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Original Article

Localization of tubulin mRNA during ascidian development

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ABSTRACT In ascidians determination of the nervous system is known to occur at the gastrula stage, when the chorda-endoderm and ectoderm come in contact. *In situ* hybridization with ß-tubulin cDNA showed that tubulin transcripts were concentrated in presumptive neural cells at the early gastrula stage and continued to increase in these cells throughout neurulation. In the young larvae, the tubulin transcripts were also detectable in the adhesive papillae cells, in mesenchyme cells, muscle cells and button cells. The results suggest that expression of tubulin mRNA could be used as a marker for neural determination at early stages and, at later stages, as a marker for all cell types that elaborate many microtubules.

KEY WORDS: *tubulin mRNA, ascidian development, neural induction, specification, in situ hybridization*

Introduction

The egg of the Phlebobranchiata solitary ascidians exhibits a typical «mosaic» development. The developmental fates of most of the embryonic cells are determined at very early stages of embryogenesis and, if lost during development, the cells cannot be replaced. Probably the most complete current evidence on the role of localization is for the determination of the ascidian muscle cell lineage. Starting from Conklin's observations, which relied on the presence of yellow pigmentation for distinguishing muscle cells and their progenitors in the embryo (Conklin, 1905), detailed investigations of the formation of muscle in the embryos of *Ciona* and *Styela*, carried out by Whittaker *et al.* (1977), used acetylcholinesterase as a marker. It has been established that isolated B4. 1 lineage blastomeres can differentiate into muscle cells autonomously. Normally the enzyme appears in the tail muscles at the neurula stage, but the transcription of the structural gene starts in the muscle lineage at the gastrula stage (Meedel and Whittaker, 1984; Meedel *et al.*, 1987).

Moreover, recent studies exploring the molecular mechanisms that underlie the regulation of muscle-specific gene expression in muscle lineage cells have investigated transcription of actin mRNA (Jeffery *et al.*, 1983), muscle actin mRNA (Tomlinson *et al.*, 1987) and myosin heavy chain mRNA (Makabe *et al.*, 1990) and have led to the conclusion that in all three cases zygotic transcription was initially observed at the gastrula stage. These experiments distinguish different kinds of developmental commitment. The cells are already specified, according to the test of isolation, by the 8-cell stage, but the nuclei are not committed to autonomous expression before the gastrula stage.

The development of the brain seems not to follow the model of «mosaic» development. The presumptive territory of the brain is located, at the 8-cell stage, mainly in the two anterior animal blastomeres a4. 2 (Conklin, 1905, Ortolani, 1954). But at this stage their fate has not yet been determined, since, when isolated, they were unable to differentiate into a neural organ and formed only ectodermal vesicles (Reverberi and Minganti, 1946). Brain, sense organs and adhesive papillae will only develop when these blastomeres are cultured in combination with the anterior vegetal pair A4. 1 (Reverberi and Minganti, 1947) or with one of their chordal or endodermal descendants of the 64-cell stage, as demonstrated in *Phallusia mamillata* and *Ascidiella aspersa* by Reverberi *et al.* (1960). Recently Nishida (1991) reported that the inducers of sensory pigment cells in *Halocynthia roretzi* are the spinal cord precursor cells. The discrepancy may be attributable to differences between species. This phenomenon has been called «evocation» rather then «induction» because, unlike in amphibians, the presumptive tail ectoderm cannot be converted into neural structures either by substitution of blastomeres or by rotation of the animal quartet, putting the b4. 2 in contact with the A4. 1 cells (Ortolani, 1987). The «evocation» process during normal development begins to function at gastrulation, as in amphibians, as a consequence of the close contact between the chorda-endodermal cells of the archenteric roof and the overlying neural ectoderm. The more recent view regarding this argument, based on studies of amphibian embryos, is that this phenomenon is a «permissive induction» (Slack, 1983; Gurdon, 1987).

