Original Article

Spatial expression of α and ß tubulin genes in the late embryogenesis of the sea urchin *Paracentrotus lividus*

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ABSTRACT In *Paracentrotus lividus* sea urchin embryos, at blastula stage, there is an abrupt increase in the abundance of α and ß tubulin transcripts in particular of the PIß1, PIß2 and PIα2 forms. In order to assign specific functions to the various embryonic tubulin genes, we have used whole-mount *in situ* hybridization to determine spatial patterns of expression of five different α and ß tubulin embryonic genes. The PIß3 transcripts, as previously shown for PIα2, start to localize in a few founder cells from which the neurogenic territory differentiates. The other four embryonic tubulin mRNAs (PIß1/2 and PIα1/10), are localized in the ciliated band- and gut-territory. These territories originate by morphogenetic processes, which occur in late embryogenesis in the sea urchin and depend on cellular interactions. In particular, the interactions between the oral and aboral ectoderm specify the position of the ciliated band, whereas the invagination of the vegetal plate forms the gut territory. We suppose that the increase in α and ß tubulin transcripts could be functionally related to these two morphogenetic events. Our results show in fact that specific tubulin isotypes, or a mix of them, are expressed in and mark the ciliated band and the neighboring oral/aboral ectoderm cells of the ciliated band, in addition to the cells of the gut territory. The same localization of all these tubulin transcripts has been confirmed by whole-mount *in situ* hybridization experiments performed on embryos treated with agents able to induce deciliation or exogastrulation. Furthermore a putative correlation of PIß2 with cilium formation has been shown by the results obtained on deciliated embryos.

KEY WORDS: *sea urchin, α and ß tubulin expression, neural territory, endoderm territory, in situ hybridization*

Introduction

Microtubules are ubiquitous eukaryotic cytoskeletal elements required for cell division, intracellular transport, cell morphogenesis and motility. *In vivo* the formation of a tubular polymer is an intrinsic property of the α/β tubulin heterodimer but also of a complex and different set of permanently or transiently associated additional proteins. It is now well established that both α and β tubulin represent families of structurally and biochemically distinguishable isoforms that have their origin in different multiple tubulin genes and in several specific forms of post-translational modifications (Gundersen and Bulinski, 1986; Sullivan and Cleveland, 1986; Murphy, 1991; Luduena *et al.*, 1992; Luduena, 1993).

The size of the expressed tubulin gene families ranges from the small families in fungi, consisting of one or two genes each for α and β tubulin, to the larger gene families typified by the vertebrate families of five to seven genes each for α and β tubulin; however, up to now, no evidence for coordinate expression of fixed α/β gene pairs has been shown (Little and Seehaus, 1988; Sullivan, 1988).

The spatial and temporal patterns of expression of different genes encoding distinct isoforms, and the regulated patterns of covalent post-transcriptional modifications suggest a functional specialization (Fulton and Simpson, 1976); this hypothesis is confirmed by the greater similarity among corresponding tubulin isoforms in different species rather than among different isoforms in the same species. Moreover it is noteworthy that even within a single cell, tubulin isotypes may be non-uniformly distributed (Lopata and Cleveland, 1987; Hoyle and Raff, 1990; Oka *et al.*, 1990; Alexander *et al.*, 1991).

Many of the differences among the different isotypes are clustered in the carboxy-terminal regions of the proteins, although other "hot spots" of diversity have been observed elsewhere. In particular the C-terminal and the N-terminal variable regions are

Abbreviations used in this paper: DIG, digoxigenin; PCR, polymerase chain reaction; Poly(A⁺), polyadenilated; SSC, sodium saline citrate; 3'UTR, 3' untranslated region; MOPS, 3(N-morpholino)propanesulfonic acid; SSPE, sodium saline phosphate EDTA; VPA, valproate.

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Fig. 1. Nucleotide sequences of the 3'UTRs of the PIβ1, PIβ2, PIβ3 and PIα1, PIα10 *Paracentrotus lividus* tubulin cDNA clones. *For each clone the position of the sense oligonucleotides is highlighted by raised type and the position of the antisense oligonucleotides is underlined (the oligonucleotide antisense sequences were the reverse complementary respect to those given in the Figure). These oligonucleotides were used for PCR amplification and for asymmetrical PCR of the 3'untranslated probes used on whole-mount in situ hybridization experiments. The comparison of the 3'untranslated nucleotide sequences (not shown) between α or β tubulin cDNA clones shows a very low sequence homology (about 25-30%) allowing identification of different genes.*

isotype specific and are related by an evolutionary covariation (Serrano *et al.*, 1984; Burns and Surridge, 1990; Fackental *et al.*, 1993, 1995).

The functional significance of isotypes includes not only structurally differentiated requirements for different microtubule arrays, but also the adaptation of the physical and biochemical properties of an isotype (or group of isotypes) to particular environments. Accordingly, a specific mix of isoforms might be required to generate the properties of the resultant hybrid polymers, emphasizing the importance of a threshold effect for each isoform in determining different microtubule arrays (for review see Raff, 1994).

