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Original Article

Behavior of endodermal «button cells» during metamorphosis of ascidian larvae

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ABSTRACT **Swimming larvae of *Phallusia mamillata* are known to have «button cells» of endodermal origin between the ventral surface of the pharynx and the epidermis, that are stainable by various techniques. By immunofluorescence with anti-tubulin antibody and confocal laser microscopy, we obtained a bright reaction at one pole of the cells, suggesting the presence of a cap of tubulin and of microtubules overlaying the nucleus. During metamorphosis the microtubule-rich pseudopods at their base reach the epidermis, especially in the area near the adhesive papillae. Then they emigrate through the epidermis and become roundish again.**

KEY WORDS: *ascidian development, endoderm, immunofluorescence, tubulin, metamorphosis*

Introduction

The cephalenteron of the swimming larvae of some species of ascidians contains a particular type of cells called «button cells.» They are arranged in a double row of about twenty on each side in the ventral region between intestine and epidermis. Ries (1939) considered them to be mesenchymal in origin in *Ascidia mentula*.

In experiments with *Ascidia malaca* and *Phallusia mamillata* involving separation and killing of blastomeres, an endodermal origin of these cells has been suggested. They are derived from the anterior vegetal blastomeres A4.1, *A4.1* of the 8 blastomere stage (Fig. 1), or, more precisely, from the blastomeres A6.1, *A6.1* and A6.3, *A6.3* of stage 32 (Materazzi and Ortolani, 1969). Since the blastomeres A 6.1 give rise only to endoderm, the origin of button cells from them seems uncontroverted. The blastomeres A6.3 are responsible for the formation of the endoderm and notochord. The separation of the endoderm (A7.5) from notochord (A7.6) takes place later at stage 64 (Ortolani, 1957). Therefore the button cells may likely originate from A7.5 since the A7.6 give rise to more dorsal cells of the notochord.

Button cells have been identified by many authors using several staining techniques: benzidine (Ries, 1939; Reverberi *et al.*, 1969), alcian blue (Materazzi, 1967), toluidine blue and Nadi reagent (Reverberi *et al.*, 1969). The reactions of these cells to various vital stains and cytochemical reactions suggested that they contained chromolipoids, especially lipofuscins, in addition to sulfated acid mucopolysaccharides (Materazzi, 1967). Using the stains listed above, Ortolani and Patricolo (1972) were able to show that at the beginning of the metamorphosis the «button cells» migrate extensively, regrouping themselves under the adhesive papillae and on the larval epidermis and, finally, within the tunic. The cells described above contain peroxidases, which, in the opinion of Reverberi *et al.* (1969) are probably implicated in the elaboration of lipids and of a mucopolysaccharide substance which is utilized for the construction of the tunic of the metamorphosing ascidia. When studied by electron microscopy, they were seen to be filled with vesicles of different sizes and with Golgian formations, frequently dome-shaped (Reverberi, 1971).

In previous research we have studied the expression and localization of tubulin mRNA as a marker of determination of the nervous system (De Bernardi *et al.*, 1991). We observed that in the button cells at the larva stage there was a pattern of expression of tubulin mRNA similar to that detectable at earlier embryonic stages in other tissues committed to dramatic modification of cell shape, such as adhesive papillae cells or muscle cells. After this observation, we decided to investigate in greater detail the behavior of these cells in various larval stages and their reaction to anti-tubulin antibody.

Results

In the swimming larvae, the metachromatic «button cells» can be clearly distinguished as roundish cells with diameters 7 to 10 μm, larger than the surrounding endodermal cells of the ventral part of the pharynx, from which they appear to sort out (Fig. 2). In some anterior cross sections a few button cells are so anterior that they are in front of the pharynx, just under the adhesive papillae (Fig. 2C). The disposition, dimension, number and metachromatic reactions perfectly resemble those described by Materazzi and Ortolani (1969) and by Reverberi *et al.* (1969). The metachromatic reaction

Abbreviations used in this paper: TLC, trunk lateral cells; PBS, phosphate buffered saline.

