Determinative mechanisms in secondary muscle lineages of ascidian embryos: development of muscle-specific features in isolated muscle progenitor cells

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Summary

Muscle cells of the ascidian larva originate from three different lines of progenitor cells, the B-line, A-line and b-line. Experiments with 8-cell embryos have indicated that isolated blastomeres of the B-line (primary) muscle lineage show autonomous development of a muscle-specific enzyme, whereas blastomeres of the A-line and b-line (secondary) muscle lineage rarely develop the enzyme in isolation. In order to study the mechanisms by which different lines of progenitors are determined to give rise to muscle, blastomeres were isolated from embryos of *Halocynthia roretzi* at the later cleavage stages when conspicuous restriction of the developmental fate of blastomeres had already occurred. Partial embryos derived from B-line muscle-lineage cells of the 64-cell embryo (B7.4, B7.5 and B7.8) showed autonomous expression of specific features of muscle cells (acetylcholinesterase, filamentous actin and muscle-specific antigen). In contrast, b-line muscle-lineage cells, even those isolated from the 110-cell embryo (b8.17 and b8.19), did not express any muscle-specific features, even though their developmental fate was mainly restricted to generation of muscle. Isolated A-line cells from the 64-cell embryos (A7.8) did not show any features of muscle differentiation, whereas some isolated A-line cells from the 110-cell embryos (A8.16) developed all three above-mentioned features of muscle cells. This transition was shown to occur during the eighth cell cycle. These results suggest that the mechanism involved in the process of determination of the secondary-lineage muscle cells differs from that of the primary-lineage muscle cells. Interaction with cells of other lineages may be required for the determination of secondary precursors to muscle cells.

The presumptive b-line and A-line muscle cells that failed to express muscle-specific features in isolation did not develop into epidermial cells. Thus, although interactions between cells may be required for muscle determination in secondary lineages, the process may represent a permissive type of induction and may differ from the processes of induction of mesoderm in amphibian embryos.

Key words: ascidian embryo, muscle differentiation, developmental autonomy, determination, cell interaction.

Introduction

The process of ascidian embryogenesis is regarded as an example of typical mosaic development. A significant body of information has accumulated about cytoplasmic determinants involved in differentiation of muscle cells (reviewed by Whittaker, 1979; Uzman and Jeffery, 1986; Satoh, 1987). Initially, it was thought that all muscle cells of the larval tail originated from a single pair of blastomeres of the 8-cell embryo (the posterior–vegetal blastomeres, B4.1) (Conklin, 1905; Ortolani, 1955; Mancuso, 1969), and the results of experiments with isolated blastomeres at the 8-cell stage were interpreted according to this hypothesis. Partial embryos derived from isolated B4.1 blastomeres of the 8-cell embryo were shown, subsequently, to express autonomously certain muscle-specific features which

could be detected histologically, histochemically and ultrastructurally (Reververi and Minganti, 1946; Whittaker *et al.* 1977; Crowther and Whittaker, 1983).

However, recent studies of cell lineage, involving intracellular injection of lineage tracer molecules have revealed that not only the B-line blastomeres (designated as the primary muscle lineage according to Meedel *et al.* (1987)) but also A-line (the anterior–vegetal) and b-line (the posterior–animal) blastomeres (designated as the secondary muscle lineages) contribute to formation of tail muscle (Nishida and Satoh, 1983, 1985; Nishida, 1987). Fig. 1 is a schematic representation of the muscle lineage of *Halocynthia roretzi* embryos.