Despite the fact that each α or β tubulin family, from different sea urchin species, span from 10 to 12 genes, only some of them are expressed (Alexandraki and Ruderman, 1983; Harlow and Nemer, 1987a; Di Bernardo *et al.*, 1989; Gianguzza *et al.*, 1989, 1990, 1992, 1995). In sea urchin, as in all metazoans, the transcription of α and β tubulin genes has been related both to the cell type specification occurring at gastrula stage, and to the formation of mitotic spindle and cilia during embryogenesis (Harkey and Whiteley, 1983; Gong and Brandhorst, 1987). Much evidence has 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, 1995).

In a previous paper (Gianguzza *et al.*, 1995) we have shown a specific localization of the PIα2 transcript in the neural structures of the sea urchin embryo. In the present paper we report the spatial expressions of the other tubulin transcripts that we have examined.

Results and Discussion

During *Paracentrotus lividus* embryogenesis, at least five α and five β tubulin mRNAs are expressed, according to a regulative program that is both maternal and embryonic. Both gene families contain members that are transcribed only during oogenesis; in addition, there are also genes whose transcripts (PIβ3 and PIα10) are detected both in unfertilized eggs and in the embryo at all developmental stages, and genes whose transcripts (PIβ1, PIβ2, PIα1) are variably modulated during embryogenesis; finally some genes are expressed only from the blastula stage (PIα2 transcript). From each of these embryonic transcripts we have isolated and analyzed the correspondig cDNAs encoding for almost the complete repertoire of the α and β tubulin isotypes expressed in late embryogenesis of *Paracentrotus lividus* embryo (Gianguzza *et al.*, 1989, 1990, 1992, 1995).

In order to investigate the spatial distribution of five of these different embryonic transcripts of α and β tubulin, we carried out whole-mount *in situ* hybridization experiments as described in Materials and Methods, with each specific antisense 3'UTR probe of PIβ1/2/3 and of PIα1/10 (Fig. 1). By using coding probes from tubulin cDNA clones we had previously seen a uniform distribution throughout the whole embryo as expected because of the high homology of coding tubulin sequences (Gianguzza *et al.*, 1995).

With the specific 3'untraslated sense probes, as expected, no hybridization was detected (data not shown). However using the specific antisense 3'untranslated probes corresponding to the different genes, we were able to observe a specific territorial localization of the corresponding transcripts. The results of the *in situ* hybridization on sea urchin embryos (Fig. 2) show, in fact, that the Plβ3 transcript, at prism stage (Fig. 2, column A), is localized in the thickened epithelium of the apical tuft and starts to appear in the ciliated band; at pluteus stage (Fig. 2, column B) it is found in all neural structures: the apical and oral ganglions and the ciliated band, consisting of neurons and tracts of axons, which outline the oral field and form the rim of the larval mouth.

All the other α and β tubulin transcripts (Plβ1/2, Plα1/10) are localized (Fig. 2, column A) in the archenteron and in all ectodermic tissue at gastrula stage. At pluteus stage (Fig. 2, column B) staining of the whole gut persists, although the Plα1 probe seems to hybridize with only a portion of the gut. Ectodermic tissue localization is present in all the oral ectoderm, while only the portions of aboral ectoderm neighboring the ciliated band are stained at pluteus stage. These results and a comparative analysis of the carboxy-terminal domain of the different tubulin isotypes of *Paracentrotus lividus* are summarized in Table 1. As previously reported, the α2 neural specific isotype differs, at the level of carboxy-terminal domain, from the other α isotypes for a second possible site of polyglutamylation (a post-translational tubulin modification which could modulate affinity with the MAPS especially of the neural isotypes by increasing the negative charge of the carboxy-terminal domain — Eddé *et al.*, 1990). It is also remarkable that, although all the β isotypes can be polyglutamylated at Glu^{435} of the canonical pentapeptide (Mary *et al.*, 1994), the β1 isotype is the most divergent, really two non-conservative substitutions and one deletion reduce the total negative charge of its carboxy-terminal domain (see Table 1).

Are tubulin isotypes involved in ciliated band formation?

In *Strongylocentrotus purpuratus* sea urchin pluteus the embryonic ectoderm is constituted of two territories: the oral and the aboral ectoderm; in both the major cell type is a squamous epithelial cell with a single cilium. These two territories are separated by a strip of columnar ciliated cells called the ciliated band, which is also the site of nerve cell differentiation and presumably the origin of neurons as well (Cameron *et al.*, 1990). Moreover, in *Strongylocentrotus purpuratus* embryos the ciliated band, which appears at late gastrula stage, is positioned through processes of intercellular signaling between the oral and aboral ectoderm and is not fixed with respect to cell-lineage (Cameron *et al.*, 1993). In fact at the 8-cell stage, when the future oral side of the embryo can be distinguished, cells whose progeny contribute to the ciliated band segregate. The progeny of the aboral animal-half blastomere Na contribute both to the squamous epithelium of the aboral ectoderm and to the columnar epithelium of the ciliated band between the oral arms while the progeny of the vegetal-half oral blastomere VO contribute to the region between the anal arms of the ciliated band, as well as to the squamous epithelium of supra-anal ectoderm and the facial oral ectoderm. The progeny of the oral animal-half blastomere No contribute to the facial oral ectoderm, and to the oral and lateral portions of the ciliated band; the animal-half lateral blastomeres NL (right and left) contribute to the aboral ectoderm and the lateral portion of the ciliated band.