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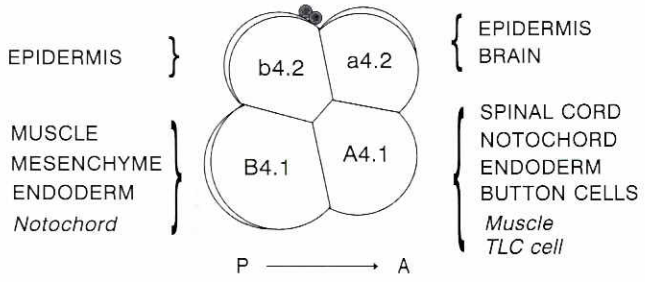

Fig. 1. Lateral view (left) of an 8-cell embryo of *Phallusia mamillata* demonstrating the orientation of blastomeres and descendant tissues *(from data of Ortolani, 1957; Reverberi et al., 1960; Materazzi and Ortolani, 1969). Italic words mark corrections made by Nishida and Satoh (1983, 1985) and Nishida (1987) for other species.*

is concentrated in granules which, at this stage, are distributed uniformly in the cells. The cells are already metachromatic when they are still interspersed in the endoderm, which distinguishes them from the cells forming the wall of the pharynx. We did not observe any mitosis in the endoderm at this stage, suggesting that the button cells derive from cells which divided in earlier stages.

In the swimming larvae stained with an FITC-conjugated anti-tubulin antibody and observed under confocal laser microscope, the brightest reaction, as expected, was in the elongated cells of the brain and of the adhesive papillae. The «button cells» can also be observed by bright fluorescence, lined up ventrally to the pharynx (Fig. 3A) or arranged under the adhesive papillae (Fig. 3B-C). The cells are usually roundish or moderately elongated at one pole, with a bright anti-tubulin reaction at one pole of the cells, suggesting the presence of a cap of tubulin and of microtubules overlaying the nucleus.

When during metamorphosis tail retraction begins, the «button cells» lose their double row arrangement and begin to scatter in the body cavity, mixing with mesenchyme cells but still distinguishable by the metachromatic reaction (Fig. 4A). At this stage many cells produce processes recognizable as pseudopodia or filopodia and the metachromatic granules are concentrated in a part of the cell. From this stage onwards, the button cells assume the shape and structure typical of migrating cells. Some pseudopodia seem to approach the cells very near the epidermis, until they reach the inner membrane of the epidermal cells (Fig. 4B). When a «button cell» is closest to the epidermal cells, the metachromatic granules become concentrated opposite to the pseudopod (Fig. 4C-D). In Fig. 4E, the cell just outside the epidermis is recognizable as a «button cell» from both its dimensions and its metachromatic reaction. The shape again becomes roundish and the metachromatic granules are again scattered in the cytoplasm. This emigration can be observed throughout the ventral epidermis, but seems to occur more frequently in the region near the palps.

In metamorphosing larvae stained by FITC-labeled anti-tubulin antibody, the button cells can be seen to approach the epidermis (Fig. 5). There is a heavy anti-tubulin reaction presumably given by microtubules at the bases of the pseudopods. In Fig. 5D, the two cells just outside the epidermis are recognizable as button cells from their marked, diffuse anti-tubulin stain. The emigrated cells do

