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α-Tubulin marker gene of neural territory of sea urchin embryos detected by whole-mount in situ hybridization

FABRIZIO GIANGUZZA*, CATERINA CASANO and MARIA RAGUSA

Dipartimento di Biologia Cellulare e dello Sviluppo, Palermo, Italy

ABSTRACT We have used Northern blot and whole-mount *in situ* hybridizations to analyze the temporal and spatial expression pattern of the *Plα2* α-tubulin gene in *Paracentrotus lividus* sea urchin embryos. The *Plα2* 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 *Plα2* transcript is localized in the neural structures of the embryo. We conclude that the *Plα2* gene is an early neurogenic territory marker. Furthermore we have observed the same localization of the *Plα2* transcript in the Zn^{++}- or phenytoin-treated embryos, confirming the animal localization of the *Plα2* transcript and its specific relation to neurogenic territory, whose differentiation starts from few founder cells present at blastula stage.

KEY WORDS: *sea urchin, α-tubulin expression, neural territory, in situ hybridization*

Introduction

Vertebrate α and β 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, α and β 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 α and β isotypes increases; this seems to be of some significance, and leads to speculation about the different functions of the different isotypes (Luduena, 1993).

The genome of different sea urchin species contains 10-12 α and β tubulin genes but the number of functional genes is lower, 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 α and β 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 β isotype (β1) 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 α and β 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 α and β tubulin mRNAs are expressed during

Abbrevations used in this paper: DIG, digoxigenin; EDTA, ethylenediaminetetracetic acid; MOPS, 3(N-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; Poly(A+), polyadenilated; RNAse, ribonuclease; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; SSPE, sodium saline phosphate EDTA; 3'(UTR), 3' untranslated region.

***Address for reprints:** Dipartimento di Biologia Cellulare e dello Sviluppo, Viale delle Scienze, Parco D'Orleans II, 90128- Palermo, Italy. FAX: 91.424337.

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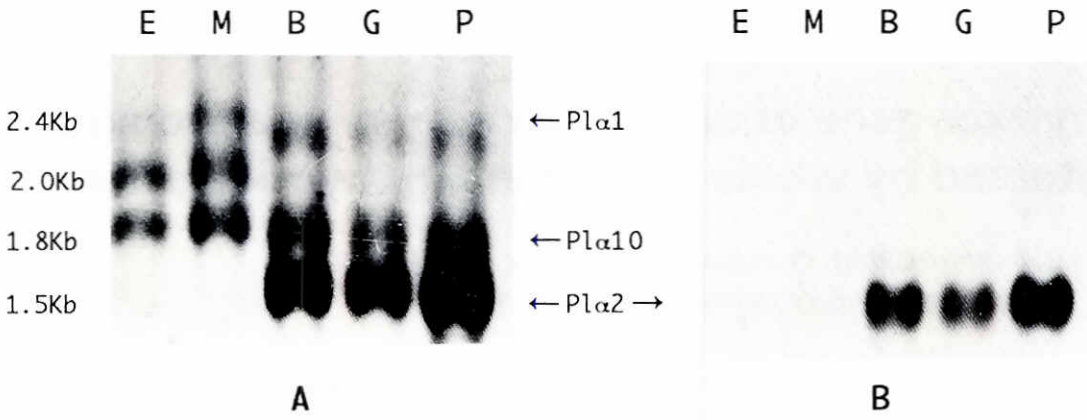

Fig. 1. Characterization of α-tubulin transcripts. *Poly(A⁺) RNAs were fractionated by formaldehyde agarose gel electrophoresis, blotted onto nylon membrane and hybridized with a coding probe corresponding to the total Plα2 cDNA* **(A)**, *or PA1 oligonucleotide probe complementary to a portion of 3'UTR* **(B)**. *The amount of RNA loaded was 1 mg for each egg (E), morula (M), blastula (B), gastrula (G), pluteus (P) samples. Hybridization and washing conditions were as described in Materials and Methods. The two autoradiograph represent an overnight exposure of the blot.*

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 *(Plα1, Plα10)*, and of three different β-tubulin cDNA clones encoding almost for two different isotypes *(Plβ1/β3, Plβ2)* (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 (Plα2)*. 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 *(Plα1)* and 1.8 Kb *(Plα10)* 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 *(Plα2)* 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 *Plα2* with the same regions of the *Plα10* and *Plα1* clones previously described (Gianguzza *et al.*, 1989, 1990) reveals a high sequence homology and also that

