

Cell lineage and determination of cell fate in ascidian embryos

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Introduction

The comparative development of different taxa and the origin of the primary germ layers were major problems in embryology at the turn of the century. The principal technique used to address these problems was direct observation of developing embryos. Painstakingly, the early embryologists followed the history of embryonic cells, from the uncleaved egg through the daughter cells of each cleavage, and sometimes as far as the rudiments of larval tissues and organs. These observations led them to recognize that in some embryos particular cell types arose from specific regions of the egg. These results were often expressed as a fate map with the future tissues superimposed on the surface of the egg or early embryo. The clonal origin and fate of each embryonic cell can also be expressed as a cell lineage. The term "cell lineage" was first used by Wilson (1892), who observed that the mesodermal derivatives of *Nereis* embryos could be traced back to a single blastomere, the mesoblast. The observations of Conklin (1905a) on the pigmented eggs of *Styela partita* provided the first definitive ascidian cell lineage. This lineage was determined from direct observations of the fate of individual cells during embryogenesis. With the advent of modern cell tracing techniques, Conklin's cell lineage has been refined and extended. In this review, we discuss features of the classic cell lineage, recent revisions to this cell lineage, and the possible mechanisms determining cell fate in ascidian embryos.

Ascidians are protochordates whose life cycle includes motile larval and sessile adult stages. The larval stage is called a tadpole because of its superficial resemblance to an amphibian tadpole. A diagram of an ascidian tailbud-stage embryo illustrating the organization of the tadpole larva (Katz, 1983) is shown in Figure 5A-B. The larva is divided into a head and a locomotory tail. The head contains a dorsal nervous system consisting of an anterior brain with two pigmented sensory cells (the ocellus and otolith), a brain stem, and a posterior spinal cord. The head also contains undifferentiated anlagen of adult tissues including the mesenchyme cells, putative precursors of adult mesodermal derivatives, and the endodermal cells, which give rise to the adult alimentary tract. The tail contains the caudal portion of the spinal cord, a central notochord, lateral bands of muscle cells, and a ventral extension of the endoderm known as the endodermal strand. The entire larva is surrounded by a layer of epidermal cells.

After a day or less of development, the larva hatches and begins locomotion. Depending on the species, the duration of larval life varies from minutes to several days. When the larva has dispersed and a suitable attachment site is selected, it attaches to a substratum and undergoes metamorphosis. During metamorphosis, the tail is retracted into the head, the differentiated tail muscle and notochord cells are resorbed, and the larval anlagen differentiate into the tissues and organs of the sessile adult. Our knowledge of ascidian cell lineage extends only to the larval stage; no cell lineage determinations have been attempted through metamorphosis.

Cell lineage

Early cell lineage studies

Although Kowalevsky (1866; 1871) was the first to provide a detailed description of ascidian development, he made no attempt to follow the fates of the embryonic cells. Shortly thereafter, however, van Beneden and Julin (1885; 1886) examined cleavage patterns in eggs of several ascidian species and determined the history of the embryonic cells up to the 44-cell stage. Their description of the cell lineage of *Clavellina rissoana* (van Beneden and Julin, 1886) revealed a clear understanding of the relationships between early cleavage and symmetry of the embryo. Ascidians cleave bilaterally (Fig. 1) with the first and second cleavages meridional (through the animal-vegetal axis) and the third cleavage equatorial, dividing the embryo into quarters of approximately equal sized cells. After the 32-cell stage, the cleavage pattern becomes asynchronous in different parts of the embryo. Gastrulation is initiated between the 64- and 76-cell stage, and morphogenetic movements of presumptive notochord and muscle cells bring about formation of the tail following neurulation. Cells formed during cleavage show a constant relationship to the animal-vegetal axis of the egg and the subsequent bilateral symmetry of the embryo. The future dorsal region of the embryo is derived from the vegetal hemisphere of the egg, while the future ventral region of the embryo is derived from the animal hemisphere (Fig. 1C).

By the turn of the century, ascidian development had been studied extensively (Kowalevsky, 1866; van Debeden and Julin, 1885; 1886; Seeliger, 1885; Samassa, 1894). However, Castle (1896) noted that considerable differences of opinion existed concerning the derivation of the primary

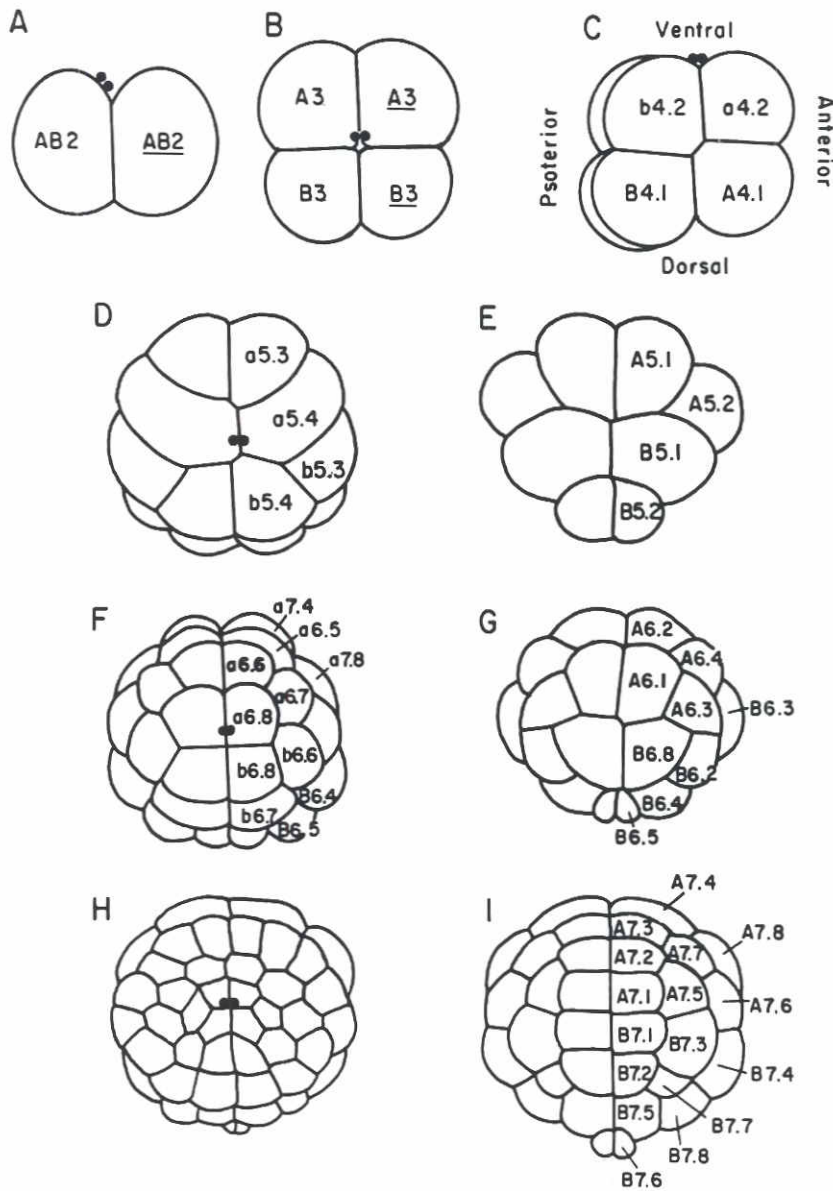


Fig. 1. Early development of the ascidian embryo showing cell lineage nomenclature. **A.** Two-cell embryo viewed from the posterior pole. **B.** Four-cell embryo viewed from the animal pole. **C.** Eight-cell embryo viewed from right side. **D-E.** Sixteen-cell embryo viewed from the animal (D) and vegetal (E) poles. **F-G.** Thirty-two-cell embryo viewed from the animal (F) and vegetal (G) poles. **H-I.** Sixty-four-cell embryo viewed from the animal (H) and vegetal (I) poles. In the 64-cell embryo only vegetal cells mentioned in the text are labeled. The cells on the right side of the embryo (C-I) are not underlined in this diagram after the 4-cell stage.

germ layers. Castle believed that this disagreement was the result of errors sustained in orienting the embryo. He examined cell fate in *Ciona intestinalis* embryos, but mistakenly identified the vegetal pole region as the future ventral side of the embryo and labeled the animal pole region as the dorsal side. His results, however, were in agreement with those of Seeliger (1885) and Samassa (1894), whose orientations of the embryo were also inverted. These inversions can be attributed to the lack of visible markers in most ascidian embryos and a shape change that occurs in animal and vegetal hemisphere cells between the 32- and 76-cell stage. At the 32-cell stage, animal pole cells are columnar, but by the 76-cell stage they flatten, and the vegetal pole cells become columnar (Conklin, 1905a). These cell shape changes were documented correctly by van Beneden and Julin, but escaped Castle, who assumed that van Beneden and Julin had inverted the embryo.