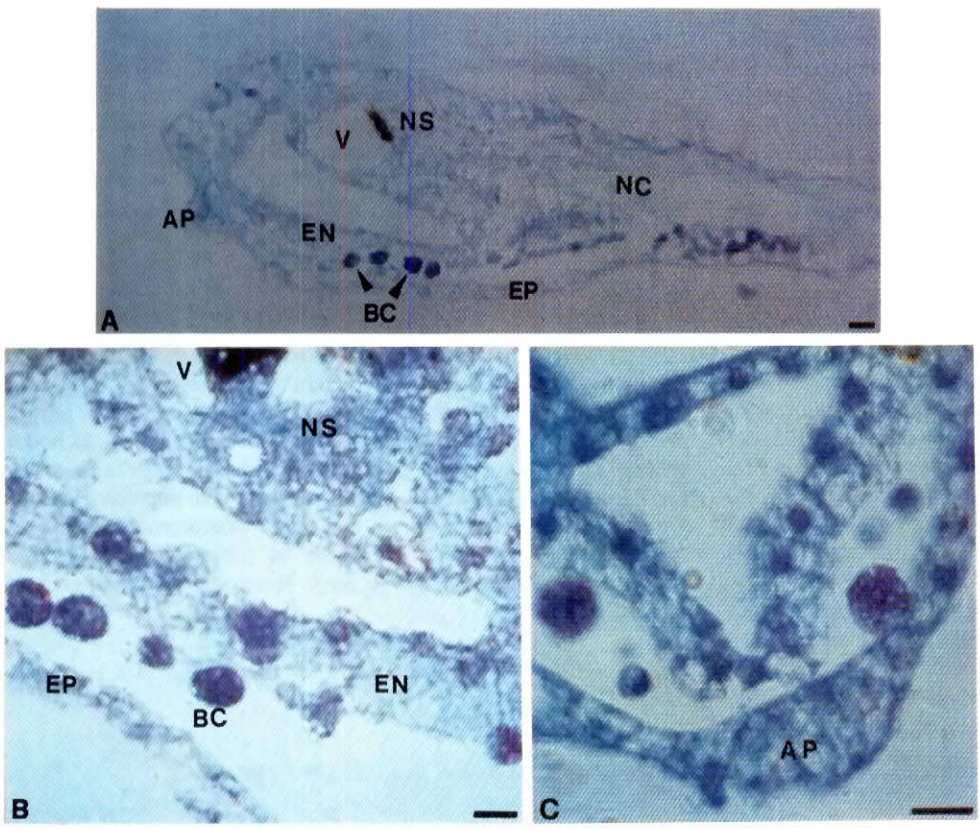

Fig. 2. Swimming larvae of *Phallusia mamillata:* sections stained by methylene blue. (A-B) *Longitudinal sections showing the metachromatic button cells (BC) in the coelomic cavity and some metachromatic cells inside the endoderm (EN).* **(C)** *Anterior cross section showing some button cells near the adhesive papillae (AP). EP, epidermis; NC, notochord; NS, nervous system; V, brain vesicle. Scale bars, 10 μm.*