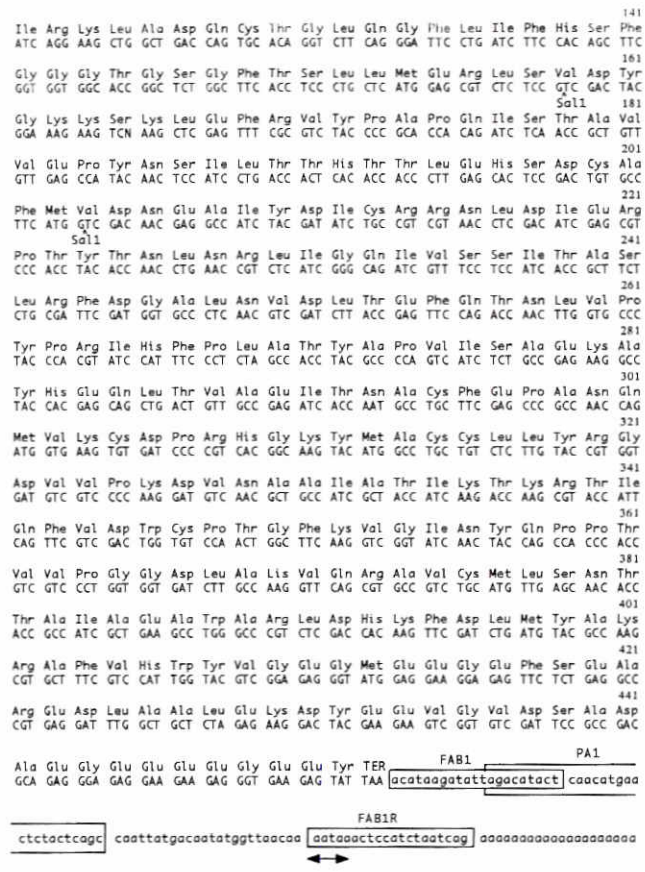

Fig. 2. Nucleotide and amino acid predicted sequences of *Paracentrotus lividus Plα2* cDNA clone. *Coding region is in capital, whereas lower case letters indicate the 3' untranslated sequences. The nucleotide sequences of PA1 oligonucleotide used as probe for Northern experiments, and of FAB1 and FAB1R oligonucleotide sequences used for amplification of the 3' untranslated probes for in situ hybridization whole-mount experiments are boxed. A putative polyadenylation signal is also underlined with a double arrow. The cutting sites of SalI restriction enzyme used for cloning the coding sequences into Bluescript vector are also indicated.*

Fig. 3. Comparison of the 3'UTR nucleotide sequences of *Plα1, Plα10* and *Plα2* 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.*

most of the nucleotide substitutions are conservative, occurring in the third base of the codons. The nucleotide sequences of the 3'UTR of the three clones are divergent instead and allow us to identify different genes (Fig. 3).

Comparison of the predicted amino acid sequence of the three α-tubulin clones (Fig. 4) reveals that Plα2 differs for four amino acid substitutions – at residues 151 (T to S), 171 (R to A), 290 (A to S), 450 (E to D) — from Plα1 and for two amino acid substitutions — at residues 171 (R to A), 450 (E to D) — from Plα10. All the replacements are conservative except the ones at positions 171 and 290. Particularly, at level of the C-terminal tail, Plα2 differs from Plα1 and Plα10 only for a Glu to Asp substitution. Although this substitution is conservative in terms of charge, it is possible that it influences posttranslational tubulin modifications. In fact glutamate is sometimes used as a primer for reversible polyglutamylation, a process discovered in nerve cells that can influence the total charge of the molecule (Audebert *et al.*, 1993). During neural development, for example, the neuron specific βIII tubulin increases its heterogeneity from one to seven isoforms, by sequential addition of glutamyl residues (Alexander *et al.*, 1991).

As shown in Fig. 1B, the PA1 oligonucleotide (corresponding to 3'UTR) specifically hybridizes to the 1.5 Kb RNA band, demonstrating that the *Plα2* clone is the corresponding gene. The temporal expression of *Plα2* 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 *Plα2* 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) prisma (5C) and pluteus (5D-E) embryos, with specific antisense 3' untranslated probe of Plα2 (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 *Plα2* 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 *Plα2* 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 prisma 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, 1986; 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 *Plα2* 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.

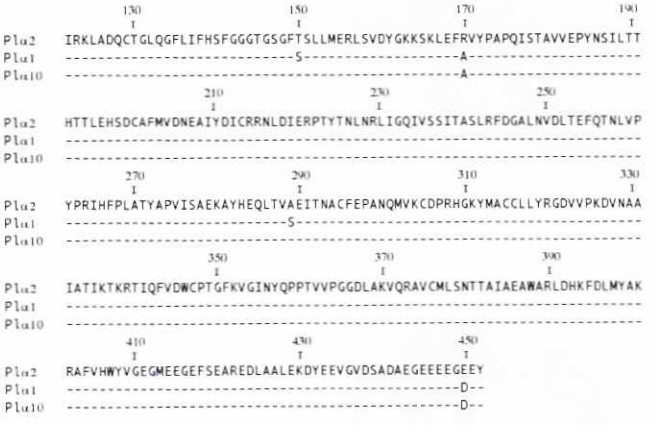

Fig. 4. Comparison of the C-terminal deduced amino acid sequences from three different cDNA of *P. lividus*. *The reference sequence is that predicted from Plα2 cDNA. Amino acids predicted from Plα10 and Plα1 are aligned and indicated only when different from those of the reference sequence.*