Conklin (1905a) was fortunate to have selected the ascidian *Styela partita* for his investigations. The eggs and early embryos of this species contain pigmented cytoplasmic regions which segregate into different blastomeres during cleavage so that the embryonic cells can be distinguished by color. Consequently, Conklin observed the developmental history of the embryonic cells with unprecedented clarity and constructed a detailed cell lineage up to the 64-cell stage. His observations also led to a theory of embryonic determination in which specific "organ-forming" substances localized in the egg were proposed to direct the differentiation of larval tissues after their segregation into the early blastomeres.

The nomenclature for ascidian cell lineages devised by Conklin (1905a) is still used by modern embryologists. Two letters are required for the entire cell lineage, A for the

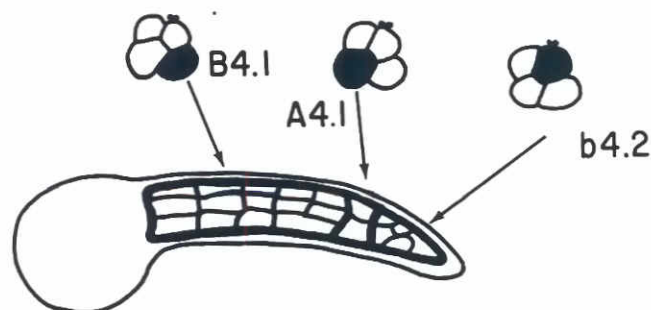


Fig. 4. A schematic diagram showing the origin of tail muscle cells of the *Halocynthia* embryo from B4.1, A4.1 and B4.2 blastomeres of the 8-cell embryo. Adapted from Nishida and Satoh (1983)

deposited in the animal hemisphere as a distinct cytoplasmic region, the ectoplasm. Following fertilization, a series of cytoplasmic rearrangements occur that result in further segregation of the ooplasm. During the first phase of ooplasmic segregation, the yellow cytoplasm and ectoplasm move toward the vegetal pole. The yellow cytoplasm eventually covers the surface of the vegetal hemisphere and the ectoplasm forms a compact zone at its periphery (Fig. 2A). The sperm nucleus migrates with these cytoplasmic regions into the vegetal region of the zygote where it forms an aster. During the second phase of ooplasmic segregation, the sperm aster extends upward along the future posterior side of the embryo accompanied by the ectoplasm and yellow cytoplasm. The yellow cytoplasm forms a sub-equatorial crescent with its center at the posterior pole and its wings extending laterally approximately halfway around the egg (Fig. 2B). This yellow crescent enters the larval tail muscle and mesenchyme cells during cleavage, and is called the myoplasm. During pronuclear fusion, the ectoplasm moves from a zone above the yellow crescent and re-enters the animal hemisphere. Throughout development the animal hemisphere of the embryo is enriched in ectoplasm, and this cytoplasmic region is incorporated into the ectodermal cells. The grey yolky cytoplasm, which was positioned centrally in the unfertilized egg, moves to the animal hemisphere during the first phase of ooplasmic segregation, and then returns to the vegetal hemisphere during the second phase of ooplasmic segregation. Conklin (1905a) observed that the grey cytoplasm consisted of two parts: a light grey chordoplasm, which is located in the future anterior region of the zygote (Fig. 2B) and enters the notochord cells during cleavage, and a dark grey endoplasm, which is located in the vegetal pole region and is distributed to the endodermal cells during cleavage. Thus, before first cleavage, the principal germ regions of the *Styela* embryo can be distinguished as 4 different cytoplasmic regions (Fig. 2B). The same cytoplasmic regions are

present in embryos of other ascidian species, but they are not as distinctly colored.

Conklin (1905a) followed the cytoplasmic regions of the *Styela* egg into different embryonic cells during early cleavages. For example, the myoplasm is located primarily in 2 cells of the 2-, 4- and 8-cell embryo (the paired AB2, B3, and B4.1 cells respectively), 4 cells of the 16-cell embryo (the paired B5.1 and B5.2 cells), and 6 cells of the 32-cell embryo (the paired B6.2, B6.3 and B6.4 cells). Conklin believed the final fate of each cell was decided by the 64-cell stage. He described the vegetal (dorsal) hemisphere of the *Styela* embryo to consist of 4 neural, 4 notochord, 10 mesenchyme, 4 muscle and 10 endodermal lineage cells and the animal (ventral) hemisphere to consist of 26 epidermal and 6 neural lineage cells. Subsequently, he reported identical cell lineages for *Ciona intestinalis* (Conklin, 1905b) and *Phallusia mammillata* (Conklin, 1911) embryos.

Refining the classic cell lineage

Conklin's (1905a) cell lineage was not significantly challenged for almost half a century. His observations were confirmed in *Asciella aspersa* embryos using vital staining methods (Tung, 1932), and an even more spectacular example of ooplasmic segregation and cytoplasmic localization was described in the brightly pigmented eggs and embryos of *Boltenia hirsuta* (Berrill, 1929). Using chalk and carbon particles to mark the surface of blastomeres, Ortolani (1955; 1959; 1962) determined the origin of the larval adhesive palps and brain sensory cells, and also found that minor details of Conklin's muscle/mesenchyme cell lineage were incorrect. According to Conklin (1905a), there are 10 presumptive mesenchyme and 4 muscle cells in the 64-cell embryo. In contrast, Ortolani identified 8 muscle (the paired B7.4, B7.5, B7.6, and B7.8 cells) and 4 mesenchyme cells (the paired B7.3 and B7.7 cells) in the 64-cell embryo. Conklin also thought that mesenchyme cells are derived from cytoplasm in the most posterior portion of the B4.1 cells. However, Ortolani (1955) showed that the posterior region of the B4.1 cells was devoted to the formation of muscle cells, while mesenchyme cells were derived from a more lateral region.