In previous studies (De Bernardi and Condorelli, 1987; De Bernardi *et al.*, 1990), we showed by dot blot and by *in situ*

Abbreviations used in this paper: EDTA, ethylene diamine tetraacetate-disodium salt; SSC, sodium chloride-sodium citrate; Tris, Tris(hydroxymethyl)-aminomethane; PBS, phosphate buffered saline.

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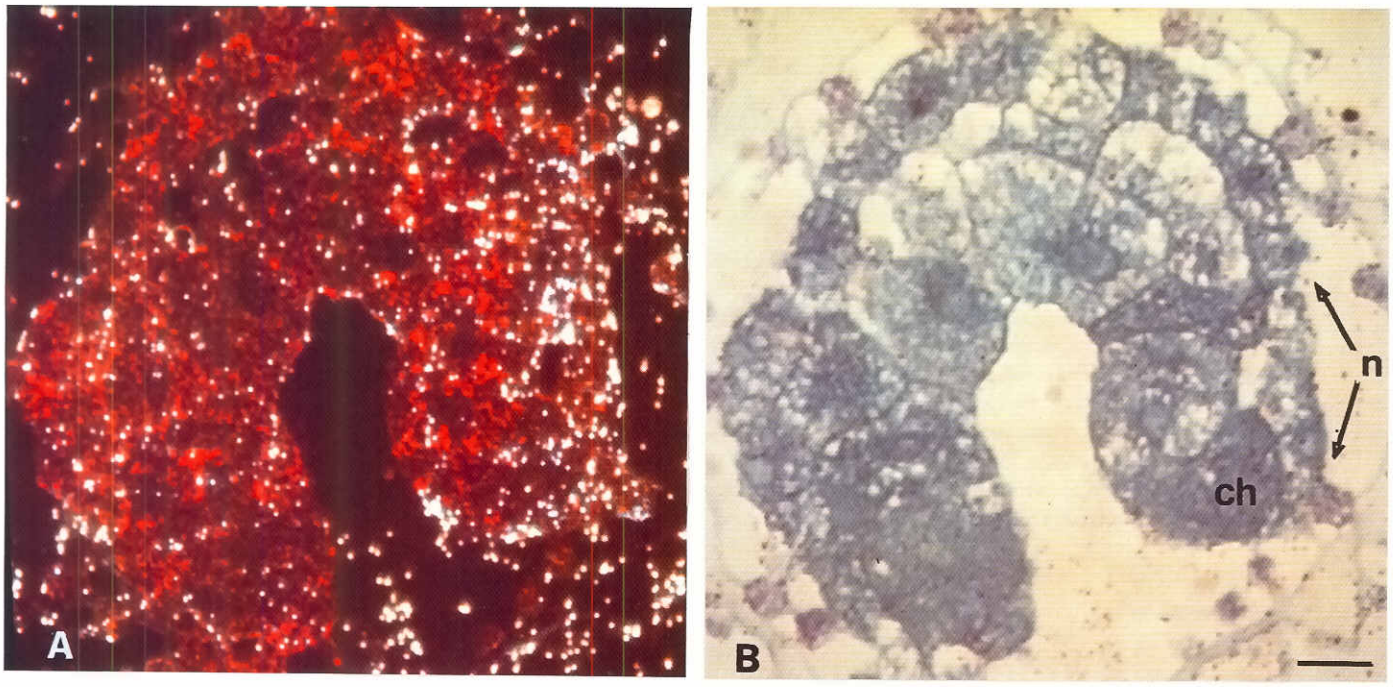

Fig. 1. *In situ* hybridization with ß-tubulin cDNA of a mid-gastrula sectioned through the animal-vegetal axis. (A) *Dark-field photomicrograph showing hybridization signals accumulated in the neural ectoderm (n).* **(B)** *Light photomicrograph of the same section; ch, chordal cells. Scale bar equals 10 µm.*

hybridization that ß-tubulin mRNA can be used as a marker for neural determination in amphibian embryos because its synthesis increases in the neural ectoderm from the gastrula stage onwards, when neural induction by the underlying chordomesoderm has occurred. These results demonstrated that an early step in neural commitment is elongation of the ectodermal cells. During neurulation, indeed, apical bundles of microtubules appear in ascidians (Mancuso, 1973), arranged parallel to the major axis of the cells as they are in Vertebrate neural plate (Karfunkel, 1974, Camatini and Ranzi, 1976).