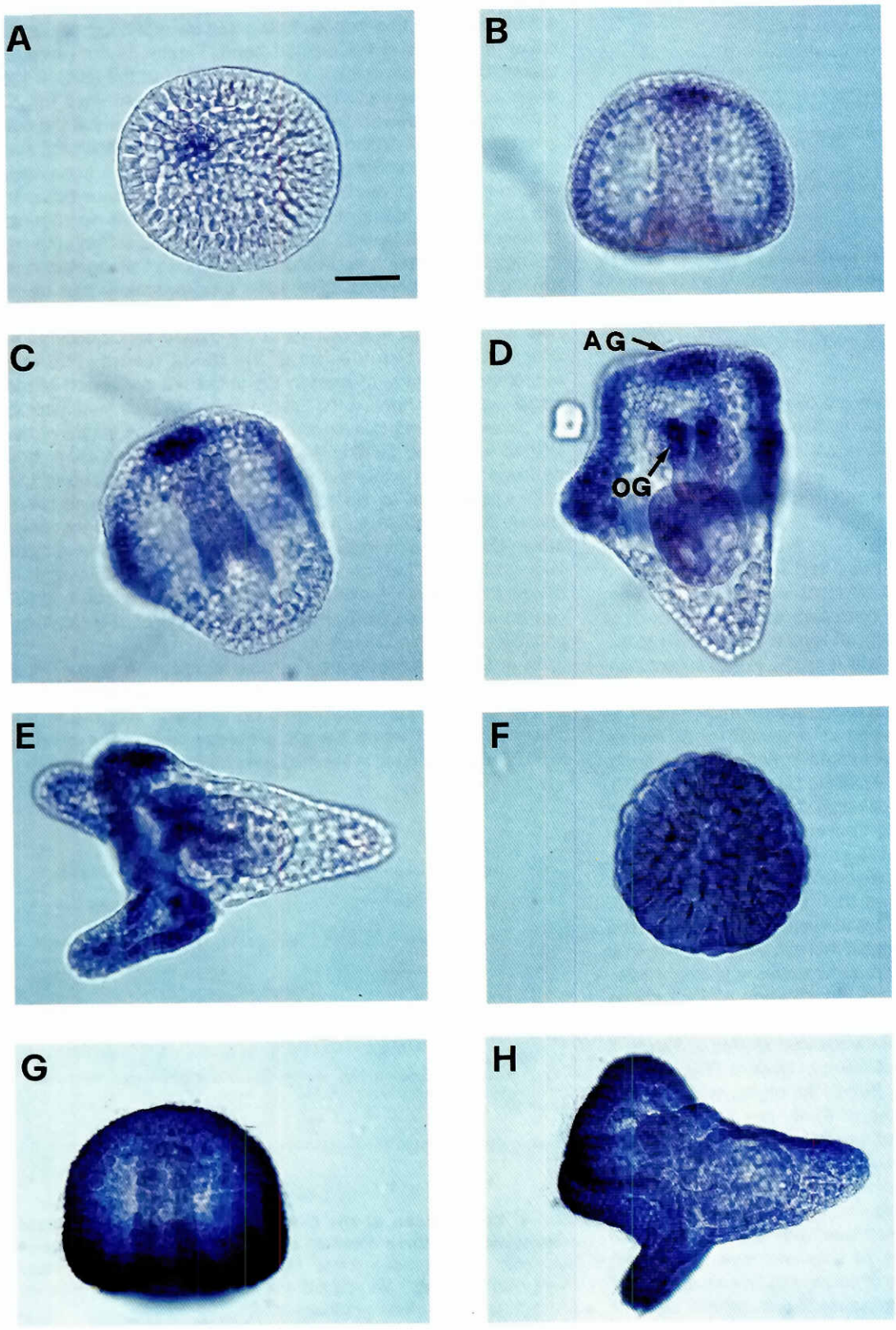

Fig. 5. Whole-mount *in situ* hybridization of various stage *Paracentrotus lividus* embryos illustrating the developmental pattern of expression of the α-tubulin transcripts (blue staining region). *Embryos were photographed with an Axioskop 20 (Zeiss) photomicroscope equipped with an automatic MC80 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 Plα2 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 Plα2 transcript;* **(C)** *prisma, 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 Plα2 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 Plα2 coding antisense α-tubulin probe. The results show the spreading distribution of all α-tubulin mRNA.*