The myoplasm of *Styela* eggs contains a concentration of mitochondria which are segregated into the presumptive muscle cells (Reverberi, 1956; Berg and Humphrey, 1960; Mancuso, 1969). Reverberi (1956) vitally stained *Phallusia* eggs with the mitochondrial dye Janus green and found that the dye was gradually segregated into presumptive muscle cells, although the details of the cell lineage were not published. Using electron microscopy, however, Mancuso (1969) reassigned the fates of certain vegetal blastomeres, based on the distribution of mitochondria. Since the B7.5 cell was found to be rich in mitochondria it was assigned to the muscle cell lineage; the B7.6 cell, however, was found to be poor in mitochondria and was assigned to the mesenchyme cell lineage. These results are in contrast to those of Ortolani (1955) who assigned both cells to the muscle cell lineage but are in agreement

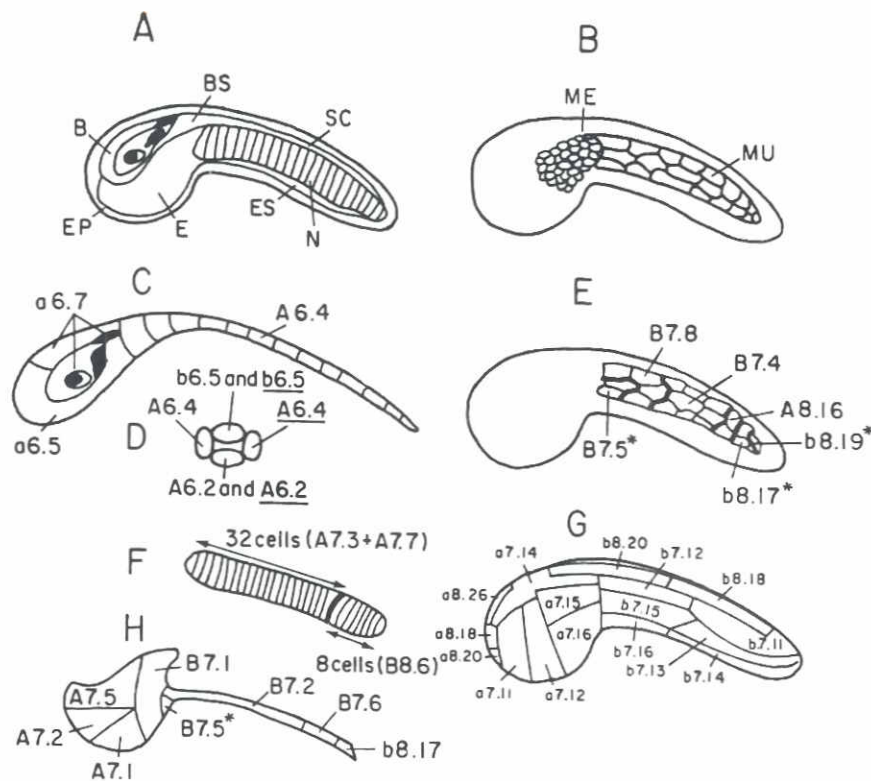


Fig. 5. Schematic diagrams of *Halocynthia roretzi* tailbud embryos illustrating the clonal origin of cells in various tissues. **A.** A mid-sagittal section through a tailbud embryo showing the position of the brain (B) with otolith and ocellus (dark spots), brain stems (BS), spinal cord (SC), notochord (N), head endoderm (E), endodermal strand (ES) and epidermis (EP). **B.** A parasagittal section through a tailbud embryo showing the position of the mesenchyme (ME) and muscle (MU) cells. **C.** Clonal organization of the brain and spinal cord, longitudinal section. **D.** Clonal origin of the spinal cord, cross section. **E.** Clonal organization of the muscle cells. Thick boundaries represent the borders of clones derived from a single precursor cell at the tissue-restricted stage. Thin boundaries represent individual cells (less than the total number of 40 is represented). **F.** Clonal organization of the notochord. Thick boundaries represent the border of clones derived from A and B blastomeres. Thin boundaries represent individual cells (less than the total number of 40 is represented). **G.** Clonal organization of the epidermis. **H.** Clonal organization of the head endoderm and endodermal strand. The asterisks indicate precursor cells that contribute to two or more tissues. Adapted from Nishida and Satoh (1983) and Nishida (1987).

with Conklin's original cell lineage. This confusion over the derivatives of the B7.5 and B7.6 cells has been resolved by recent cell lineage analyses using intracellular markers (see below).

Revising the classic cell lineage

Recent experiments carried out by Nishida and Satoh (1983; 1985), Nishida (1987) and Zalokar and Sardet (1984) have used intracellular marking to investigate ascidian cell lineage; others (Satoh, 1979; Nishida, 1986; Nicol and Meinerzhagen, 1988a, b) have used scanning electron microscopy (SEM). These studies have resulted in further revisions and extensions to Conklin's original cell lineage. The most important changes are discussed below, and Figure 3 shows the current *Halocynthia roretzi* cell lineage.

Muscle cell lineage

According to Conklin (1905a), larval muscle cells are derived exclusively from B cells of the 8-cell embryo. However, it has now been shown that descendants of the b and A cells also contribute to larval muscle.

Using the fluorescent dye 3,3'-diethyloxycarbocyanine (DiOC₂), a vital stain for mitochondria, Zalokar and Sardet (1984) traced the muscle cell lineage up to the 180-cell stage in *Phallusia* embryos. Although every cell originally described as a presumptive muscle cell by Conklin was stained with DiOC₂, other cells were fluorescent. One fluo-

rescent pair that was suspected to be part of the muscle cell lineage is b8.17. This cell is located in the animal hemisphere and its progeny became associated with the other muscle cell precursors during gastrulation. Zalokar and Sardet believe that the contribution of b8.17 to larval tail muscle was observed earlier by Mancuso (1969), but that this cell was mislabeled as B8.8. Strong fluorescence was also observed in A6.4. This cell is located in the neural plate on the vegetal side of the embryo and had previously been assigned to the neural lineage (Ortolani, 1962).

By microinjecting horseradish peroxidase (HRP) into *Halocynthia roretzi*, *Ciona intestinalis*, and *Ascidia ahodori* embryos and examining the subsequent distribution of the enzyme in presumptive tissues at the tailbud stage, Nishida and Satoh (1983; 1985) also concluded that ascidian muscle cells have multiple origins. Their initial results, in which blastomeres up to the 8-cell stage were injected, showed that B, A and b cells all have progeny that form tail muscle cells. Subsequent studies, in which blastomeres of 16- to 32-cell embryos were injected, showed that B cells contribute muscle cells in the anterior and middle regions of the tail, A cells contribute muscle cells in the posterior region of the tail (via their A5.2 and A6.4 progeny), and b cells contribute muscle cells at the caudal tip of the tail (via their b5.3 and b6.5)(Fig. 4). These results are in agreement with those of Zalokar and Sardet (1984) who assigned A6.4 and b8.17 (a derivative of b6.5 progeny) to the muscle cell lineage. Nishida (1987) has continued this work by microinjecting blastomeres of 64-cell and later *Halocynthia*

embryos with HRP. These studies have shown that b8.19 (another derivative of b6.5) is also a muscle cell precursor. Nicol and Meinertzhagen (1988a, b), who have studied the cell lineage of the neural plate by SEM, confirm most of the results of Nishida and Satoh, but there is still uncertainty from their studies concerning the identity of some of the progeny arising from the A cells. However, it is clear from three independent kinds of cell lineage analyses that muscle cells arise from A and b cells as well as B cells. The clonal origin of tail muscle cells is summarized in Figure 5E.

The number of tail muscle cells varies in different ascidian species. In species with large larvae (many compound ascidians), the number of tail muscle cells is often doubled or tripled. This increase in cell number has been attributed to extra divisions of the B cells in the bulk of the tail musculature (Berrill, 1945), but this question is deserving of re-examination using modern cell lineage tracing methods. In those species having smaller larvae (*Styela*, *Ciona* and *Halocynthia*), however, it is evident that a few muscle cells are added at the tip of the tail by increasing the contribution of b cells (Nishida and Satoh, 1983; 1985). For instance, *Ciona intestinalis* and *Ascidia ahordori* tadpoles have a total of 36 tail muscle cells, 18 on each side, while *Halocynthia roretzi* has a total of 42 cells, 21 on each side of the tail. In these species, 14 of the tail muscle cells on each side of the tadpole are derived from the B cells, and 2 muscle cells are provided by A cells. In *Halocynthia*, 5 b cells become muscle cells at the caudal tip of the tail (Fig. 4E), whereas in *Ciona* and *Ascidia* only two of these become caudal muscle cells. Interestingly, somites at the caudal tip of the tail of urodele amphibians also appear to be derived secondarily from cells located in the neural plate (Woodland and Jones, 1988). The recruitment of ectodermal cells into muscle tissue may be a general mechanism used to extend the tail in chordates.