For the reasons given above, the ascidian embryo is particularly attractive for examining the pattern of gene expression during development, to compare Protochordata and Vertebrata and traditionally considered «mosaic» development with traditionally considered «regulative» development. The results presented here indicate that the dichotomy between these two definitions should no longer be emphasized.

Results

The source of neural cells in ascidians is well known to be from descendants of the a4. 2 blastomeres of the 8-cell embryo. In the experiments described here, the ß-tubulin cDNA probe was used to examine the distribution of the corresponding transcripts from the gastrula stage onwards, following the results obtained by Reverberi *et al.* (1960). At the gastrula stage, the distribution of the autoradiographic grains showed a slight elevation in the presumptive neural cells (Fig. 1A). At this stage, the presumptive neural cells can be distinguished by their position at the anterior lip of the blastopore, overlapping the big chordal cells (Fig. 1B). In animal-vegetal section, it can be seen better that neural cells accumulate tubulin transcripts more than other ectodermal cells. There were also a number of transcripts in all the cells of the embryo, due to the ubiquitous distribution of the tubulin, but this labeling was barely above background (Fig. 1A).

At the neurula stage, the tubulin transcripts appeared to be concentrated only in the neural plate cells in both transverse and in parasagittal sections (Fig. 2A-C). The hybridization in non-neural ectodermal cells had dropped to the background level. The hybridization marker was particularly evident in the cytoplasmic area, and was absent from the nuclei. Even in a parasagittal oblique section, a characteristic anterior neural groove appeared to be labeled (Fig. 2C). In some sections (not shown) also spinal cord cells were labeled. In a frontal section of an early neurula, the neural cells of the anterior row of the ectoderm and the mesenchyme cells were labeled. The two rows of cells of the primary muscle lineage, posterior to the mesenchyme cells, were unlabeled (Fig. 2E-F).

At the young larva stage, most of the hybridization was located in the neural cells forming the wall of the brain vesicles, in the sense organs and in the neural tube, as can be seen in the longitudinal sections dark-field photographed (Fig. 3A-C). The autoradiographic grains can also be seen in the elongated cells of the larval adhesive papillae and in some cells located ventrally to the pharynx. Some hybridization signals can also be seen in some nuclei of the endodermal cells of the pharynx, probably related to their mitotic activity (Fig. 3A.)

In a cross section of the larva, the hybridization was clearly concentrated in the dorsal neural tube. Other dorsolateral