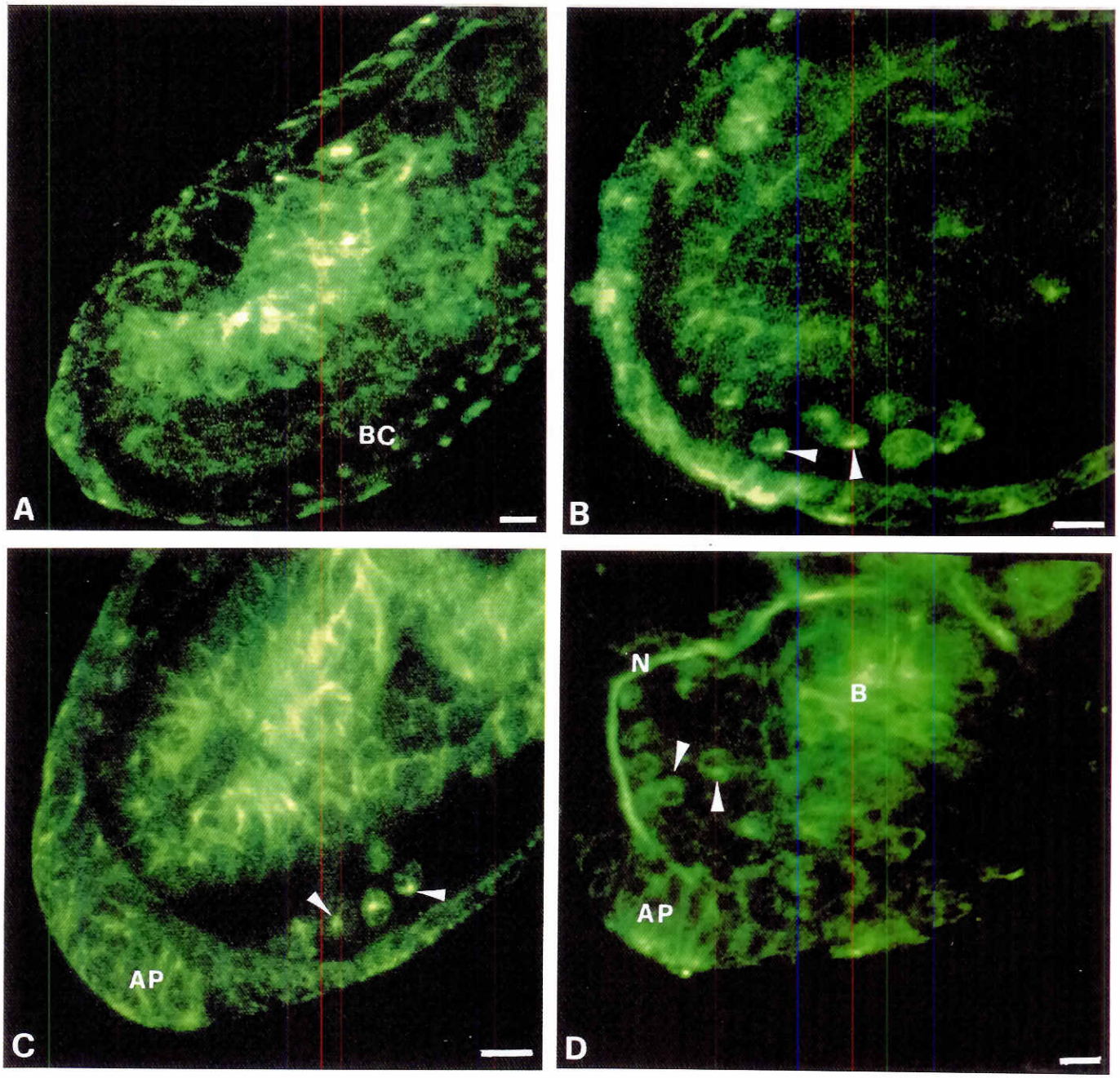

Fig. 3. Swimming larvae of *Phallusia mamillata* stained by FITC-conjugated anti-tubulin antibody and observed under the confocal microscope. **(A)** *The button cells (BC) are aligned ventrally to the pharynx, between endoderm and epidermis.* **(B)** *Higher magnification of the line of roundish button cells showing a bright anti-tubulin reaction at one pole of the cells (arrows).* **(C-D)** *Roundish button cells near the adhesive papillae (AP). B, brain; N, nerve. Scale bars, 10 μm.*

not show anti-tubulin fluorescence concentrated in the pseudopods, which have disappeared as the cells become round.

Discussion

The results presented here show that the button cells contain large quantities of tubulin distributed at one pole of the cells or near the point of cell process formation. In absence of electron microscopy evidence we can at this moment only argue on the basis of the brightness and shape of the reaction that anti-tubulin fluorescence may be concentrated in microtubules forming a cap overlaying the nucleus or in bundles of microtubules at the bases of the pseudopods. Moreover these results confirm our previous study in which we had shown, by *in situ* hybridization with tubulin cDNA, that tubulin mRNA accumulates in the button cells of the larva as well as in other cell-types (De Bernardi *et al.*, 1991). The present data show clearly that