Deno, Nishida and Satoh (1985) reinvestigated the autonomy of muscle differentiation in partial embryos derived from each pair of blastomeres of the 8-cell
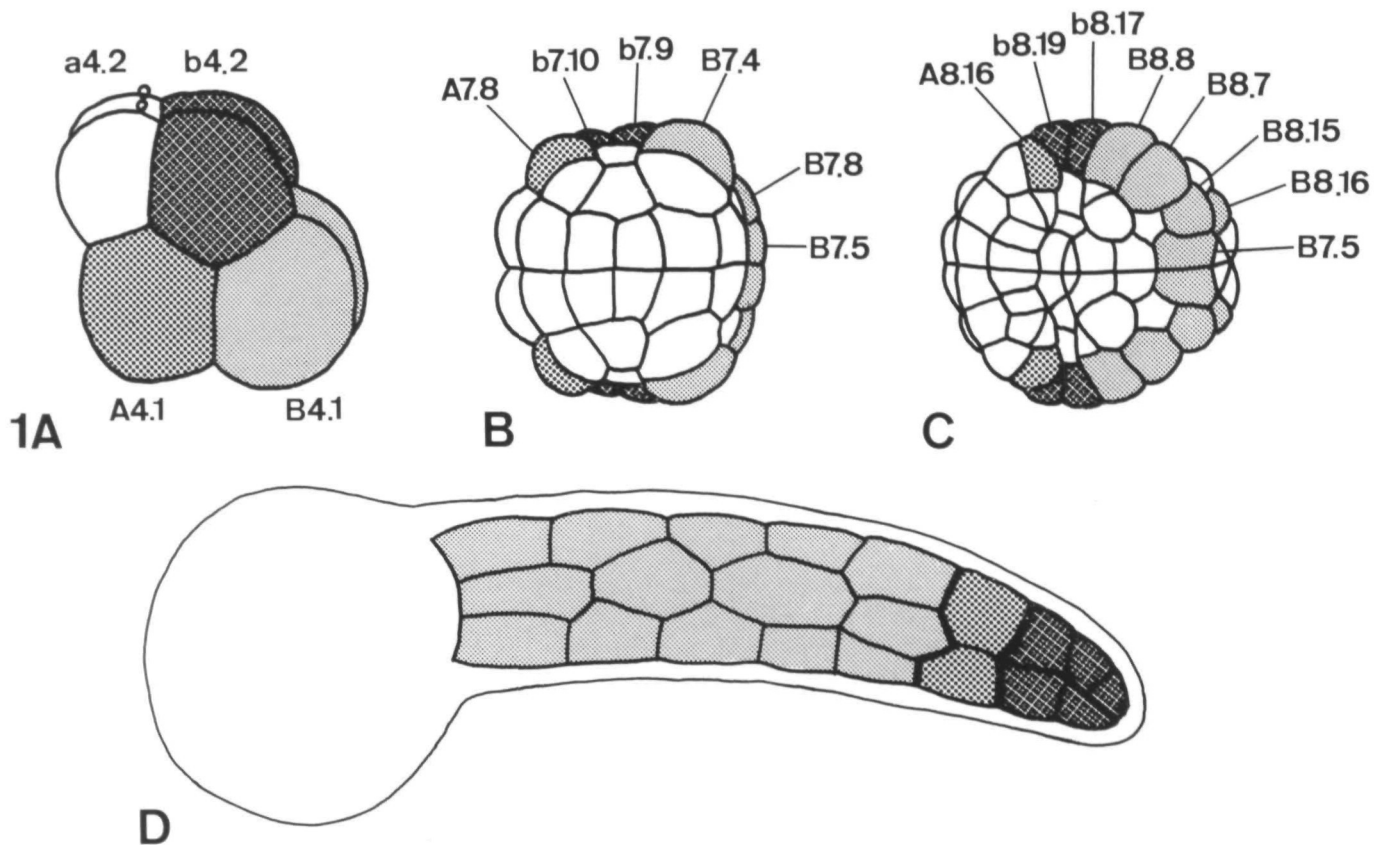
Fig. 1. Muscle cell lineage of the *Halocynthia roretzi* embryo. The nomenclature of blastomeres follows Conklin (1905). All diagrams are oriented anterior, left and posterior, right. B-line (primary), and A-line and b-line (secondary) muscle-lineage cells of the embryos and muscle cells of the tadpole are distinguished by the density of shading. The lightest shading indicates the B-line, the medium shading indicates the A-line, and the darkest shading indicates the b-line. Ascidian embryos are bilaterally symmetrical. (A) Lateral view of the 8-cell embryo. The animal pole is uppermost. a4.2 is the anterior-animal blastomere of the 8-cell embryo and b4.2 is the posterior-animal blastomere. A4.1 is the anterior-vegetal blastomere and B4.1 is the posterior-vegetal blastomere. For example, the letter 'a' is inherited by every descendant of the a4.2 blastomere after the 8-cell stage. The first digit denotes the generation of the cell, with the unsegmented egg as the first generation. The second digit gives the cell number, which doubles at each division (e.g., A7.8 divides into A8.15 and A8.16). (B,C) Vegetal view of the 64-cell embryo (B) and of the 110-cell embryo (C). The cleavage pattern and fates of blastomeres are bilaterally symmetrical. Muscle-lineage cells lie in the equatorial region at these stages. There are six bilateral pairs of muscle-lineage cells at the 64-cell stage: the B7.4, B7.5, B7.8, A7.8, b7.9 and B7.10 pairs, and there are eight pairs at the 110-cell stage: the B8.7, B8.8, B8.15, B8.16, B7.5, A8.16, b8.17, and b8.19 pairs. (D) Left-side view of a tailbud larva. The number and position of differentiated muscle cells in the larval tail are shown. On each side there are 21 muscle cells. Among them, 14 muscle cells in the anterior and middle part of the tail belong to the B-line, 2 cells in the posterior part belong to the A-line, and 5 cells in the caudal tip belong to the b-line. The number and relative positions of B-line, A-line, and b-line muscle cells are invariant in any individual, and they do not change after the tailbud stage.