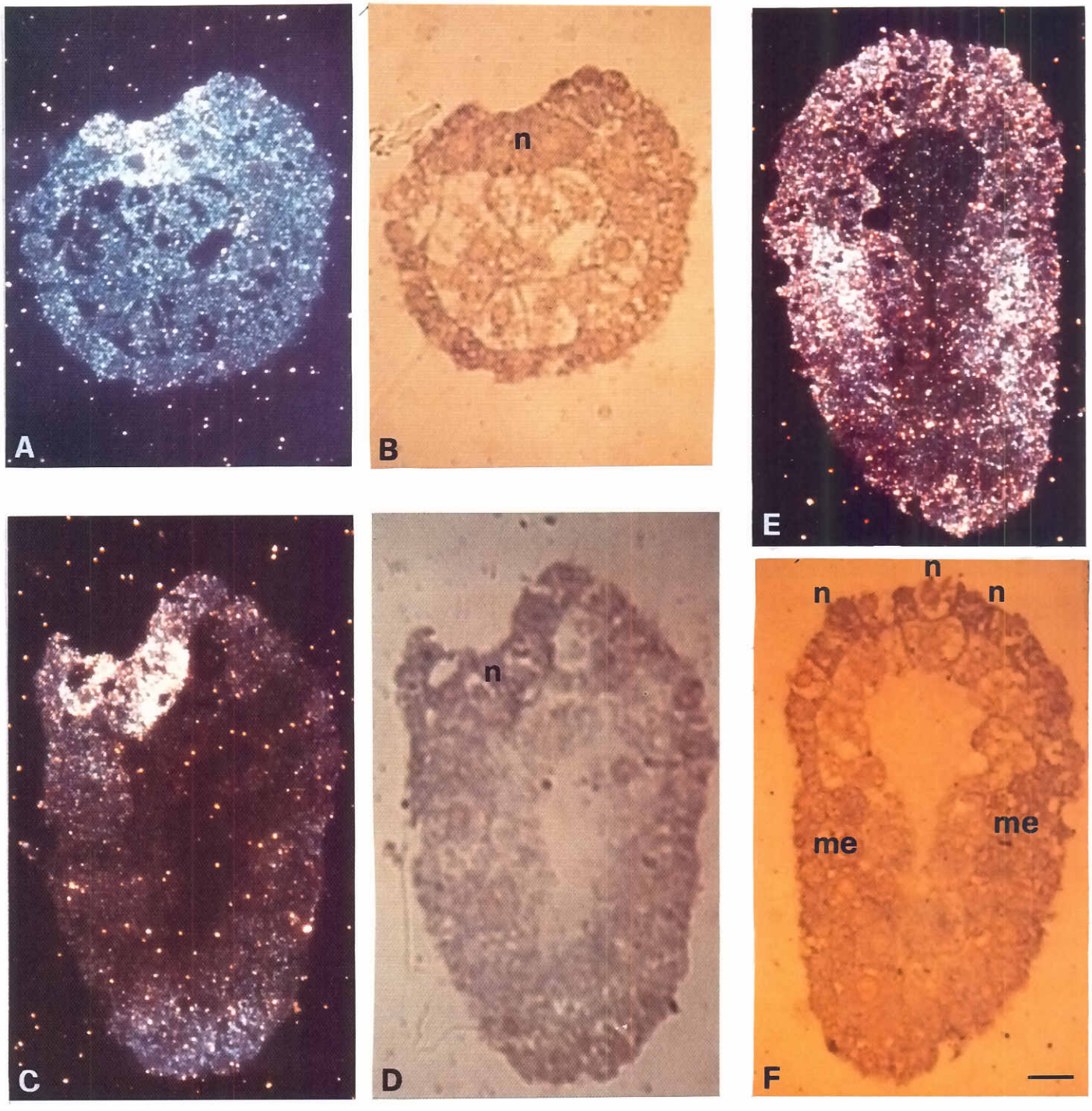

Fig. 2. *In situ* hybridization of neurula-stage embryos with ß-tubulin cDNA. (A-C) *Dark-field photomicrograph showing ß-tubulin mRNA concentrated in neural plate cells (n) in transverse (A) and parasagittal oblique section (C).* **(B-D)** *Light photomicrograph of A and C.* **(E-F)** *Frontal sections through the anterior-posterior axis showing two rows of labeled mesenchyme cells (me). Scale bar equals 10 μm.*

mesenchymal cells appeared to be hybridized and, more evidently than in longitudinal sections, the cells under the pharynx (Fig. 3C-D). These cells, as judged from the section in Fig. 3 E-F, appeared highly metachromatic when stained with 0.1% methylene blue and very similar to the «button cells» that have been shown to be endodermal in origin (Materazzi and Ortolani, 1969). Some cross sections of the tail showed a number of grains inside the muscle cells surrounding the unlabeled notochord, although the muscle