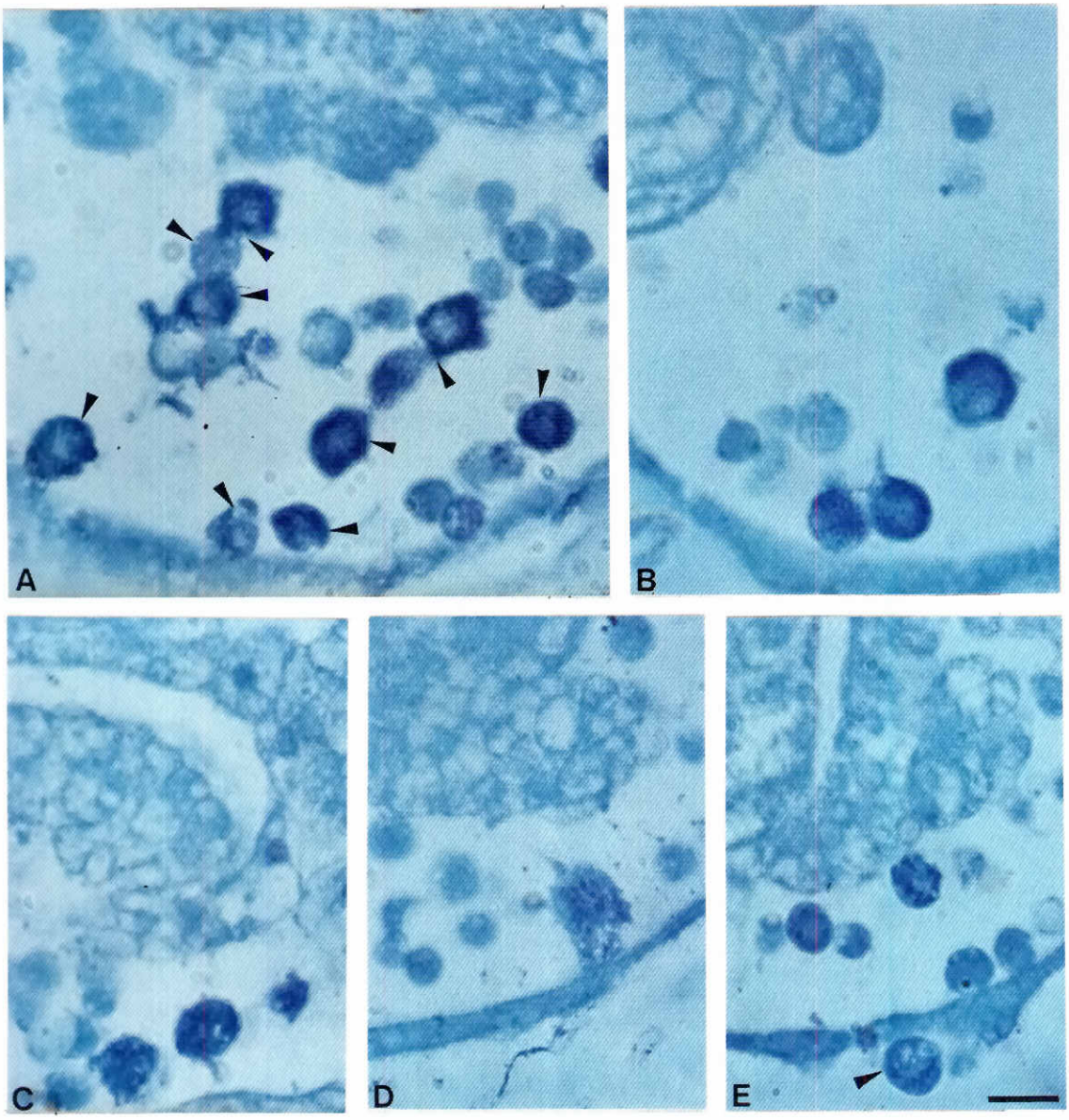

Fig. 4. Metamorphosing larvae of *Phallusia mamillata* sections stained by Nile blue sulphate. (A) *Metachromatic button cells (arrows) mixed with non-metachromatic, smaller mesenchyme cells.* **(B)** *Metachromatic button cells near the epidermis showing filopodia.* **(C)** *Button cells approaching the epidermis with their pseudopods.* **(D)** *Button cell with a less metachromatic region in close contact with epidermis.* **(E)** *Metachromatic, roundish button cell outside the epidermis (arrow). Scale bar, 10 μm.*

tubulin gene expression in these cells coincides with the prediction of a synthesis of tubulin, like in other tissues committed to dramatic modification of cell shape, such as adhesive papillae cells (see Figs. 3C and 5A).

Reverberi *et al.* (1969) reported finding button cells with different species-specific distribution in two species of Mediterranean ascidians, *Ascidia malaca* and *Phallusia mamillata*, belonging to the Ascidiae family of the Phlebobranchiata suborder.

Numakunai *et al.* (1965) have found similar cells in *Halocynthia roretzi* containing hydrolytic enzymes, located in the middle part of the trunk of the larva, and they considered these to be mesodermal. In a recent study of *Halocynthia roretzi* embryos, Nishida (1987) found a group of about 16 cells, derived from A6.3 (st. 32) and from A7.6 blastomeres (st. 64), situated laterally and posteriorly to the brain stem, which he named «trunk lateral cells» (TLC). The TLC were also stained by a specific monoclonal antibody. When the fate of TLCs after metamorphosis was followed with the TLC antibody, the antibody stained coelomic cells of juveniles. Since coelomic cells are thought to be derived from embryonic mesenchyme cells, these results suggested that TLCs are related to mesenchyme cells (Nishikata and Satoh, 1991). These cells share with our button cells the property of not dividing after the middle-tailbud stage until immediately before hatching, but they are different in position and probably in the origin. In fact, we have never observed button cells