6A) clearly show that the expression of the mRNA coding for *Plα2* 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 *Plα2* transcript in the thickened epithelium of apical tuft. Furthermore, to exclude the possibility that the initial localization of *Plα2* transcript was related to events of gastrulation, per se, we analyzed embryos cultured in the presence of Phenytoin 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 *Plα2* 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 is, to our knowledge, 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 assembly 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 *Plα2* encode for a neurospecific α-tubulin-isotype, and since the transcription of *Plα2* 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 $ZnSO_4$ 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). Phenytoin (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 $MgCl_2$, incubated with 50 μg/ml of

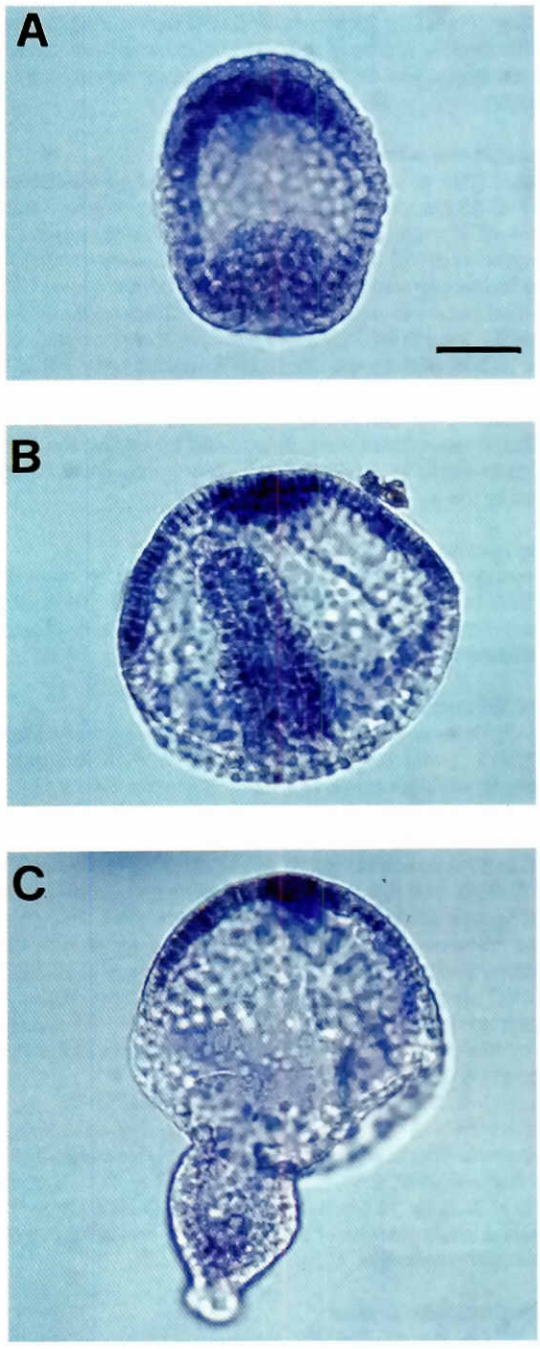

Fig. 6. Expression pattern of *Plα2* in *Paracentrotus lividus* embryos Zn^{++}- or phenytoin- treated. *The whole-mount in situ hybridizations are performed as in Fig. 4 using as DIG-probe the 3'UTR antisense of the Plα2.* **(A)** *22 h animalized gastrula-equivalent showing that, after treatment with $ZnSO_4$, the localization of the Plα2 transcript is restricted and enhanced in the animal pole;* **(B-C)** *36 h prisma-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 DNaseI 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 (Huynh *et al.*, 1985). The α tubulin clones were isolated from non-amplified libraries by plaque hybridization (Benton and Davis, 1977) with a ^{32}P-labeled α-tubulin coding probe from *Paracentrotus lividus*, and, subsequently, the clones *Plα10* and *Plα1* previously isolated, subtracted by hybridization with its specific 3' UTR labeled with ^{32}P 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 NaOCOCH3, 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 ^{32}P-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' untranslated specific probe we utilized the 5'-^{32}P PA1 oligonucleotide labeled by Kinase at the conditions suggested by Biolabs supplier. The sequence of PA1 is the following:

GCTGAGTAGAGTTCATGTTGAGTATGTC,

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 ^{32}P-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.

Digoxygenin-labeled probes

Plα2 3'UTR was amplified by polymerase chain reaction technique. Two PCR primers were prepared according to the sequence of the cDNA clone: FAB1 (ACATAAGATATTAGACATACT) and FAB1-R (CTGATTAGATGGAGTTTATT). 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 Plα2 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 hr at 50°C in 5X SSC, 20 mM Tris pH 7.5, 500 μg/ml yeast t-RNA, 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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