These results suggest that ascidian muscle cells are derived from three different quadrants of the 8-cell ascidian embryo. The muscle cells that arise from the B.4.1 cells are designated primary muscle cells, and those that arise from the A4.1 and b4.2 cells are designated secondary muscle cells. The adult muscle cells, whose origin remains to be determined, are designated tertiary muscle cells.

Endodermal cell lineage

The results of Conklin (1905a) suggested that endodermal cells arise from A and B cells in the vegetal hemisphere. The recent HRP microinjection studies (Nishida and Satoh, 1983; 1985; Nishida, 1987) have confirmed this assignment and revealed the following additional details. The derivatives of B7.5 and B7.6 have always been questionable (see above), but were formerly assigned to either muscle or mesenchyme cells. It is now known that B7.5 gives rise to muscle and endoderm, while B7.6 gives rise to part of the endodermal strand. The remainder of the endodermal strand is formed by derivatives of B7.2 and b8.17, with the latter also contributing to muscle or in some

instances (see below) to spinal cord. The clonal origin of the endodermal cells is summarized in Figure 5H.

Notochord cell lineage

It was assumed previously that all notochord cells originate from A blastomeres (Conklin, 1905a). However, HRP microinjection (Nishida and Satoh, 1983; 1985; Nishida, 1987) shows that B cells also contribute to the notochord. In *Halocynthia roretzi*, there are a total of 40 notochord cells; the anterior 32 cells arise from the derivatives of A7.3 and A7.7, while the posterior 8 cells are formed from the derivatives of B8.6. Interestingly, notochord cells located at the caudal tip of the tail in *Ciona* and *Styela* larvae express alkaline phosphatase, an enzyme otherwise restricted to the endodermal cell lineage (Whittaker, 1977; Bates and Jeffery, 1987a). These caudal notochord cells may have originated from endodermal cells (Crowther and Whittaker, 1986) and thus may represent another instance of cell lineage conversion during evolution. The clonal origin of the notochord is shown in Figure 5F.

The notochord consists of a single row of disc-shaped cells. An interesting phenomenon has been noted by injecting HRP into either the right (A3) or left (A3) blastomeres of 4-cell embryos and examining the distribution of the enzyme in notochord cells at the tailbud stage

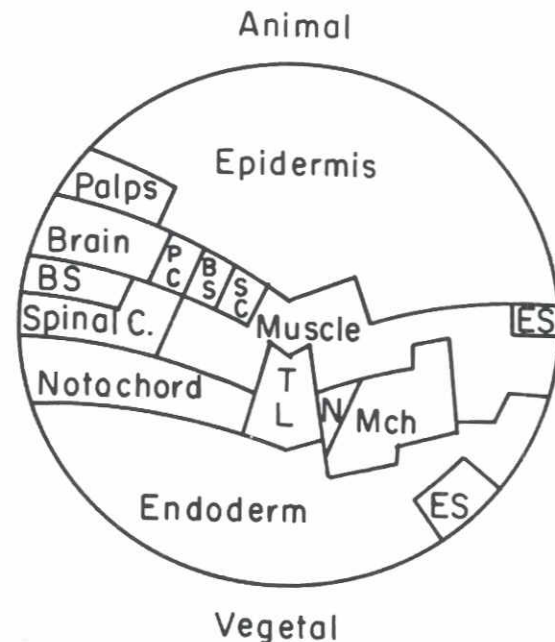


Fig. 6. A lateral view of the *Halocynthia blastula* fate map. BS: brain stem. ES: endodermal strand. Mch: mesenchyme. N: notochord. PC: pigmented sensory cells. TL: lateral trunk cells. The anterior pole is on the left and the posterior pole is on the right. From Nishida (1987).

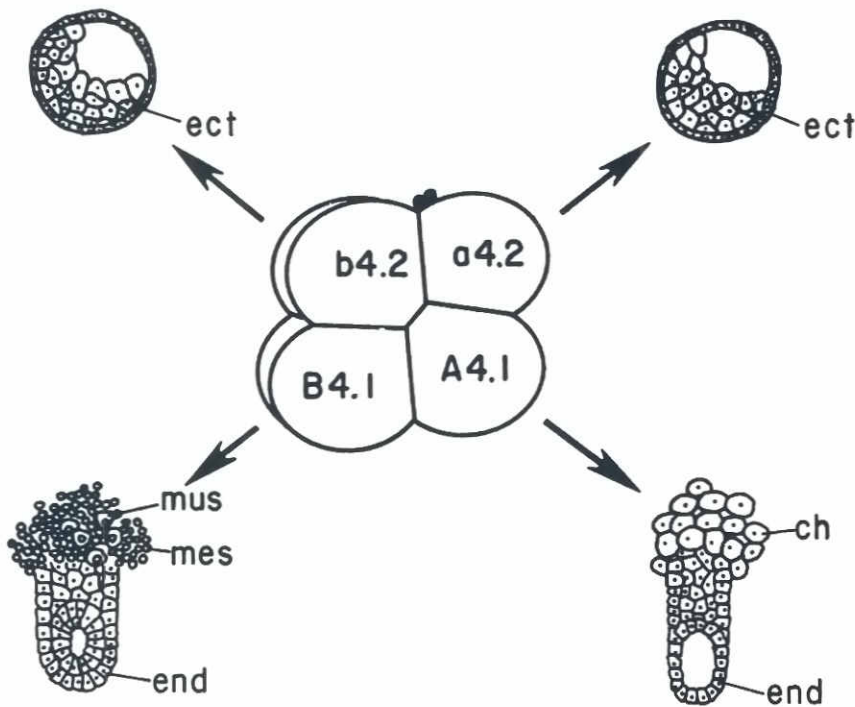


Fig. 7. A schematic diagram illustrating the derivatives of partial embryos obtained from a4.2, b4.2, A4.1 and B4.1 blastomere pairs of the 8-cell embryo. ect: ectoderm. mus: muscle cells. mes: mesenchyme cells. end: endoderm. ch: notochord. Adapted from Reverberi and Minganti (1946).

(Nishida and Satoh, 1983). As expected, derivatives of both cells contribute progeny to the notochord. Surprisingly, however, the cells derived from A3 and A3 do not always appear in a regular alternating sequence in this structure. Similarly, two of the b8.17-derived muscle cells may exchange their positions from right to left, and *vice-versa*. These results suggest that presumptive notochord and caudal tip muscle cells from either side of the embryo can mix randomly at the midline without affecting the morphogenetic processes associated with tail formation.

Neural cell lineage

Previously it was reported that the neural cell lineage is derived exclusively from a and A cells (Conklin, 1905a; Ortolani, 1962). HRP microinjection (Nishida and Satoh, 1985; Nishida, 1987) and SEM analysis (Nicol and Meinertzhagen, 1988a, b) confirm this assignment, but also demonstrate that b cells make a minor contribution to the neural cell lineage. The brain is derived from a6.5 and a6.7 (Ortolani, 1962), but the brain stem is derived not only from A6.2 but also from progeny of the b6.5 cell, which forms the roof of this structure. A detailed analysis of the neural plate cell lineage can be found in Nicol and Meinertzhagen (1988a, b). The clonal organization of larval neural tissue is shown in Figure 5C-D.

Cell lineage analyses indicate that cell fate is usually identical on either side of the bilaterally symmetric embryo. An exception to this is the development of the otolith and ocellus, the pigmented sensory cells in the larval brain.