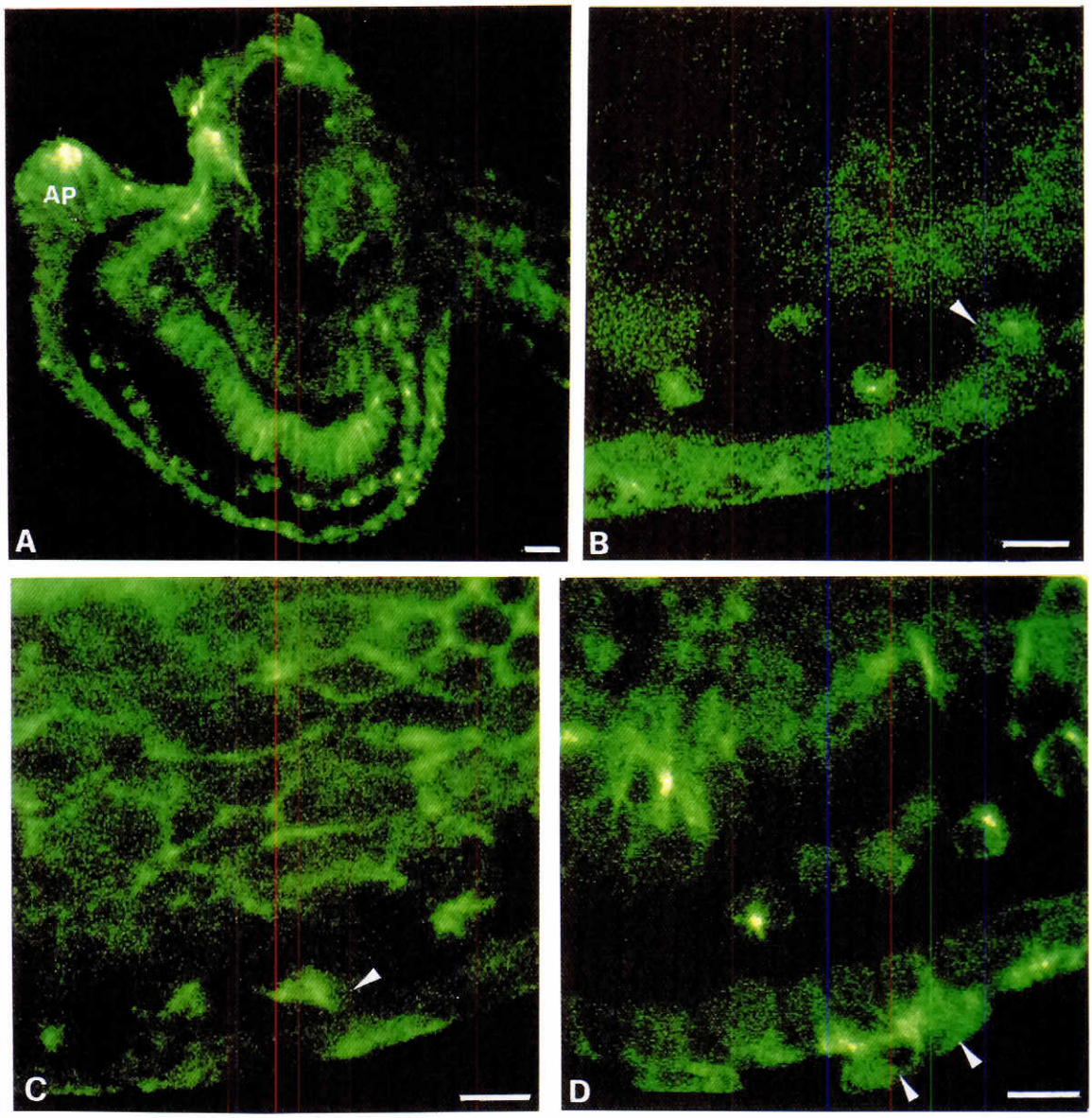

Fig. 5. Metamorphosing larvae stained by FITC-conjugated anti-tubulin antibody. (A) *The button cells are scattered and approaching the epidermis.* **(B-C)** *The bases of the pseudopods show a bright anti-tubulin reaction. Arrows mark two button cells contacting the epidermis.* **(D)** *Button cells just outside the epidermis (arrows). The anti-tubulin reaction is diffused in the cytoplasm. Scale bars, 10 μm.*

dorsally in swimming larvae, but frequently we observed mesenchyme cells in that position.

There appears to be a marked difference between different species in presence and position of button cells and these differences are more striking in non-related species. Button cells with the same stain properties have never been found in species belonging to other families. In *Ciona intestinalis*, a cosmopolitan species belonging to the Cionidae family of the same Phlebobranchiata suborder, cells similar to button cells were found containing neutral mucopolysaccharides (Materazzi, 1967). Mancuso (1986) describes the ultrastructure of mesenchymal cells of *Ciona intestinalis* as containing B vesicles, i.e., vesicles whose interiors are subdivided by dense septa of more lucent regions in which thin fibrils are seen. During metamorphosis these cells spread throughout the body cavity and some of them migrate outside the tunic, crossing the epidermal mantle and the cuticle. *Halocynthia roretzi* belongs to a different order Pleurogona, and therefore the different positions and origin of the button cells and of the TLC cells are not surprising.