Halocynthia embryo. Results clearly showed that most of the B-line partial embryos autonomously developed acetylcholinesterase, the putative marker of muscle differentiation, whereas few of the A-line and b-line partial embryos expressed the enzyme. The proportion of the A-line and b-line quarter embryos that expressed the enzyme was only about 3 %. Essentially similar results were obtained with *Ciona* by Deno *et al.* (1985) and Meedel *et al.* (1987). From these observations, Meedel *et al.* (1987) suggested that interactions between cells are involved in the process of determination of the secondary muscle progenitor cells.

Isolation of blastomeres from cleavage-stage embryos and examination of the developmental autonomy of the cells is a powerful method for the investigation of

the determinative state of embryonic cells, which is not evident during normal development. However, several factors make it difficult to interpret the results of differentiation of muscle in partial embryos derived from secondary-lineage blastomeres isolated from the 8-cell embryo: (a) the low proportion of positive specimens; (b) only a small number of muscle cells is derived from secondary lineages during normal development, as compared with the number of cells derived from primary lineage; and (c) the developmental fate is only partially segregated at the 8-cell stage, and each muscle-lineage blastomere gives rise to various kinds of larval tissue in addition to muscle.

In this study, muscle-lineage cells of three different lines were isolated from embryos at later cleavage

stages, when restriction of their developmental fate to muscle was already advanced in A-line and b-line muscle-progenitor cells. If the failure of muscle development in A-line and b-line quarter embryos were due to a weaker restriction of their developmental fate, then A-line and b-line muscle-progenitor cells isolated from the 64-cell or 110-cell embryo might possibly develop features characteristic of differentiation to muscle. However, if such failure were due to the interruption of the intercellular interactions with other blastomeres that occur late in development, the secondary muscle-lineage cells isolated even at the late-cleavage stage might not develop muscle-specific features.

Furthermore, since mesodermal induction is involved in the formation of mesodermal tissues, including muscle, during amphibian embryogenesis, the possibility that mesodermal induction may play some role in ascidian embryogenesis was also investigated.

Materials and methods

Embryos

Adults of the ascidian, *Halocynthia roretzi* (Drasche), were collected in the vicinity of Asamushi Marine Biological Station, Aomori, Japan. They spawned yellowish, translucent eggs, about 280 μm in diameter exclusive of the chorion. The translucency and large size of the eggs facilitated identification and isolation of each blastomere. Naturally spawned eggs were fertilized artificially with a dilute suspension of sperm from other individuals. Embryos were reared in Millipore-filtered seawater (MFSW) at 13°C, at which temperature embryos hatched about 35 h after fertilization.

Isolation of blastomeres

Fertilized eggs were allowed to develop until the 16-cell stage. The 16-cell embryos were manually dechorionated with sharpened tungsten needles and reared in 0.9 % agar-coated plastic dishes filled with MFSW that contained 50 μg ml^{-1} streptomycin sulphate. At desired stages between the 64-cell and the 110-cell stage (8–9 h after fertilization), identified blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope (OLYMPUS SZH-121). Isolated blastomeres were cultured separately in agar-coated plastic dishes filled with MFSW that contained streptomycin. Isolates were cultured until control larvae hatched, and they were then prepared for histochemical and immunohistochemical analysis.

Histochemistry and immunohistochemistry

Vital staining for mitochondria

3,3'-diethyloxacarbocyanin [DiOC$_2$ (3)] is a fluorescent probe for vital staining of mitochondria and its validity in ascidian embryos has been demonstrated (Zaloker and Sardet, 1984). Dechorionated embryos were incubated in MFSW that contained 0.5 μg ml^{-1} DiOC$_2$ (3) (Molecular Probes Inc. Eugene) for 1 h at 13°C during the 16-cell and 32-cell stages, and then they were transferred to MFSW. The drug was not washed out in MFSW. At the 64-cell and 110-cell stages, blastomeres of various lineages were isolated from the embryos and then their mitochondria were examined with a Nikon Labophoto equipped with an epifluorescence optic unit EFD and a B2 excitation filter cassette (excitation wavelength 460–485 nm). In this system, mitochondria were visualized by green fluorescence emitted by the DiOC$_2$ stain.