Nishida and Satoh (1985) report that the otolith and ocellus are derived from either the a6.7 or the a6.7 blastomere in a complementary manner. Thus, if the otolith is formed from a6.7, then the ocellus forms from a6.7, and *vice-versa*. Nishida (1987) and Nishida and Satoh (1989) have confirmed this result, showing that these sensory cells are formed from the 8.25 and a8.25 cells of the 110-cell embryo in the same complementary fashion. A similar relationship was found at the caudal tip of the larval tail where derivatives of the b8.17 or b8.17 cells form either spinal cord or endodermal strand in a complementary manner (Nishida, 1987). These results suggest that the sensory cell precursors are members of an equivalence group: sibling cells that assume different fates through hierarchical interaction. Equivalence groups have been identified previously in *Caenorhabditis elegans* (Sulston and White, 1980), leech (Shankland and Weisblat, 1984), and grasshopper (Kuwada and Goodman, 1985) embryos.

Epidermal cell lineage

Epidermal cells were fate mapped to a and b cells of the animal hemisphere by Conklin (1905a). This general assignment has been confirmed by more recent cell lineage determinations. Minor differences in the assignments of the ectodermal cells, however, have been obtained by using different cell lineage tracing methods. The larval adhesive palps were described previously as having been derived from the a7.11 blastomere in *Phallusia*, *Ascidia* and *Ascidella* embryos (Ortolani, 1962). HRP microinjection suggests that these palps are derived instead from the

a8.18 and a8.20 cells (descendants of a7.9 and a7.10 respectively) in *Halocynthia* embryos (Nishida, 1987). At present, this discrepancy cannot be explained, but there may be variation in the cell lineages that form palps in different ascidian species. The clonal organization of the epidermis is shown in Figure 5G.

Restriction of cell fate during embryogenesis

Conklin (1905a) thought that the fate of each blastomere was decided as early as the 64-cell stage. In contrast, Nishida and Satoh (1985) found that many of the blastomeres of the 32-cell stage *Halocynthia* embryo form at least three different kinds of tissue, indicating that all blastomeres are not restricted to form a single tissue type at the 64-cell stage. Nishida (1987) traced the fates of different embryonic cells to the stage at which they generate a single tissue type. He found that there are 2 tissue-restricted blastomeres at the 16-cell stage, 12 at the 32-cell stage, 44 at the 64-cell stage, and 94 at the 110-cell stage. Nicol and Meinertzhagen (1988a,b) also found that *Ciona* embryos show mixed cell fates beyond the 64-cell stage. The cell lineage shown in Figure 3 illustrates the stage at which various blastomeres become tissue restricted. Based on these findings, Nishida (1987) devised a fate map for the *Halocynthia roretzi* embryo (Fig. 6). The fate map shows that most of the animal hemisphere gives rise to epidermis, the marginal zone gives rise to ectodermal and mesodermal derivatives, and the vegetal hemisphere contains a central area that becomes endoderm, a posterior zone that gives rise to mesodermal derivatives and an anterior zone which contributes to ectodermal derivatives.

Determination of cell fate

Cell lineages are indicative of cell fate *in situ*, but do not provide information about the developmental potential of embryonic cells. Experimental manipulations are required to determine if cells are developmentally restricted or are potentially able to express other fates. The invariant cell lineage of ascidians has been attributed to the inheritance of localized cytoplasmic determinants by specific blastomeres. Our current understanding suggests that while cell fate specification appears to involve the activity of cytoplasmic determinants in some lineages, cell interactions are required in others.

Mosaic development

The first experimental studies on ascidian development were designed to determine if these organisms were mosaic or regulative. The procedure used to assign a species to one of these categories was to destroy or remove specific blastomeres and determine how this affects the overall development of the embryo. If embryos missing blastomeres form incomplete larvae or if isolated blastomeres develop only into the derivatives expected from the cell lineage, then development is considered mosaic. Alternatively, if partial embryos or isolated blastomeres compensate and produce more than the structures expect-

ed from the cell lineage, then development is considered regulative.

After killing one blastomere of the *Ascidella aspersa* embryo at the 2-cell stage, Chabry (1887) found that the remaining blastomere continued to develop as if it were part of the whole embryo and eventually formed a half larva. Similarly, destruction of a blastomere in the 4-cell embryo led to the development of an incomplete larva. Thus, Chabry concluded that ascidian embryos do not compensate for missing parts, and are mosaic. This was the first demonstration of mosaic development in any species, having been published a year before Roux's classic experiments on amphibian embryos (Roux, 1888). Chabry's results were criticized by Driesch (1895), who deleted blastomeres of 2- and 4-cell *Phallusia* embryos and described the partial embryos as cleaving normally, gastrulating, and forming complete larvae. Chabry's half larvae were interpreted by Driesch as incomplete larvae lacking only minor features. Thus, Driesch concluded that ascidian embryos could compensate for missing parts, and are regulative.

In an attempt to resolve these contradictory results, Crampton (1897) examined the development of partial embryos of *Molgula manhattensis*. He agreed that partial embryos cleave as if the blastomeres are still part of the intact embryo, but also concluded that missing parts are gradually supplied by the cells that are already present. Of particular importance in these studies is the question of what constitutes a complete larva. While Driesch and Crampton both stated that nearly complete larvae were formed during their experiments, they provided no compelling evidence that development was normal, except that the larvae possessed a head and a tail. Neither investigator examined the internal structure of the larvae. Conklin (1905c; 1906; 1911) conducted similar experiments with embryos of *Styela partita* and *Phallusia mammillata*. He concluded that while partial embryos sometimes develop into larvae that appear to be complete superficially, they always lack the tissues or organs normally produced by the missing blastomeres. Thus, Conklin concluded that ascidian embryos were mosaic.

Subsequent studies on partial embryos and isolated blastomeres (also see below) have confirmed the mosaic development of ascidian embryos. For instance, Berrill (1932) and Cohen and Berrill (1936) carefully isolated individual blastomeres from *Ascidella* embryos. By counting the number of notochord and muscle cells that developed in larvae obtained from these embryos, they demonstrated that only half larvae develop from isolated AB2 blastomeres of 2-cell embryos. What had appeared as a complete complement of muscle cells to earlier investigators, was actually a rearrangement of muscle cells from one side of the embryo to fill the space normally occupied by muscle cells from the other side. Significantly, half-larvae are able to undergo metamorphosis and develop into complete functional adults (Nakauchi and Takeshita, 1983). Thus, development of ascidian larval structures is mosaic, but adult development is regulatory.

Autonomous development

Once it was established that ascidian embryos are mosaic, embryologists focused on understanding the mechanisms that determine cell fate. Development is said to be autonomous when isolated blastomeres develop as they would in the intact embryo. Autonomy implies that cell fate can be established without the intercession of other cell types and involves intrinsic factors: cytoplasmic determinants. When isolated blastomeres do not develop as they would in the intact embryo, development is said to be non-autonomous. Lack of autonomy suggests that cell fate depends on extrinsic factors, probably requiring cell interactions (although other interpretations are also possible). A major question that remained to be established was whether the cell types of ascidian embryos develop autonomously.

Reverberi and Minganti (1946) examined cell autonomy in ascidian embryos by determining the ability of a4.2, b4.2, A4.1, and B4.1 blastomere pairs isolated from 8-cell *Ciona* embryos to form various larval tissues (Fig. 7). As expected from previous studies, none of these blastomeres developed into complete larvae. Instead, each blastomere divided and usually formed only the tissues expected from Conklin's cell lineage, including muscle, mesenchyme, notochord, endoderm and ectoderm. It was concluded that each of these tissues develops autonomously, and may be specified by cytoplasmic determinants. However, while the cell lineage predicted that neural tissue would develop from the a4.2 and A4.1 cells (Figs. 2 and 4), none of the isolated blastomeres developed a brain or sensory cells. Therefore, Reverberi and Minganti concluded that neural tissue does not show autonomy and may require inductive cell interactions. Subsequently, they and others have provided more direct evidence that brain development requires induction (see below).