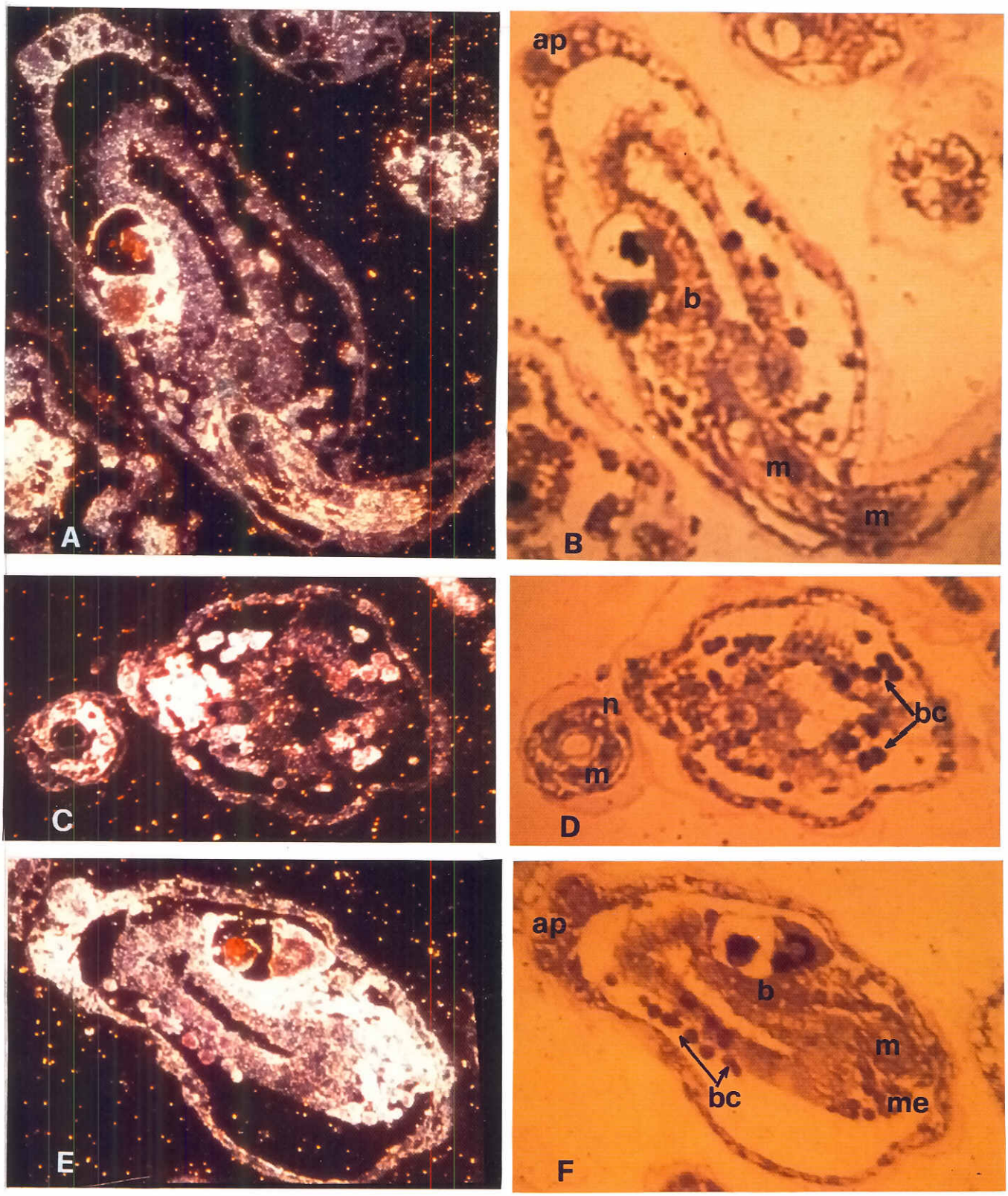

Fig. 3. *In situ* hybridization of young larva-stage sections with ß-tubulin cDNA photographed in dark-field and light optics. (A-B) *Sagittal section.* **(C-D)** *Transverse section of the trunk and of the tail.* **(E-F)** *Parasagittal section of the trunk. Autoradiographic grains are concentrated in brain vesicles (b), neural tube (n), and adhesive papillae (ap) and are also evident in mesenchyme cells (me), muscle cells (m) and button cells (bc). Scale bar equals 20 µm.*

cells contained fewer hybridization grains than the neural tube cells (Fig. 3C.)