Histochemical staining for acetylcholinesterase (AchE)

Histochemical detection of AchE was carried out by the method described by Karnovsky and Roots (1964). Partial embryos were fixed for 10 min in 5 % formalin in seawater and, after several washings with distilled water, they were incubated in the reaction mixture for 1–2 h at 30°C. Specimens were then washed and observed with Nomarsky differential interference optics (Nikon Optiphoto with NT equipment). Histochemical localization of AchE activity in larvae is shown in Fig. 2A. Most of the activity is restricted to the tail muscle. At the larval stage, all of the muscle cells, including secondary muscle cells, show the same intensity of activity. The primordial pharynx and several cells in the trunk region also show evidence of the enzymatic activity, as described by Deno *et al.* (1985).

Staining for actin with NBD-phallacidin

7-nitrobenz-2-oxa-1,3-diazole(NBD)-phallacidin is a drug that binds specifically to filamentous actin and a emits yellow fluorescence. Partial embryos were placed on glass coverslips and fixed for 7 min at 20°C in 4 % formalin dissolved in Ca^{2+}-free artificial seawater buffered with 10 mM Tris–HCl (pH 7.0). Embryos were then extracted with acetone for 1 min at −20°C, and rinsed with buffered seawater. Extracted embryos were stained with a solution of 330 ng ml^{-1} NBD–phallacidin (Wako Chemicals Ltd.) in buffered seawater for 30 min at 20°C, rinsed in buffered seawater, mounted in 50 % glycerol and observed with a fluorescence microscope equipped with a B2 excitation filter cassette. When the larvae were stained with this drug, all tail muscle cells emitted an intense fluorescence (Fig. 2B). At high magnification, each myofibril was visible. Myofibrils were located only in the peripheral cytoplasm of muscle cells, and they ran at a slightly oblique angle to the anterior–posterior axis. The boundaries of non-muscle cells also emitted a weak fluorescence, most likely indicative of the presence of cytoskeletal actin. Fluorescence from cytoskeletal actin was faint and no filamentous structure was visible, so that such actin was easily distinguishable from myofibrils.

Immunohistochemistry with monoclonal antibodies Mu-2 and Epi-2

In order to examine the expression of antigens recognized by monoclonal antibodies in partial embryos, specimens were placed on glass coverslips, fixed for 10 min in methanol at −20°C, washed with phosphate-buffered saline (PBS) and incubated with hybridoma culture fluid for 1 h at room temperature. After washing with PBS, specimens were stained for 30 min with FITC-conjugated rabbit serum raised against mouse IgG (Miles-Yeda Ltd) diluted 1:60 in PBS. Specimens were washed again, mounted in 50 % glycerol and observed with a fluorescence microscope equipped with a B2 excitation filter cassette.