Whole-mount *in situ* hybridization experiments performed with the Plβ3 3'UTR antisense specific probe (see Materials and Methods) show that, starting from the blastula stage, the Plβ3 transcript is localized in only a few cells of the animal pole, corresponding to the thickened epithelium of the apical tuft (data not shown). At early gastrula stage (Fig. 3, column A) this restricted pattern of localization is more evident, whereas at prism stage (Fig. 2, column A), the Plβ3 transcript is present in the thickened epithelium of apical tuft (apical ganglion), and also in the ciliated band. Finally, at pluteus stage (Fig. 2, column B) hybridization is evident in the apical ganglion, in the ciliated band, and in the oral cavity, at the level of the oral ganglion neighboring the esophageal muscles. Notably this spatial localization is quite similar to that of Plα2 transcript (Gianguzza *et al.*, 1995).

TABLE 1

SUMMARY OF THE TEMPORAL AND SPATIAL EXPRESSION OF α AND β EMBRYONIC TUBULIN TRANSCRIPTS AND COMPARATIVE ANALYSIS OF THE CARBOXY-TERMINAL DOMAIN OF THE DIFFERENT DEDUCED TUBULIN ISOTYPES

cDNA clones	Developmental mRNA expression							Deduced Isotype	Spatial expression at pluteus stage	Carboxy-terminal domain
	E	4/8	M	B	G	Pr	Pl			
Plβ1			+-	+	+	+	++	β1	ciliated band and gut	DATAEEEGEFEEEEEEDQE*V
Plβ2			++-	+	+	+	++	β2	ciliated band and gut	DATAEEEGEFDEEEEGDEEAA
Plβ3	+	+	+	+	+	+	+	β3	apical ganglion, oral ganglion and ciliated band	DATAEEEGEFDEEEEGDEEAA
Plα1	+	+	+	+-	+	+	+	α1	ciliated band and foregut	VDSADAEGEEEEGDEY
Plα2				+	+	+	++	α2	apical ganglion, oral ganglion and ciliated band	VDSADAEGEEEEGEEY
Plα10	+	+	+	+	+	+	+	α10	ciliated band and gut	VDSADAEGEEEEGDEY

The developmental expression has been previously determined by Northern blot hybridization using specific probes corresponding to each cDNA. Developmental stages indicated are: E, eggs; 4/8, 4/8 blastomeres; M, morula; B, blastula; G, gastrula; Pr, prism and Pl, pluteus stages. The comparison of the carboxy-terminal domains of β (made from aa 427 to aa 447) and α (made from aa 437 to aa 452) tubulin deduced isotypes of *Paracentrotus lividus* are shown. The consensus pentapeptide probable substrate of glutamylation (Mary *et al.*, 1994) and the glutamyl residue (in bold face) at positions 435 for β isotypes, at 445 and at 450 for α isotypes are underlined. The comparative analysis of the three β isotypes shows two non-conservative substitutions and one deletion (indicated by *) in the β1 reducing the negative total charge of the domain. Among the α isotypes, only in the α2 there is a second probable site of glutamylation (underlined and in bold ; Eddé *et al.*, 1990).