Discussion

The use of cloned probes corresponding to a specific transcript is a more direct way to measure specific gene expression, it is not complicated by the possibility of regulation at the level of translation and it is detectable in earlier stages of development than the corresponding protein. For this reason the transcription of a particular gene can be shown long before the histological modification of the cells leading to the terminal differentiation, and the intermediate stages of the commitment can be directly evaluated. Moreover, *in situ* hybridization, with which one can detect the intracellular localization of mRNA, seems to be particularly suitable to small embryos, such as those of ascidian.

Detailed studies with ascidian (Mancuso, 1973), amphibian and chick embryos by electron microscopy and by immunofluorescence showed that there is an increase in microtubules with the elongation of the neural plate cells (Burnside, 1971; Karfunkel, 1974; Camatini and Ranzi, 1976) even after artificial induction (Cigada Leonardi *et al.*, 1987). Therefore, the synthesis of a mass of tubulin mRNA could reasonably be the first step of neural commitment. As we showed in previous work on amphibian embryos (De Bernardi *et al.*, 1990), ß-tubulin mRNA can be used as a molecular marker of the neural cell-fate as early as the gastrula stage, when a concentration of tubulin transcripts was observed in presumptive neural plate cells. Since electron microscopy showed a similar morphology of the differentiation of the nervous system in Protochordate and in Vertebrate embryos, the intense transcription of tubulin mRNA in presumptive nervous cells could be considered an early marker for neural specification, despite the wide distribution of tubulin.

In addition, a widely expressed tubulin mRNA in the cells (ß1-tubulin) has been used as a marker in *Drosophila* embryos too (Buttgereit *et al.*, 1991). Its expression starts shortly after the commitment of neuroblast cell to its fate and it has been proposed as a model for the identification of neural-specific transcription factors. Another example of a widely distributed protein as a marker of early determination is the increased F-actin density in the dorsal 3D macromere of the molluscan *Patella vulgata* embryo following the inductive interactions that establish the dorsal-ventral axis (Serras and Speksnijder, 1990). Moreover, the first evidence for the cytoplasmic localization of a specific mRNA class during early ascidian development was the finding by Jeffery *et al.* (1983) that actin mRNA sequences localized in the myoplasm of the eggs were concentrated in the mesodermal cell lineages during early embryogenesis.

Perhaps a general conclusion can be drawn from our own and the cited experiments. The more specific proteins and their mRNAs start to be transcribed from the determination step onwards but some widely distributed proteins (and their mRNAs) that are in a certain way characteristic of a cell type start to be transcribed in larger quantity as the first step of commitment (specification) begins.

In our experiments a remarkable concentration of tubulin mRNA appeared in muscle cells of the larval tail (Fig. 3 A-C), but the primary muscle lineage cells in earlier stages never showed more autoradiographic grains than the background level (Fig. 2E). We can conclude that the presence of tubulin mRNA is connected with the modification of cell shape at the beginning of the cellular differentiation. On the other hand, mesenchyme cells showed a hybridization signal at the neurula and larval stages (Figs. 2E and 3A-C). In this case the presence of detectable quantities of tubulin mRNA could be connected to the remarkable modification of cell shape these cells undergo in both early and larval stages. The same could be true for the endodermal button cells of the larva. They indeed become amoeboid and migrate in anterior-dorsal direction reaching the base of the palps of the swimming larva (Reverberi *et al.*, 1969). At the beginning of tail reabsorption, they cross the ectodermal layer and thereafter can be seen within the tunic (Ortolani and Patricolo, 1972).

The pattern of expression of tubulin mRNA could suggest that these transcripts are more frequent in epidermal and neural precursor cells, which are characterized by rapid cell divisions even after this process has ceased in most other parts of the embryo (Nishida, 1986, 1987; Nicol and Meinertzhagen, 1988), like the cytoskeletal actin genes Cy I and Cy IIb of sea urchin embryos, found to be expressed exclusively in rapidly dividing cell lineages (Cox *et al.*, 1986). Our results, however, showed most evidently labeled those tissues which were committed to build many microtubules during differentiation, such as neural cells, palp cells and, in lesser quantity, muscle cells, mesenchyme cells and «button cells,» which are committed to dramatic modifications of cell shape. The specific accumulation of tubulin mRNA in these cells or in their precursors appears to be achieved by two processes: first localization and differential segregation of maternal mRNA and then activation of gene transcription in particular cells.