The monoclonal antibody Mu-2 specifically recognizes differentiated muscle cells of *Halocynthia* larvae (Nishikata *et al.* 1987a) and it binds with the myosin heavy chain (Makabe and Satoh, 1989). In the larvae stained indirectly with the Mu-2 antibody, fluorescence from FITC was completely restricted to muscle cells and no other cells emitted comparable fluorescence (Fig. 2C). With the exception of the nucleus, the entire cytoplasm of muscle cells was stained with this antibody. Even at high magnification, no filamentous structures of myofibrils were visible, possibly because of the method used for fixation. In all, three kinds of histochemical and immuno-
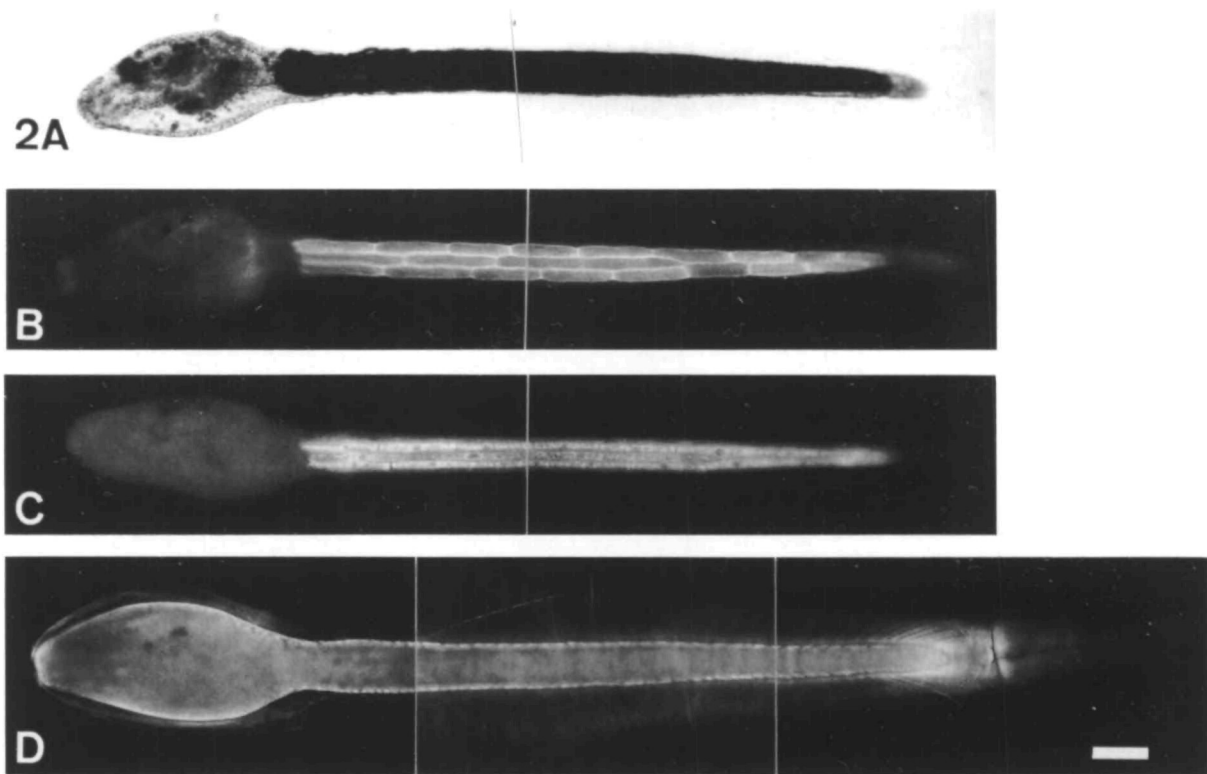
Fig. 2. Markers for expression of differentiation of muscle cells (A–C) and epidermis cells (D) of *Halocynthia* embryos. (A) A larva stained histochemically for AchE. (B) A larva stained with NBD-phallacidin. (C) A larva indirectly stained with the monoclonal antibody Mu-2. In these three larvae, the staining is localized in each case mainly in muscle cells in the tail. (D) A larva stained indirectly with the monoclonal antibody Epi-2. Epidermis cells and larval tunic are stained with this antibody. Bar, 100 μm.

histochemical technique were used to evaluate the differentiation of muscle cells in partial embryos.

The monoclonal antibody Epi-2 recognizes cells of the larval epidermis and the larval tunic material which is secreted by the epidermal cells (Nishikata *et al.* 1987*b*). Figure 2D shows a larva stained with Epi-2 antibody.

Results

Verification of identification and isolation of blastomeres

The large size of *Halocynthia* eggs and use of a high-resolution stereomicroscope permitted the identification and isolation of all blastomeres up to the 110-cell stage. Each muscle-progenitor blastomere was carefully isolated from embryos with a fine glass needle. Since embryos are bilaterally symmetrical (Fig. 1), the left and/or right blastomere(s) was isolated from each embryo.

The reliability of the identification and isolation of muscle lineage cells was examined in two ways. First, numbers of mitochondria in isolated muscle-lineage blastomeres were examined. Mitochondria in ascidian egg cytoplasm segregate preferentially to the muscle-lineage blasomeres, and ultimately they segregate to larval muscle cells (Reververi, 1956). DiOC$_2$ is a vital stain for mitochondria and is a useful marker for muscle-lineage cells of ascidian embryos (Zalokar and Sardet, 1984). In *Halocynthia* embryos, staining with DiOC$_2$ occurs mainly in muscle-lineage cells (Fig. 3A). Cells of the spinal cord and brain lineage contain some mitochondria, but fewer than muscle-lineage cells. The cells of other lineages contain few mitochondria. Embryos were stained with DiOC$_2$ at the 16-cell and 32-cell stages, and each muscle-lineage cell was then isolated at the 64-cell and 110-cell stage. The number of mitochondria in each isolated cell was immediately determined. Every isolated muscle-lineage cell (B7.4, B7.8, A7.8, b7.9 and b7.10 of the 64-cell embryo, and A8.16, b8.17 and b8.19 of the 110-cell embryo) was found to be rich in mitochondria when compared with isolated cells of other lineages (Fig. 3B and C).