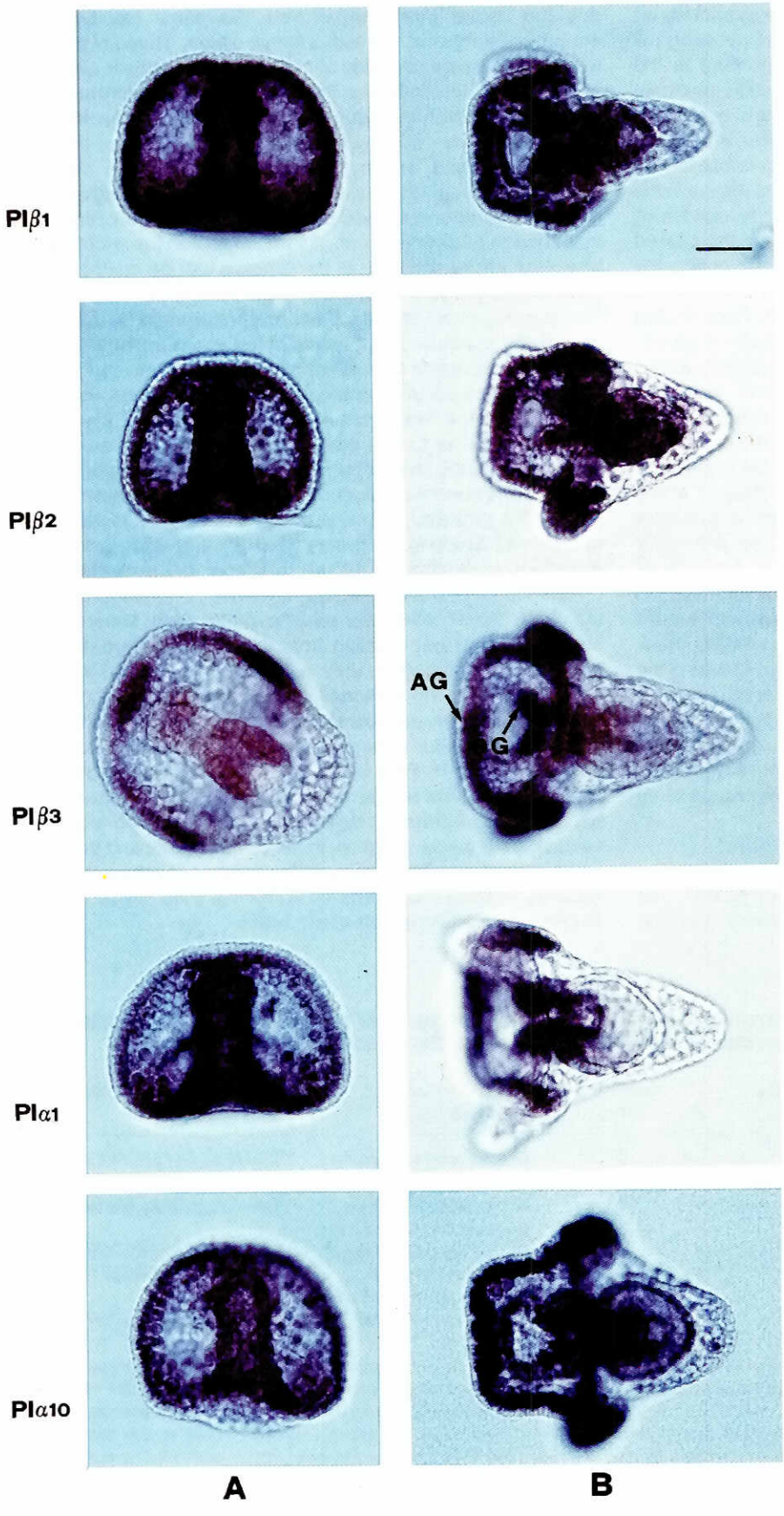
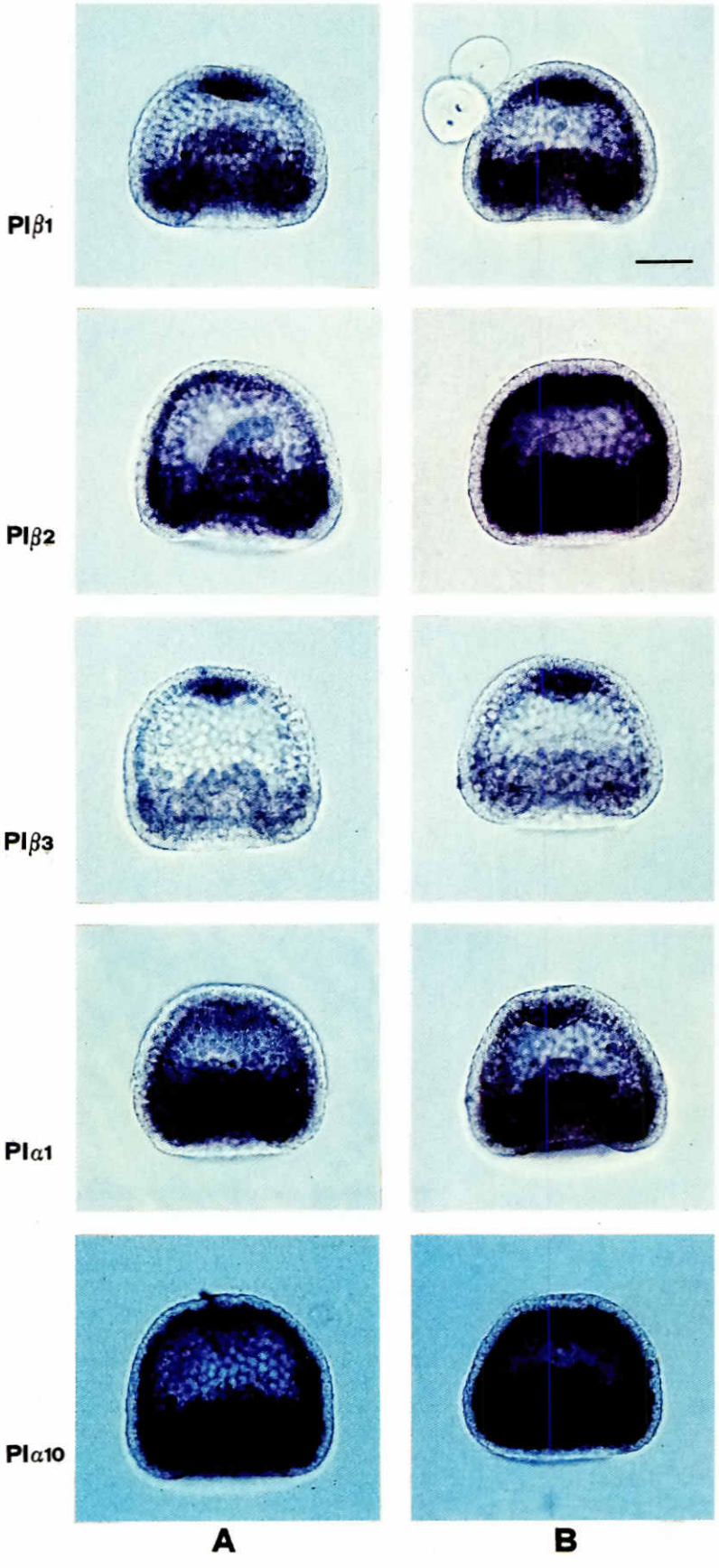