The pioneering studies of Reverberi and Minganti (1946) have been confirmed and extended in recent years using molecular and ultrastructural markers for the various cell types. Notochord cells can be identified ultrastructurally by specific vacuolar inclusions and extracellular specializations; these structures developed only in the progeny of isolated B4.1 and A4.1 blastomeres (Crowther and Whittaker, 1986), as predicted by the revised cell lineage (Fig. 3). Similarly, epidermal cells can be identified by specific monoclonal antibodies (Mita-Miyazawa *et al.*, 1987), and epidermal antigens develop exclusively in isolated a4.2 and b4.2 blastomere pairs of 8-cell *Halocynthia* embryos (Nishikata *et al.*, 1987b). Thus, in contrast to neural tissue, notochord and epidermal cells appear to be specified autonomously, possibly by cytoplasmic determinants.

The development of muscle cells is more complex. These cells can be identified histochemically by the presence of acetylcholinesterase (AChE), an enzyme that is produced almost exclusively in the tail muscle cells of ascidian embryos (Durante, 1956); ultrastructurally by the presence of myofilaments and myofibrils (Crowther and

Whittaker, 1983); and by specific monoclonal antibody and cloned gene probes (Nishikata *et al.*, 1987a; Tomlinson *et al.*, 1987; Makabe and Satoh, 1989). Blastomere isolation studies with *Ciona* embryos have shown that AChE or myofilaments develop in isolated B4.1 blastomere pairs, but not in three-quarter embryos lacking the B4.1 cells (Whittaker *et al.*, 1977; Whittaker, 1982; Crowther and Whittaker, 1983). These results are supported by cleavage arrest experiments (Whittaker, 1973; Crowther and Whittaker, 1984), in which embryos arrested at various cleavage stages by treatment with the microfilament inhibitor cytochalasin developed AChE or myofilaments only in the maximal number of B line blastomeres expected from Conklin's cell lineage. These results suggest that only primary muscle cells develop muscle cell features autonomously.

In contrast to the results of Whittaker and colleagues, Reverberi and Minganti (1946) sometimes found muscle cells in three-quarter embryos lacking B cells (not shown in Fig. 7). Similar results had previously been reported by Von Ubisch (1939), who destroyed the three pairs of B line cells (B6.2, B6.3 and B6.4) in 32-cell embryos and observed muscle cells in the resulting larvae. More recently, Deno *et al.* (1985) have shown that partial *Ciona* embryos from which B4.1 cells are removed at the 8-cell stage produce AChE and myofibrils in almost all cases. These results support the idea that B cells are not the sole source of muscle cells in ascidian embryos. The important question of secondary muscle cell autonomy will be considered further below.

Cytoplasmic determinants

The early blastomere deletion and isolation studies highlighted the importance of cytoplasmic determinants in specifying cell fate in ascidian embryos, especially in the primary muscle cells. Cytoplasmic redistribution and transfer experiments have provided further evidence for the existence of cytoplasmic determinants in these cells. Morgan (1910) conducted the first cytoplasmic redistribution experiments in ascidian embryos. He investigated the effects of altering the position of the cleavage planes by compressing *Ciona* embryos. As shown in Figure 1C, the third cleavage of ascidian embryos is equatorial, distributing myoplasm mainly into the B4.1 cells of the 8-cell embryo. If embryos are compressed perpendicular to the animal-vegetal axis, however, the third cleavage can be made meridional instead of equatorial and myoplasm can be distributed to as many as 4 cells of the 8-cell embryo. Morgan found that compressed embryos develop abnormally, do not gastrulate, and sometimes show duplication of certain organs. These experiments were repeated and extended by Whittaker (1980), who compressed *Styela plicata* embryos at third cleavage causing myoplasm to be partitioned to 3 or 4 cells. Whittaker then cleavage arrested the compressed 8-cell embryos with cytochalasin and determined the number of cells that express AChE. As discussed previously, a maximum of 2 cells normally express AChE in 8-cell cleavage-arrested embryos. By

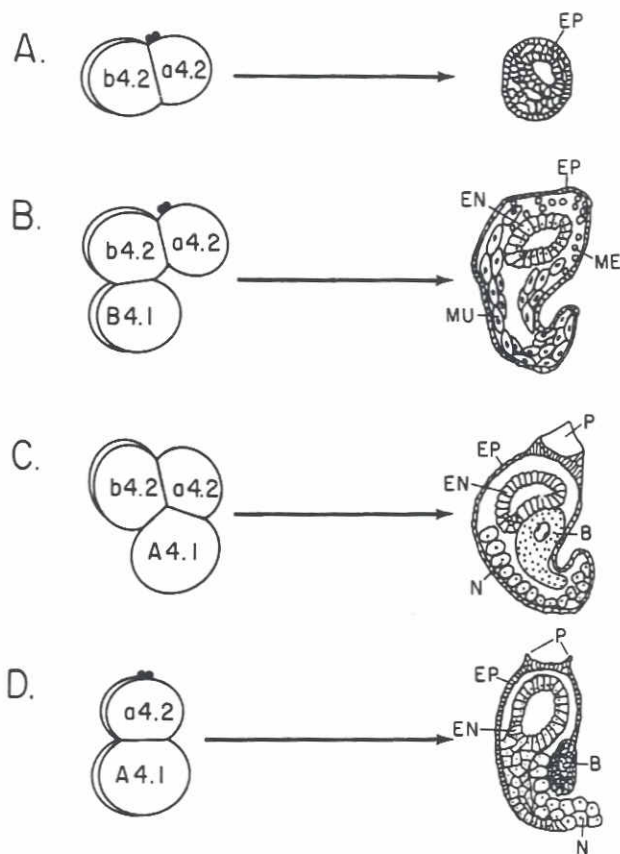


Fig. 8. Microsurgical experiments that provide evidence that inductive interactions are required for brain to form from the *a* cell derivatives in ascidian embryos. **A.** Animal half-embryos (*a4.2* and *b4.2* cells) develop epidermis (EP). **B.** Three-quarter embryos lacking *A4.1* cells develop muscle (MU), mesenchyme (ME), epidermis (EP), and endoderm (EN). **C.** Three-quarter embryos lacking *B4.1* cells develop epidermis (EP), endoderm (EN), notochord (N), palps (P), and brain (B). **D.** Anterior half-embryos (*a4.2* and *A4.1*) develop palps (P), epidermis (EP), endoderm (EN), notochord (N) and brain (B). Adapted from Reverberi and Minganti (1946).

examining the distribution of the yellow cytoplasm in compressed embryos, it was shown that the extra cells expressing AChE were those that received myoplasm. In a related series of experiments, the position of the third cleavage furrow of *Ascidia nigra* embryos was altered with a micro-needle so that part of the myoplasm from a *B4.1* cell was moved to a *b4.2* cell (Whittaker, 1982). Subsequently, *b4.2* cells in some of the operated embryos produced AChE. Finally, Deno and Satoh (1984) have shown that AChE expression can be stimulated in a small proportion of *A4.1* cells of 8-cell *Halocynthia* embryos microinjected with cytoplasm from *B4.1* cells. These cytoplasmic redistribution and transfer experiments have important implications for the control of muscle cell development. First, since nuclear assignments are not changed during compression,

the factors responsible for specifying muscle cells must be cytoplasmic. Second, the results are consistent with (but do not prove) the possibility that muscle cell determinants are localized in the myoplasm. Third, since extra cells are stimulated to produce AChE, these muscle-determining factors must act in a positive fashion. However, the following caveats must be considered. Since it is now known that the *A* and *b* cells contribute secondary muscle cells to the tadpole tail, these experiments would be more meaningful if *a4.2* cells of the 8-cell embryo were employed as recipients of myoplasm, for they have no progeny that normally develop into muscle cells. Furthermore, in some cases, redistribution of myoplasm to other cells did not cause notable effects on development. For example, Bates (1988) redistributed myoplasm to non-*B4.1* cells of 8-cell *Styela* embryos, and the larvae did not develop muscle cell features in atypical locations. The relatively low levels of response obtained in myoplasm transfer experiments may be explained if sub threshold levels of myoplasm are transferred.

