even if the two species diverged 65 Myr ago.

In conclusion, we identified and isolated an α-tubulin gene specifically expressed in the presumptive neurogenic territory of sea urchin P. lividus, in which the nervous system controls the swimming and feeding responses of the pluteus, and the process of the metamorphosis (Burke, 1983). It is already known that tubulins together with the neurofilament subunits are the main cytoskeletal proteins of the axon and participate directly in axonal growth and transport, contributing to neuronal plasticity in Metazoa. It is also known that specific tubulin isotypes are used preferentially to assemble the neurite microtubules in mammals (Burgoyne et al., 1988; Joshi and Cleveland, 1989) and that the touch receptor neurons of Caenorhabditis elegans, which possess a structurally and functionally distinct class of microtubules, express specific β-tubulin gene (Hamelin et al., 1992).

As the Pk2 encode for a neurospecific α-tubulin-isotype, and since the transcription of Pk2 is drastically activated during the sea urchin embryogenesis, this gene deserves a molecular analysis of the promoter region to determine the cis and trans controlling elements involved in its transcriptional regulation.

Materials and Methods

Embryo culture and RNA extraction

Adult sea urchins of the species Paracentrotus lividus were collected along the west Sicily coast. The eggs were fertilized and cultured at a concentration of 10,000/mL in Millipore filtered sea water containing antibiotics. Some embryo cultures were treated with animalizing (Zn+++) or exogastrulating (Phenytoin: G. Sconzo, personal communication) agents. Zn++ was added as ZnSO4 1 mM 30 min after fertilization, and embryos were further cultured for 22 h, which corresponds to late gastrula stage in the control embryos (Lallier, 1975). Phenyltoin (Finnel, 1981) was added at a concentration of 20 mM 30 min after fertilization and the embryos were then cultured for 36 h, which corresponds to the pluteus stage in the controls.

Total RNA was extracted from eggs and embryos by homogenization in 7 M urea, 2% SDS, 0.35 M NaCl, 10 mM Tris-HCl, pH 8.0 (Holmes and Bonner, 1973) and several phenol-chloroform extractions. RNA samples were recovered by ethanol precipitation followed by centrifugation at 10,000g for 15 min at 4°C. RNA pellets were resuspended in 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM MgCl2, incubated with 50 μg/ml of

Fig. 6 Expression pattern of Pla2 in Paracentrotus lividus embryos Zn++- or phenytoin-treated. The whole-mount in situ hybridizations were performed as in Fig. 4 using as DIG-probe the 3'UTR antisense of the Pla2. (A) 22 h animalized gastrula-equivalent showing that, after treatment with ZnSO4, the localization of the Pla2 transcript is restricted and enhanced in the animal pole; (B-C) 36 h prism-equivalent phenytoin-treated embryos, showing that the hybridization is restricted in the oral ectoderm thickened by apical tuft both if the archenteron invagination is deviated from its normal axis and if the archenteron is everted. Scale bar, 20 μm.
RNase-free DNase I for 10 min at 37°C and then extracted with phenol-chloroform. In order to prepare the RNA polyadenylated fraction, samples were chromatographed on oligo (dT)-cellulose as described by Aviv and Leder (1972).

Construction and screening of cDNA library
Poly(A\(^{+}\)) RNA of blastula embryos was used as a template for the synthesis of double stranded cDNA, which was performed according to Sambrook et al. (1989). This cDNA was used to construct a library in λgt10 (Huyrhy et al., 1985). The α-tubulin clones were isolated from non-amplified libraries by plaque hybridization (Benton and Davis, 1977) with a 32P-labeled α-tubulin coding probe from Paracentrotus lividus, and, subsequently, the clones PkX10 and PkX1 previously isolated, subcloned by hybridization with its specific 3' UTR labeled with 32P by random priming (Feinberg and Vogelstein, 1983). Phage DNA from the remaining positive plaques was purified as described by Sambrook et al. (1989). Restriction enzyme maps were determined by cutting the DNAs, after subcloning in a pUC vector, with restriction enzymes under the conditions suggested by the supplier.

DNA sequencing
The sequence was obtained by the chain termination method (Sanger et al., 1977) using the chemically modified phage T7 DNA polymerase (Tabor and Richardson, 1987), from overlapping fragments of recombinant cDNA subcloned into pUC derived vectors (Zhang et al., 1988).