Fig. 2. Whole-mount *in situ* hybridization of *Paracentrotus lividus* embryos illustrating the developmental pattern of expression of the α and β tubulin embryonic transcripts (blue staining region). *Embryos were photographed with an Axioskop2O (Zeiss) photo microscope equipped with an automatic MC8O exposure system at a magnification of x40. Bar, 20 μm. The DIG-probes used are the 3'UTR antisense of the α and β tubulin cDNA clones, at a concentration of 10-15 ng/ml. The Plβ3 transcript is localized, at prism stage* **(column A)**, *in the thickened epithelium of the apical tuft, as well as in the ciliated band. At early pluteus stage* **(column B)**, *the localization of the Plβ3 transcript in the apical ganglion (AG, arrow), in the ciliated band and in the oral ganglion (OG, arrow) is clearly visible. All the other α and β tubulin transcripts (Plβ1/2, Plα1/10) seem to be localized at gastrula stage* **(column A)** *in the archenteron and in all the ectodermic tissues of the embryo. At pluteus stage* **(column B)**, *the localization of all the transcripts on the whole gut is confirmed, although the Plα1 probe seems to hybridize only with a portion of the gut (the esophagus), while, in the oral and aboral ectoderm, we notice a more specific localization, in fact, the ciliated band and/or the portions neighboring the ciliated band are stained.*



We have also carried out *in situ* hybridization experiments on deciliated embryos. Cilia, removed from gastrula by brief treatment with hypertonic sea water, start to regenerate within 15 min after deciliation continuously increasing their length. After 2 h, cilia regain their original length over the entire embryo surface but apical tuft cilia continue to elongate. Deciliation also results in a temporary enhancement of synthesis of total tubulin mRNA with a maximum at 30 min after deciliation. After 1 h, transcriptional activity returns to levels observed in ciliated control embryos (Auclair and Siegel, 1966; Gong and Brandhorst, 1987). The results obtained by *in situ* hybridization experiments performed on deciliated early gastrula embryos (fixed 20 min after deciliation) using the specific antisense probe corresponding to the 3'UTR of Plβ3 (Fig. 3, column B; see column A as control), confirm the localization of the Plβ3 mRNA in the animal pole at the level of the thickened epithelium of the apical tuft and show great similarity with the control embryo. Furthermore, *in situ* hybridization experiments on embryos cultured in the presence of VPA (which induces exogastrulation or deviation of the axis of the archenteron invagination — Sconzo *et al.*, 1996 — Fig. 4, columns A-B) also confirm the localization of Plβ3 transcript in the apical tuft thickened epithelium and in the ciliated band; these latter results also suggest that the Plβ3 localization is correlated neither with the archenteron invagination nor with the formation of the mouth.

These results seem remarkable because the Plβ3 probe specifically marks both ectoderm-derived neurons and their founder cells, just like Plα2 probe (Gianguzza *et al.*, 1995). It is also remarkable that despite this same transcript localization, *Plα2* gene is clearly transcriptionally activated only at specific stages, whereas *Plβ3* seems to be expressed in all stages of embryogenesis. It would be of some interest to discover whether this common localization is correlated to the existence of specific transcriptional factors which regulate the expression of both genes in the neural cells, or whether the localization of Plβ3 transcript is related to a post-transcriptional mechanism that specifically regulates its stability in neural cells.

The results of whole-mount *in situ* hybridization experiments on gastrula (column A) and pluteus (column B) embryos with specific antisense 3'UTR probes of Plβ1/2, and of Plα1/Plα10 are shown in Figure 2. By using these

Fig. 3. Whole-mount *in situ hybridization* of the α and β tubulin transcripts on *Paracentrotus lividus* deciliated embryos. *The whole-mount in situ hybridization experiments were performed as described in Figure 2, using the same amount of antisense DIG-probes and the same time of staining both for deciliated* **(column B)** *and normal embryos* **(column A)**. *The results of in situ hybridization on the deciliated gastrula-equivalents embryos* **(column B)** *clearly show for Plβ3 transcript the same specific localization in the thickened epithelium of apical tuft as in the control. All other transcripts, after deciliation, are principally localized, as in controls, in the vegetal plate progeny and in the ectodermic tissues of the embryos. The oral localization is clearly enhanced for the Plβ2 probe on deciliated embryos. Bar, 20 μm.*