Cytoplasmic determinants have classically been thought to act at the level of cell fate specification in ascidian embryos. Bates and Jeffery (1987b), however, have shown by microsurgery that these factors may also be involved in specifying the site of gastrulation and dorsoventral axis determination. In these experiments, small defined regions of cytoplasm were deleted from *Styela* eggs before fertilization or during the first or second phase of ooplasmic segregation following fertilization. Specific defects in development were obtained only when cytoplasm was deleted from the vegetal pole between the first and the second phases of ooplasmic segregation. Zygotes lacking the vegetal pole region cleaved normally and developed markers for muscle and endodermal cells, but did not gastrulate or develop dorsoventral axes. Based on these results, Bates and Jeffery concluded that axial determinants may be localized transiently at the vegetal pole of the uncleaved zygote. Since this region was fate mapped to four endodermal cells (Bates and Jeffery, 1987b), which are the first cells to invaginate during gastrulation, these determinants are thought to define the site of gastrulation.

Although there is strong evidence that ascidian eggs contain cytoplasmic determinants, these factors have yet to be characterized, and their manner of localization and mode of action remain to be elucidated. Centrifugation experiments with *Styela partita* embryos (Conklin, 1931), in which pigment granules and associated mitochondria were displaced from the myoplasm into atypical regions of the egg without affecting subsequent muscle cell development, demonstrate that these organelles are not determinants. Cytoplasmic determinants are more likely to be macromolecules, such as RNAs or proteins, which are localized in one of the unique cytoplasmic regions of the egg and segregated into specific blastomeres during cleavage.

Maternal mRNA is a popular candidate for a cytoplasmic determinant (Davidson and Britten 1972; Jeffery, 1983) and

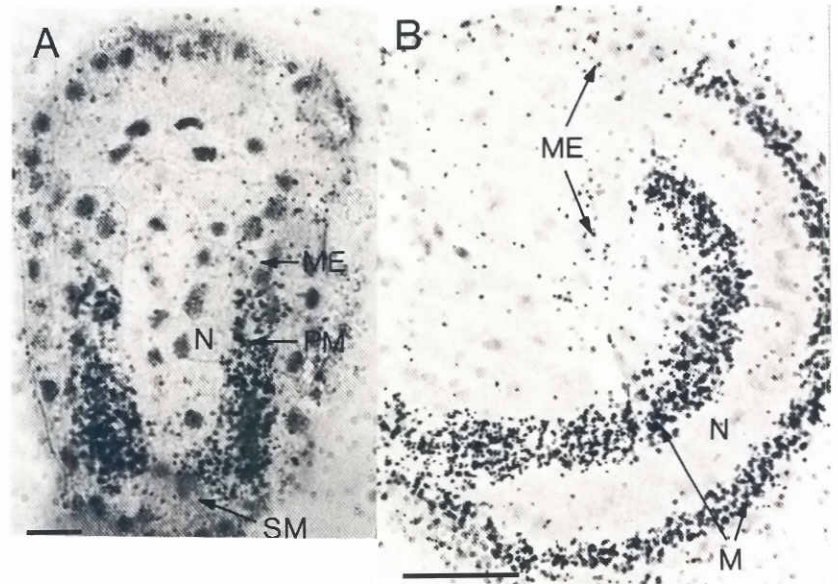


Fig. 9. Differential expression of the muscle actin gene in primary and secondary muscle cells of *Styela* embryos as determined by in situ hybridization with a cloned muscle actin probe. A. Early tailbud embryo showing muscle actin transcripts in primary (P) but not secondary (S) muscle cells. B. Late tailbud embryo showing muscle actin transcripts in primary and secondary muscle cells. The magnification bars represent 10 μm . From Tomlinson et al. (1987).

may be localized in the egg cytoplasm in a non-translatable form that is translationally activated only after segregation into specific cell lineages. Translation products could function to promote gene expression or act directly as determinants of differentiated cell types. Current investigations are concerned with determining whether pre-formed mRNAs corresponding to tissue-specific proteins pre-exist in eggs or if their production requires gene expression during embryogenesis.

The role of embryonic gene activity in muscle AChE development has been determined by treatment of embryos with transcriptional inhibitors, by examining anucleate egg fragments, and by monitoring the level of AChE mRNA at various stages of development. AChE is not produced in embryos treated with transcriptional inhibitors (Whittaker, 1973) or in anucleate egg fragments (Bates and Jeffery, 1987a) suggesting that enzyme production requires new gene expression. Determination of the level of AChE mRNA during *Ciona* development (Meedel and Whittaker, 1983) has shown that translatable AChE mRNA is first detected in early gastrulae and continues to be present between the early gastrula and tailbud stages. These results suggest that pre-formed AChE mRNA is not present in the egg and that embryonic gene expression is required for the production of this enzyme. If an AChE determinant exists, then it is not maternal mRNA coding for this enzyme, but rather a factor that promotes AChE gene expression in the muscle cell lineage.

In contrast to AChE, the production of alkaline phosphatase (AP) in the endodermal cell lineage is independent of new transcription. AP is synthesized principally in the endodermal cells beginning at about the time of gastrulation (Minganti, 1954; Whittaker, 1977). The development of this

enzyme appears to be specified by a cytoplasmic determinant localized in the endoplasm and segregated to endodermal cells. The appearance of AP is not affected by treating embryos with transcriptional inhibitors (Whittaker, 1977; Bates and Jeffery, 1987a) and is presumed to be specified by a pre-formed mRNA that is localized in the egg. However, AP does not develop in anucleate egg fragments (Bates and Jeffery, 1987a) suggesting that some sort of nuclear activity, perhaps DNA replication (Satoh, 1982), may be required for the expression of this enzyme.

Jeffery and Capco (1978) and Jeffery *et al.* (1983) have tested for localized maternal mRNAs in *Styela* eggs by *in situ* hybridization with poly (U) and cloned probes. They found that localizations of total and specific mRNAs occur in these eggs. While most of the maternal mRNA is localized in the ectoplasm, actin mRNA is enriched in the myoplasm. If other localized mRNAs exist in the myoplasm, they must be rare species since analysis of the mRNA composition of yellow crescents isolated from *Styela* embryos indicates that there are no major differences in the abundant messages present in this region compared to the remainder of the egg (Jeffery, 1985).

Messenger RNA localization in the myoplasm and other cytoplasmic regions appears to be mediated by the binding of these molecules to specialized domains of the egg cytoskeleton. Jeffery (1984) showed that mRNA is localized to the same regions of intact *Styela* eggs and eggs extracted with detergent. In addition, the myoplasm contains a unique cytoskeletal domain consisting of a peripheral layer of actin filaments closely associated with the plasma membrane, and a deeper zone of thicker filaments, perhaps intermediate filaments, associated with pigment granules and mitochondria (Jeffery and Meier,

1983). The different parts of the myoplasmic cytoskeletal domain co-migrate during ooplasmic segregation, due to contraction of the peripheral actin filament network (Sawada and Osanai, 1981; 1986; Jeffery and Meier, 1984; Sawada and Schatten, 1989), and are segregated together into the B cells. It is possible that the myoplasmic cytoskeletal domain localizes determinants and thereby establishes the fate of the primary muscle cells.