RNA blot hybridization
Poly(A\(^{+}\)) RNAs dissolved in Mops-acetate buffer (20 mM Mops, 5 mM NaOCOCH\(_3\), 1 mM Na\(_2\)EDTA, pH 7.0), 50% formamide, 2.2 M formaldehyde, were denatured at 65°C for 5 min, run onto a 1.5% agarose slab gel, and transferred onto nylon membranes as already described (Gianguzza et al., 1989, 1992). The RNA blots were prehybridized for 6-12 h at 42°C in 50% formamide, 5X SSPE, 5X Denhardt's solution (Denhardt, 1966), 1% SDS, and then hybridized in the same solution containing 50 µg/ml of sonicated denatured salmon sperm DNA and 10 ng/ml of denatured 32P-labeled α-tubulin coding fragment, for 48 h at 42°C. After hybridization, the filters were washed in three changes of 2X SSPE, 0.5% SDS at 60°C and in three changes of 0.1X SSPE, 0.5% SDS at 60°C. As 3' untranscribed specific probe we utilized the 5-32P PA1 oligonucleotide labeled by Kinase at the conditions suggested by Biolabs supplier. The sequence of PA1 is the following: GCGAGATCGTTGCTGGATGTC, and it is complementary to that indicated by the box in Fig. 2. The hybridization conditions were the following: prehybridization in 5X SSPE, 1X Denhardt's solution, 0.5% SDS at 37°C for 4 to 12 h; hybridization in 5X SSPE at 37°C for 12 h with 2 ng/ml of 32P-labeled probe. The filters were washed in two changes of 5X SSPE, for 15 min at room temperature and in the same solution for 15 min at 37°C.

Digoxigenin-labeled probes
Plz2 3'UTR was amplified by polymerase chain reaction technique. Two PCR primers were prepared according to the sequence of the cDNA clone: FAB1 (ACATAAGATATTAGACGACT) and FAB1-R (CTGATTAGATGGATTATTT). The location of primers is indicated in Fig. 2. Denaturation for 3 min at 95°C was followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. The 84 bp DNA fragment, corresponding to 3'UTR, was recovered from agarose gel and then reamplified. Labeled single-stranded DNA probes were generated by asymmetric PCRs (Tautz et al., 1992), in the presence of DIG-dUTP. The primer utilized for sense strand synthesis was FAB1, whereas the one for antisense was FAB1R. The conditions were as follows: 25 cycles of 94°C for 45 sec, 50°C for 30 sec, 72°C for 60 sec. The coding probes, sense or antisense, were prepared by classical DIG-labeling in vitro transcription reaction with T3 or T7 RNA polymerase (Boehringer Mannheim), using, as template the Plz2 coding region between the Sal I sites indicated in Fig. 1, after its cloning in bluescript vector. The conditions were those suggested by the supplier.

Whole-mount in situ hybridization
Whole-mount in situ hybridizations were performed according to Harkey et al. (1992) and Lepage et al. (1992) with the following modifications. Fixed embryos were treated with 20 µg/ml proteinase-K and the incubation time increased to 20 min at 37°C. Only when we had used the sense or antisense 3' untranslated probe the hybridization conditions were changed from these of Lepage et al. (1992) as follows: the prehybridization was performed for 2 h at 50°C in 5X SSC, 20 mM Tris pH 7.5, 500 µg/ml yeast tRNA, 500 µg/ml heparin, 10% PEG, 0.3% Tween 20, 1X Denhardt, 5 mM EDTA. After the prehybridization step, embryos were washed in hybridization solution (5X SSC, 20 mM Tris pH 7.5, 500 µg/ml heparin, 0.3% Tween 20, 5 mM EDTA) and resuspended in the same mixture containing DIG-labeled probe at 5 ng/ml. The hybridization step was performed in the capillary pipette at 50°C overnight. After hybridization, the embryos were washed at 50°C twice for 15 min in 5X SSC, 0.1% Tween 20, and twice at 45°C for 15 min in 2X SSC, 0.1% Tween 20.

The staining reaction was allowed to develop 2-4 h in the dark with shaking.

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