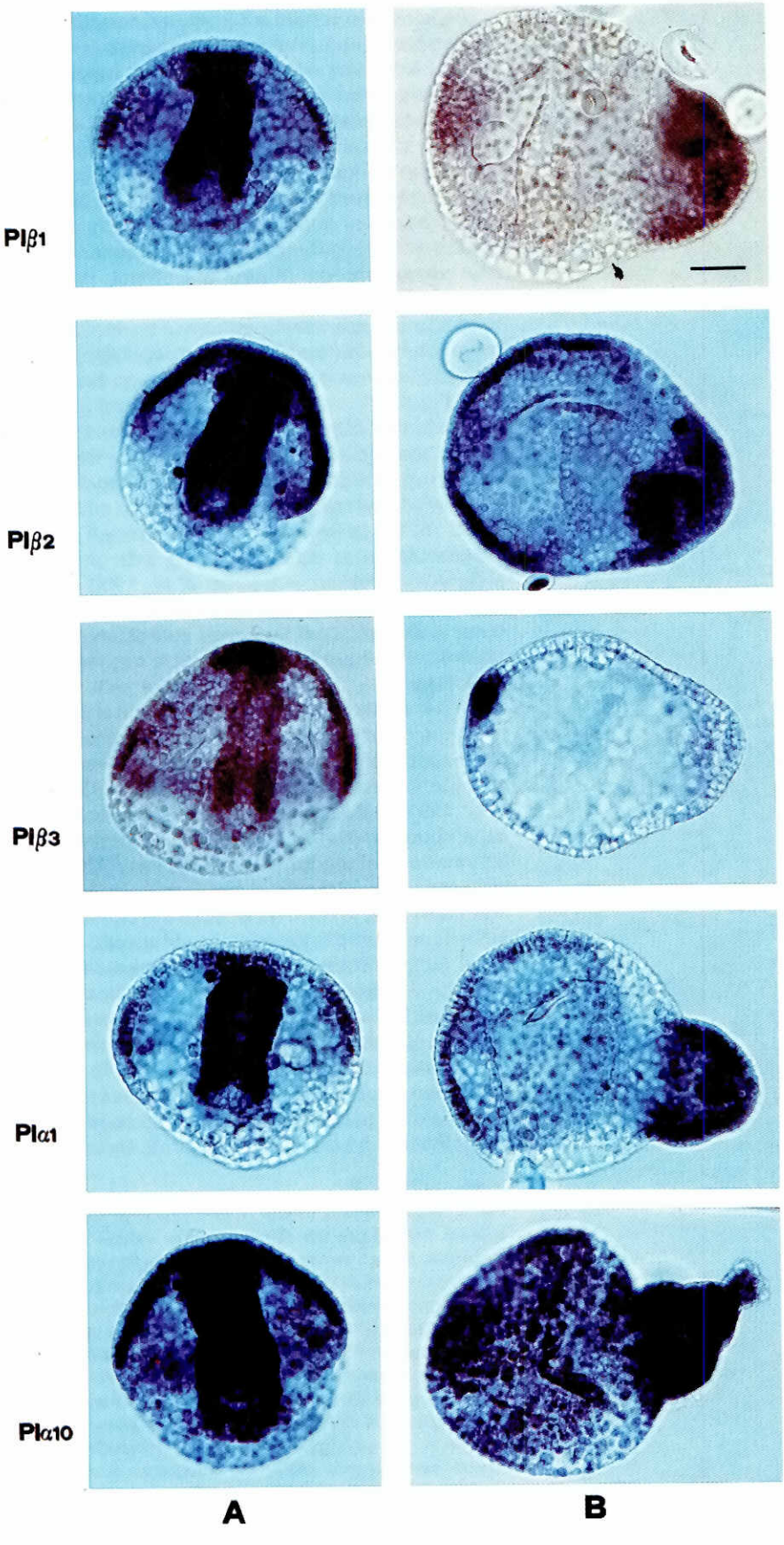


Fig. 4. Whole-mount *in situ* hybridization on 36 h prism-equivalent VPA-treated embryos. *Plβ3 transcript is localized in the thickened epithelium of the apical tuft and in the ciliated band* **(column A)**, *or restricted to the thickened ectoderm of the apical tuft when the archenteron is everted* **(column B)**. *All the other probes hybridize with the whole archenteron* **(column A)** *or, in the exogastrula* **(column B)**, *with the everted archenteron, confirming the results obtained with normal embryos. Moreover, the Plβ1 and Plα1 probes seem to hybridize preferentially with the ectodermal portion delimited by the ciliated band (oral ectoderm), whereas the Plβ2 and Plα10 probes seem to hybridize with the ectodermal portion surrounding the ciliated band (probably a portion of the aboral ectoderm;* **column A**). *Bar, 20 μm.*

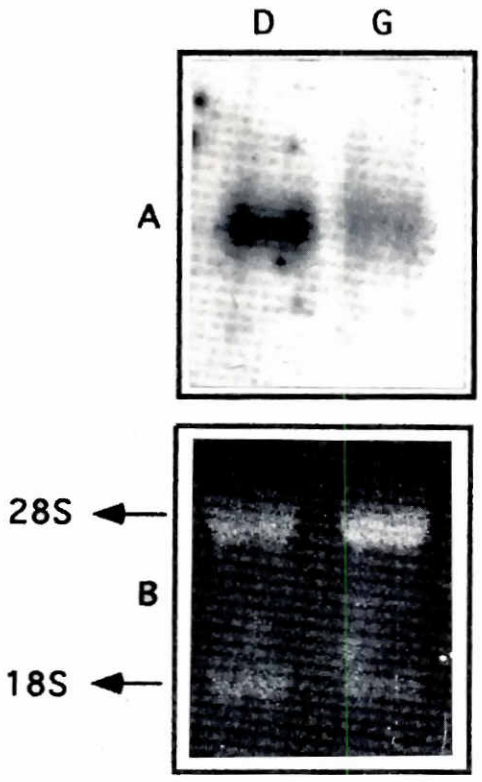


Fig. 5. Northern blot comparative analysis. (A) *10 μg of total RNA extracted from gastrula (G) and deciliated gastrula (D) embryos, were fractionated by formaldehyde-agarose gel electrophoresis, blotted onto nylon membrane and hybridized with a specific 3' UTR oligonucleotide antisense probe of Plβ2 clone (see Fig. 1). Labeling, hybridization and washing conditions were as described in Materials and Methods. The autoradiography represents a 2-day exposure of the blot.* **(B)** *Ethidium bromide staining of the total RNA gel before the blot shows that quite similar amount of total RNA loaded onto the gel.*

specific probes, one by one, we were able to observe, especially at pluteus stage, a territorial localization in the ciliated band and/or in the oral/aboral ectoderm neighboring the ciliated band. Both differences and similarities thus seem to exist among the spatial localization of the Plβ1, Plβ2, Plα1 and Plα10 transcripts.