The last point we can mention about tubulin mRNA in ascidian embryos is that at the gastrula stage there is an increase in its synthesis, as has been demonstrated for muscle actin (Tomlinson *et al.*, 1987) and for the myosin heavy chain (Makabe *et al.*, 1990). This increase is probably related to an analogous increase in amphibian embryos at the gastrula stage. In these embryos, indeed, the «mid-blastula transition» has been precisely recognized as a boundary between the exclusive use of maternal transcripts and the beginning of zygotic gene expression. Further research could probably lead to a similar definition for ascidian embryos, thus emphasizing the similarity of the two embryos, already suggested by Reverberi *et al.* (1960) despite the dichotomy between «mosaic» and «regulative» embryos.

Materials and Methods

Phallusia mamillata eggs were obtained from oviducts of dissected animals and were collected in filtered sea water. The majority of them were dechorionated by sharpened needles and were fertilized with a suspension of self and non-self sperm. The fertilized eggs were allowed to develop until they reached the required stages. Embryos from gastrula to tadpole stage were fixed in 5% formaldehyde-PBS and embedded in methacrylate, cut in 2-µm sections and processed for in *situ* hybridization. .

ß-tubulin cDNA from plasmid pT2 (Lopata *et al.*, 1983), kindly provided by D.W. Cleveland, contains the complete coding region of a chick brain ß-tubulin mRNA and is also useful for ascidian embryos, because of the widely recognized similarities in the coding sequences of all Vertebrate tubulin genes (Sullivan and Cleveland, 1986; Sullivan, 1988). The ß-tubulin cDNA recognizes both ß2, which appears to be the predominant ß-tubulin gene expressed in neurons and brain, and ß1 isotype, which is expressed, as a minor species, in many cell types (*e.g.*, skeletal muscle cells). From further data it recognizes even the ß4-tubulin mRNA, afterwards classified as class III isotype, minor neuronal (Sullivan, 1988) and greatly expressed in many adult tissues, but in minor proportion in the embryonic ones (Havercroft and Cleveland, 1984).

The plasmid was obtained and purified from *E. coli* by large scale extraction and CsCl gradient centrifugation (Maniatis *et al.*, 1982). The ß-tubulin cDNA was prepared by Hind III restriction of plasmids and was separated by agarose gel electrophoresis.

Tritium-labeled cDNA probes were prepared by random priming reaction, using 50 µCi of each nucleotide. The Multiprime kit and labeled nucleotides were from Amersham.

In situ hybridization and autoradiography were carried out as previously described (De Bernardi *et al.*, 1990) based on standard procedures (Jamrich *et al.*, 1984). Postfixed sections were prehybridized for 3 hours at 40°C and then hybridized under coverslips with the labeled probe (about $1x10^6$ cpm for each slide) in 5 µl of hybridization buffer (10 mM Tris-HCl, pH 8, 0.3 M NaCl, 5 mM EDTA, 40% formamide) for 17 hours at 45°C. The slides were then washed in hybridization buffer, twice in 2xSSC, then in 0.3 M ammonium acetate-70°ethanol, and rinsed in absolute ethanol. Slides were dipped in Kodak NTB2 liquid emulsion and exposed for 6 weeks. After development, the sections were heavily counterstained with methylene blue to identify the different cell types even in dark-field pictures, in which the autoradiographic granules appear bright and the underlying tissues appear light blue. For negative controls some slides were incubated at 37°C for 20 h in 50 µg/ml pancreatic RNase A prior to in *situ* hybridization. Other slides were hybridized with tritium-labeled pBR322. In each experiment the hybridization signals were at the background level.

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