Both the traditional and more recent cell lineages agree in considering that the B4.1 cells develop into posterior and ventral endoderm as well as the more anterior A4.1 (Nishida and Satoh, 1983). Perhaps the B4.1 cells do contain the material that gives rise to the button cells, but only after an inductive signal from the A4.1 cells or from their descendants. It has already been shown that

intercellular communication plays a significant role in many differentiation processes of ascidian embryos, such as that of the neural system (Reverberi *et al.*, 1960) and of the pigment cells (Ortolani *et al.*, 1979). Moreover, recent results indicate that negative cell interactions may restrict the number of cells differentiating into pigment cells in the ectoderm of cleaving ascidian embryos (Jeffery, 1993). All these results indicate evidence of the existence in ascidian embryo development of conditional mechanisms based on cell interactions, in addition to autonomous mechanisms.

The «button cells» have peculiar behavior: from a morphological point of view, they are derived from endoderm, but they migrate throughout the epidermis like mesenchyme cells do. Cloney and Grimm (1970) described the massive emigration of one type of granulocyte across the epidermis into the tunic of *Amaroucium constellatum*, adding electron microscopy evidence to some of the classical observations made in the last century about mesenchyme cells (reported by Cloney, 1978). Immediately after the beginning of tail resorption, about 2 minutes after settlement, the blood cells begin to emigrate across the epidermis into the tunic along a transcellular pathway. The area near the base of the papillae is the most favorable place to observe the phenomenon, but it may occur throughout the trunk. The pattern of emigration of the button cells might be similar to that described by Cloney and Grimm (1970), since the preliminary steps we observed are very similar: the button cells form pseudopods, the more slender of which could be better defined filopodia. Neither of them contains metachromatic granules. These pseudopods keep contact with epidermal cells and move out of them into the tunic (Ortolani and Patricolo, 1972).

These observations raise the question of the function of the button cells. Reverberi (1971) thought that they were involved in synthesizing mucopolysaccharides and lipids for construction of the tunic. Ortolani and Patricolo (1972) suggested that they were related to the process of larval transformation, since the lipofuscin pigments may be derived from lysosomes. An alternative hypothesis that we can derive from our observation is that they may be in some way involved in the exploration of the environment, perhaps to select sites for settlement. We observed that the emigration is seen particularly frequently in the region of the adhesive papillae, which are the organs specialized for contact with the environment (Cloney, 1978; Svane and Young, 1989). We observed a lot of button cells approaching the epidermis during the early steps of the metamorphosis determined by tail retraction. At present, from the data presented here we cannot say anything about the total amount of button cells sorting out from epidermis during the last steps of metamorphosis.

Materials and Methods

Histology

Eggs of *Phallusia mamillata* were obtained from oviducts of dissected animals and were collected in Millipore filtered sea water. They were fertilized with a suspension of self and non-self sperm and were allowed to develop until hatching. Larvae were collected at the swimming stage and during metamorphosis at stages judged by tail retraction. Larvae were fixed in 5% paraformaldehyde in PBS, embedded in JB 4 (Polyscience) and 2 μm sections were cut. Metachromatic staining of the button cells was obtained with 10% methylene blue or 0.1% Nile blue sulphate. Specimens were rinsed in distilled water, dried and permanently mounted in Neu-Entellan (Merck).

Immunofluorescence

Swimming and metamorphosing larvae were fixed and processed by the method of Elinson and Rowning (1988) modified by E. Houliston (personal communication). Briefly they were fixed at -20°C in methanol containing 1% formaldehyde (from a 37% formaldehyde solution) for 2 h or longer. They were gradually rehydrated to PBS, extracted for 20 minutes with 0.25% Triton X-100 in PBS, rinsed in PBS and then incubated in anti-ß-tubulin mouse monoclonal N 357 (Amersham) diluted 1:500. After repeated rinsing in 0.1% Tween-PBS, larvae were incubated with FITC-conjugated anti-mouse IgG diluted 1:50. They were then rinsed in PBS and mounted in Citifluor. The larvae were observed under a Leica confocal laser scanning microscope equipped with an argon/krypton laser by courtesy of Dr. C. Sardet at the Station Zoologique de Villefranche s/mer. 40x and 100x objectives were used and images were obtained from 8 scans of laser beam.

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