In the embryos cultured in the presence of VPA for 36 h, corresponding to the prism stage in control (Fig. 4, columns A and B), the Plβ3 probe preferentially stains the ciliated band position only, whereas the other probes stain the ciliated band and/or the neighboring ectoderm. Plβ1 and Plα1 seem to be preferentially expressed in the ectoderm portion delimited by ciliated band (oral ectoderm; NO-VO progeny), while Plβ2 and Plα10 are preferentially localized in the ectoderm portion surrounding the ciliated band (a portion of the aboral ectoderm; Na-NL progeny). Thus our results show that specific tubulin isotypes, or a mix of them, are expressed in, and mark the ciliated band and the oral/aboral ectoderm neighboring cells of the ciliated band.

The experiments performed on deciliated early gastrula embryos (Fig. 3, column B; in column A the *in situ* hybridization of the corresponding normal stages are shown as controls) clearly show that the Plβ1/2, Plα1/10 mRNAs are localized in the vegetal plate (see below) and in the entire ectoderm. Nevertheless, comparing the results obtained in control and deciliated embryos for each probe, a specific enhancement in deciliated embryos for Plβ2 probe is clearly evident. In order to confirm the increase of Plβ2 transcript by deciliation, we carried out a comparative analysis by Northern blot hybridization of total RNA extracted from embryos deciliated at gastrula stage and, as control, from untreated embryos at the same stage, using as a probe the specific antisense oligonucleotide of Plβ2 (Fig. 5). The result shows an enhancement of Plβ2 mRNA of about 2.5 times (densitometrically fixed) on deciliated embryos, whereas using the specific antisense oligonucleotide of Plβ3 as a probe a substantial similarity is found between controls and deciliated embryos (data not shown). The remarkable increase of Plβ2 mRNA shown by whole-mount *in situ* hybridization and by Nothern blot performed on deciliated embryos, along with the identity between Plβ2 and the *Strongylocentrotus purpuratus* specific isotype Spβ1 (Gianguzza *et al.*, 1992), whose expression is temporally coordinated with ciliogenesis (Harlow and Nemer, 1987b), lead us to propose a functional role of Plβ2 in ciliogenesis; on the other hand, it is remarkable that both the squamous epithelial cells and the columnar cells possess cilia.

The same isotypes are also expressed in the archenteron

Among the cells that constitute the five territories known in the early sea urchin embryo of *Strongylocentrotus purpuratus*, those of the vegetal plate are pluripotent to the highest degree. This territory will not only produce the entire endoderm, but also most of the coelomic pouches founder cells and the true mesoderm which will differentiate into pigment-, muscle- and larval mesenchyme- or basal- cells (Cameron and Davidson, 1991). Thus, vegetal plate invagination contributes to the formation of all the internal cell types of the pluteus larva, except those formed by descendants of the small micromeres, and plays a central role in sea urchin embryogenesis. It derives entirely from the "Veg2 tier" (Horstadius, 1939), whose constituents are now known as VAM1 and 2, VLM1 and 2, VOM1 and 2 blastomeres and their progeny (Cameron *et al.*, 1990). The vegetal plate is so called because in the early blastula the progeny of its founder cells form a flattened disc in the vegetal hemisphere of the embryo, concentrically surrounding the progenitors of the skeletogenic mesenchyme and the eight "small micromeres", which have different lineage and fates. The cells of this layer invaginate during gastrulation and form a single cell thick tube (the archenteron), whose distal tip consists of motile secondary mesenchyme (mesoderm) cells, themselves probably pluripotent since they will later participate in the formation of the coelomic pouches, circumoesophageal muscles and migratory pigment cells (Cameron and Davidson, 1991). The archenteron invagination occurs by convergent cell intercalation and rearrangement, and involves extensive changes in cytoskeletal organization, cell shape, and polarized cell motility (Hardin, 1989, 1994). In this process microtubules seem to be involved in the stabilization of the cell shape (Hardin, 1987). Once specified, the morphogenetic development of the vegetal plate appears to continue autonomously, leading to the formation of fore- mid- and hind-gut.

Whole-mount *in situ* hybridization experiments (Fig. 3) show that at early gastrula stage (column A), Plβ1/2, Plα1/10 3'UTR antisense specific probe marks, in addition to the animal pole (previously discussed), the progeny of the vegetal plate, that, at late gastrula stage, is found in the whole archenteron (Fig. 2, column A). At pluteus stage, Plβ1, Plβ2 and Plα10 transcripts are

diffused in the whole gut and probably in the oesophageal muscles, whereas Plα1 seems to be only localized in the foregut and the oesophageal muscles (Fig. 2, column B).

These findings are confirmed by *in situ* hybridization of the prism-equivalent VPA-treated embryos. In fact, the whole archenteron (column A) or everted archenteron (column B) in the exogastrula are stained blue (Fig. 4).