Inductive cell interactions

Brain sensory cells

Although brain sensory cells are derived from the a line blastomeres (Fig. 3), isolated a4.2 blastomere pairs from 8-cell embryos produce neither ocellus nor otolith (Reverberi and Minganti, 1946; Vanderbroek, 1938; Rose, 1939) (see Fig. 7). Thus, the development of these cells is non-autonomous and may require cell interactions.

Reverberi and Minganti (1946) conducted a series of microsurgical experiments to define the cell interactions necessary for brain sensory cell development. First, they showed that isolated animal halves (a4.2 and b4.2 cells) of 8-cell embryos form only epidermis (Fig. 8A). Next, they produced three-quarter embryos consisting of a4.2, b4.2, and B4.1 cells (Fig. 8B) or a4.2, b4.2, and A4.1 cells (Fig. 8C) and showed that only the latter developed an ocellus and otolith. Finally, they found that half-embryos containing only a4.2 and A4.1 cells could form these sensory cells (Fig. 8D). These results indicate that an interaction between the a and A cells is required to form an ocellus and otolith.

Subsequent studies by Reverberi and Minganti (1947) and Reverberi *et al.* (1960) more precisely defined which A cells were responsible for inducing the development of the ocellus and otolith and determined whether all ectodermal cells can respond to this inductive stimulus. By removing various A cells from 16-, 32-, and 64-cell embryos and transplanting them to isolated animal half-embryos, it was found that only A6.1, A7.3, A7.5, A7.6 and A7.7 or their derivatives (presumptive endodermal and notochord cells) are able to induce brain sensory cells. Removal or transplantation of A7.4 and A7.8, which normally contribute to brain stem, spinal cord, and muscle (Fig. 3), had no effect on the ability of the cells to form sensory cells. These results suggest that head endoderm and/or notochord cells can induce the formation of the ocellus and otolith. However, it seems likely the notochord cells are responsible for sensory cell induction because they underlie the neural plate during normal embryogenesis.

Cell transplantation experiments suggested to Reverberi *et al.* (1960) that only the a cells could form sensory cells, whereas similar experiments conducted by Rose (1939) and Ortolani (1961) indicated that either a or b cells are competent to form these cells when exposed to the inductive influence of A cells. More recent studies in which sensory cells developed after a cells were deleted from the embryo (Nishida and Satoh, 1989) support the results of

Rose (1939) and Ortolani (1961). Thus, it appears that a or b cells can be induced to form sensory cells by notochord cells and that induction of neural tissue in ascidians is similar to that which occurs in amphibian embryos. At present, the molecular mechanisms involved in brain sensory cell induction in ascidians are unknown.

Muscle cells

While primary muscle cells differentiate autonomously, cell interactions may be required for secondary muscle cell development. Evidence comes from those experiments discussed above in which partial embryos lacking B blastomeres do not develop AChE or myofibrils (Whittaker *et al.*, 1977; Whittaker, 1982; Crowther and Whittaker, 1983). However, more recent experiments complicate the question of autonomous differentiation of the secondary muscle cells. Deno *et al.* (1984) investigated AChE expression in isolated a4.2, b4.2, A4.1 and B4.1 blastomere pairs of 8-cell *Ciona* and *Halocynthia* embryos. As expected, AChE was detected in a high percentage of the partial embryos derived from isolated B4.1 cells. However, none of the embryos obtained from isolated A4.1 cells, and only a small number of partial embryos that developed from isolated b4.2 blastomeres expressed the enzyme (a low level of AChE was also observed in embryos obtained from isolated a4.2 blastomeres; see below). Similar results were obtained using a muscle-specific monoclonal antibody to identify differentiated muscle cells (Nishikata *et al.*, 1987a). These results prompted Meedel *et al.* (1987) to reinvestigate the autonomy of AChE and myofibril development in isolated blastomeres. They found that isolated A4.1 blastomeres from 8-cell *Ciona* embryos did not produce AChE autonomously, however, isolated A4.1 blastomere pairs of *Ascidia ceratodes* embryos showed 100% autonomous enzyme production. These results, together with experiments showing that animal half-embryos of each species do not develop AChE or myofibrils (Meedel *et al.*, 1987), suggest that depending on species (i.e. *Ascidia ceratodes* but not *Ciona intestinalis*), A cells may develop muscle cell features autonomously, and in both species b cells require inductive cell interactions for muscle cell determination. The earlier results of Deno *et al.* (1984) also support this idea since much higher levels of AChE activity were detected in three-quarter embryos consisting of a4.2, b4.2, and A4.1 blastomeres than in partial embryos obtained from isolated b4.2 cells. It has been proposed that the low level of muscle cell features sometimes seen in embryos developing from isolated b4.2 cells (Deno *et al.*, 1984; Nishikata *et al.*, 1987a) may be a stress response caused by damage during microsurgery (Meedel *et al.*, 1987). This explanation is consistent with the finding that a low level of AChE can be produced even in isolated a4.2 cells (Deno *et al.*, 1984), which have no progeny that contribute to muscle cells.

The relative roles of cytoplasmic determinants and cell interactions in specifying the secondary muscle cells remain uncertain. What is clear is that the timing of muscle differentiation in secondary muscle cells is different from

that in primary muscle cells. When Nishida and Satoh (1983) stained tailbud stage *Halocynthia* embryos for AChE, only B cell derivatives in the anterior and middle region of the tail showed enzyme activity. The b and A cell derivatives in the posterior region of the tail did not produce AChE until later in development. A delay in AChE production was also observed in partial embryos lacking B cells (Meedel *et al.*, 1987). When Tomlinson *et al.* (1987) subjected *Styela* embryos to *in situ* hybridization with a cloned muscle actin probe, transcripts were observed in primary muscle cells at the gastrula stage, but secondary muscle cells did not synthesize muscle actin mRNA until the tailbud stage (Fig. 9). Differences in the timing of AChE production and muscle actin gene expression in the different muscle lineages suggest that secondary muscle cell formation may occur by one of two possible mechanisms. The first possibility is that cell interactions take place between presumptive secondary muscle cells and unknown inducing cells. The second is that secondary muscle cells are specified by cytoplasmic determinants but receive fewer of these factors (Whittaker, 1983) than the primary cells, and thus differentiate later in development. The latter possibility is consistent with the recent observation that the myoplasm is not segregated entirely into the posterior region of the zygote during the second phase of ooplasmic segregation (Sardet *et al.*, 1989). It now appears that a small proportion of myoplasm is translocated to the anterior region during ooplasmic segregation. The anterior myoplasm is in a position to enter the A cell line during cleavage and may thus be responsible for specifying the secondary muscle cells that arise from this lineage. Similarly, it has been proposed that during the third cleavage a portion of myoplasm at the animal margin of the crescent may be distributed to the b4.2 cells (Sardet *et al.*, 1989).

Summary

A detailed cell lineage of ascidian embryos has been available since the turn of the century. This cell lineage was deduced from the segregation of pigmented egg cytoplasmic regions into particular blastomeres during embryogenesis. The invariant nature of the cell lineage, the segregation of specific egg cytoplasmic regions into particular blastomeres, and the autonomous development of most embryonic cells suggests that cell fate is determined primarily by cytoplasmic determinants. Modern studies have provided strong evidence for the existence of cytoplasmic determinants, especially in the primary muscle cells, yet the molecular identity, localization, and mode of action of these factors are still a mystery. Recent revisions of the classic cell lineage and demonstrations of the lack of developmental autonomy in certain embryonic cells suggest that induction may also be an important mechanism for the determination of cell fate in ascidians. There is strong evidence for the induction of neural tissue and indirect evidence for inductive interactions in the development of the secondary muscle cells. In contrast to the long-accepted dogma, specification of cell fate in ascidians appears to be established by a combination of cytoplasmic determinants and inductive cell interactions.

KEY WORDS: cell lineage, cell determination, embryology, ascidian embryos.

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