Conclusions

As previously shown (Alexandraki and Ruderman, 1985), in sea urchin there is a 2- to 5-fold increase in the overall amounts of α and β tubulin mRNAs between blastula stage and pluteus stage, and this effect is mainly due to an increase in specific tubulin mRNAs classes. We have shown that in blastula stage *Paracentrotus lividus* this abrupt increase in the α and β tubulin transcripts is mainly due to the increase of Plβ1, Plβ2 and Plα2 mRNAs (Gianguzza *et al.*, 1990, 1992, 1995); here we show that an α/β pair of tubulin transcripts (Plα2/Plβ3) is specifically expressed during embryogenesis in the neurogenic territory of the sea urchin *Paracentrotus lividus*. The other embryonic isotypes of α and β tubulin should be functionally related to two of the well-documented morphogenetic processes which occur at gastrula stage and depend entirely on intercellular interactions. In late embryogenesis, the interactions between the oral and aboral ectoderm specify the position of the ciliated band (Cameron *et al.*, 1993) and the invagination of the vegetal plate forms the gut territory (Davidson, 1993). Moreover we provide indications that the Plβ2 isotype may be functionally related to ciliogenesis.

Our results are consistent with a cell lineage model similar to that described in detail by Cameron and Davidson (1991) for *Strongylocentrotus purpuratus*, although paleontologic and molecular studies show that these two species diverged some 65 Myr ago (Busslinger *et al.*, 1982).

Materials and Methods

Embryo culture

Adult sea urchins of the species *Paracentrotus lividus* were collected along Sicily's western coasts. The eggs were fertilized and cultured at a concentration of 10,000/ml in millipore filtered sea water containing antibiotics. In some experiments the embryos were cultured in the presence of agents able to induce deciliation or exogastrulation. Deciliation of early gastrula embryos was obtained by adding 0.12 volumes of 4.45 M NaCl to the embryo cultures; after 1 to 2 min, 1.2 volumes of special sea water were added, which lacks NaCl but includes other salts of normal sea water at a concentration 10% higher. After settling, embryos were suspended in normal sea water. Twenty minutes after restoration (Gong and Brandhorst, 1987), embryos were collected either to be fixed for whole-mount *in situ* hybridization or to total RNA extraction, for Northern blot experiments. The embryos for deciliation experiments were cultured as follows: the embryo culture was divided at early gastrula stage, one half was then deciliated, and the other provided the control. Both the control and the deciliated embryo cultures were collected at the same time (20 min after deciliation).

Exogastrulation was obtained by adding 20 mM VPA 30 min after fertilization (Sconzo *et al.*, 1996). The embryos were allowed to develop for 36 h, which corresponds to pluteus stage in the controls.

Whole-mount in situ hybridization

Embryo fixation and whole-mount *in situ* hybridization were performed as previously described (Gianguzza *et al.*, 1995). The hybridization step was performed in the micro Eppendorf tube (0.5 ml) at 50°C overnight. After hybridization, the embryos were washed twice for 15 min at 45°C in 5xSSC, 0.1% Tween 20, twice for 15 min at 45°C in 2xSSC, 0.1% Tween 20, and twice for 15 min at 45°C in 0.2xSSC, 0.1% Tween 20.

The staining reaction was allowed to develop for 3 to 5 h at room temperature in the dark with shaking.

Digoxigenin-labeled probes

3'UTRs was amplified by polymerase chain reaction technique. Two PCR primers were made according to the sequence of cDNA clones. Location and sequence of primers are indicated in Figure 1. The selection of these oligonucleotides was based both on their melting temperature and on the length of amplified fragments. The cycling conditions were the following: denaturation at 95°C for 3 min was followed by 30 cycles of denaturation at 95°C for 1 min, annealing at a temperature specific for the pair of primers for 1 min (54° or 56°C, typically), and extension at 72°C for 1 min. The DNA fragment 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 conditions were as follows: 25-30 cycles of denaturation at 94°C for 45 sec, annealing at a specific temperature for each primer (54° or 56°C, typically) for 30 sec, and extension at 72°C for 60 sec.

RNA extraction and blot hybridization

Total RNA was extracted from normal- and deciliated-gastrula stage embryos as previously described (Gianguzza *et al.*, 1995).

10 μg of total 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 specific probe we utilized was the Plβ2 antisense oligonucleotide corresponding to 3'UTR (see Fig. 1) ^{32}P-labeled with kinase under the conditions suggested by the supplier (Biolabs). The hybridization conditions were the following: prehybridization in 5xSSPE, 1xDenhardt's solution, 0.5% SDS at 37°C for 4 to 12 h at 37°C; hybridization in 5xSSPE at 37°C for 12 h with 2-3 ng/ml of ^{32}P-labeled probe. The filters were washed twice at room temperature for 15 min in 5xSSPE and for 5 min at room temperature in 2xSSPE, and then autoradiographed with Kodak films for two days.

Note

In this and in our previous paper (Gianguzza *et al.*, 1995) we have used, as exogastrulating agent, only the VPA drug. In our previous paper (Gianguzza *et al.*, 1995) this compound has been indicated erroneously as phenytoin instead of VPA. The two drugs are both used as anticonvulsant in human therapy, but only VPA is an effective exogastrulating agent in sea urchin embryos.

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