After the 64-cell stage, the divisions of blastomeres become asynchronous. However, the timing of cell division is invariant in any individual, and the length of the cell cycle of each blastomere is unique and specific (Nishida, 1986). In a second examination, the timing of the divisions of isolated blastomeres was compared with that of blastomeres in normal whole embryos. In every case, at least one subsequent division of isolated cell occurred simultaneously with the division of their counterparts in normal embryos. Together, the results of these two kinds of test indicated the reliability of the identification and isolation of each muscle-lineage cell at the 64-cell and 110-cell stages.
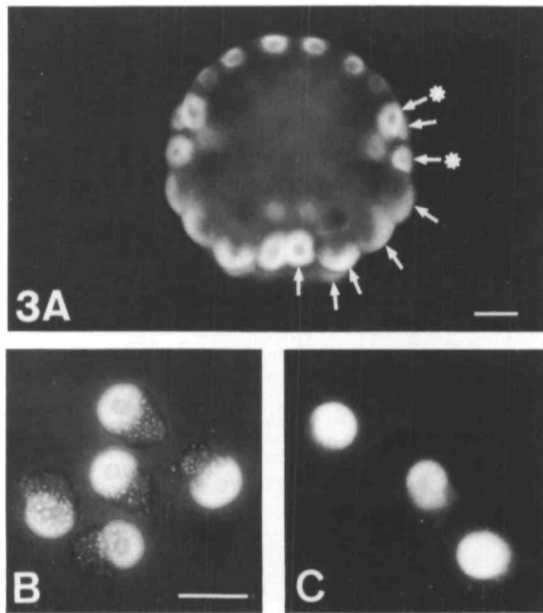
Fig. 3. Distribution of mitochondria in the *Halocynthia* embryo and in isolated muscle-lineage blastomeres. (A) Vegetal view of 110-cell embryo stained with $DiOC_2$. Anterior is uppermost. $DiOC_2$ staining is localized mainly in muscle-lineage cells (arrows on the right side of the embryo). The six stained cells lying at the anterior edge are cells of spinal cord lineage. (B,C) Isolated A8.16 (B) and b8.17 cells (C) (which correspond to the cells with asterisks in A). These cells show intensive fluorescence to indicate that they are rich in mitochondria. In B, cells are also illuminated by transmitted light to demarcate their outlines. Mitochondria are localized around a nucleus and in the yolkless area within the cell. Bar, 50 μm.

Expression of features of muscle-specific differentiation in partial embryos

Muscle differentiation in partial embryos was evaluated by histochemical staining for AchE (Fig. 4) and filamentous actin (Fig. 5A–C), and immunohistochemical staining for myosin heavy chains (Fig. 5D–F). Muscle-lineage blastomeres were separately isolated from 64-cell embryos and 110-cell embryos and cultured until control larvae hatched. Each partial embryo was then examined for expression of features of muscle-specific differentiation. The results are summarized in Table 1. In the 64-cell embryos, almost all of the isolates of the B-line (B7.4, B7.8, and B7.5) developed three kinds of muscle-specific feature, but these features were not evident in isolates of the A and b-line (A7.8, b7.9, and b7.10). At the 110-cell stage, some isolated A8.16 cells developed the muscle-specific features, but none of the isolates of the b-line did so. Consistent results were obtained using the three kinds of marker for differentiation of muscle.

(i) B-line partial embryos

In normal embryogenesis, the B7.4 cell gives rise to eight muscle cells (Nishida, 1987). The partial embryos derived from the B7.4 cells consisted of eight AchE-positive cells (Fig. 4A) in most cases, which coincided with the developmental fate of this blastomere. When stained with NBD–phallacidin, filamentous actin was visible in each specimen (Fig. 5A). Myosin heavy chain (Mu-2 antigen) was also expressed in these partial embryos (Fig. 5D).

The developmental fate of the B7.8 cell in normal embryogenesis is four muscle cells. The partial embryos derived from this blastomere consisted of four cells,
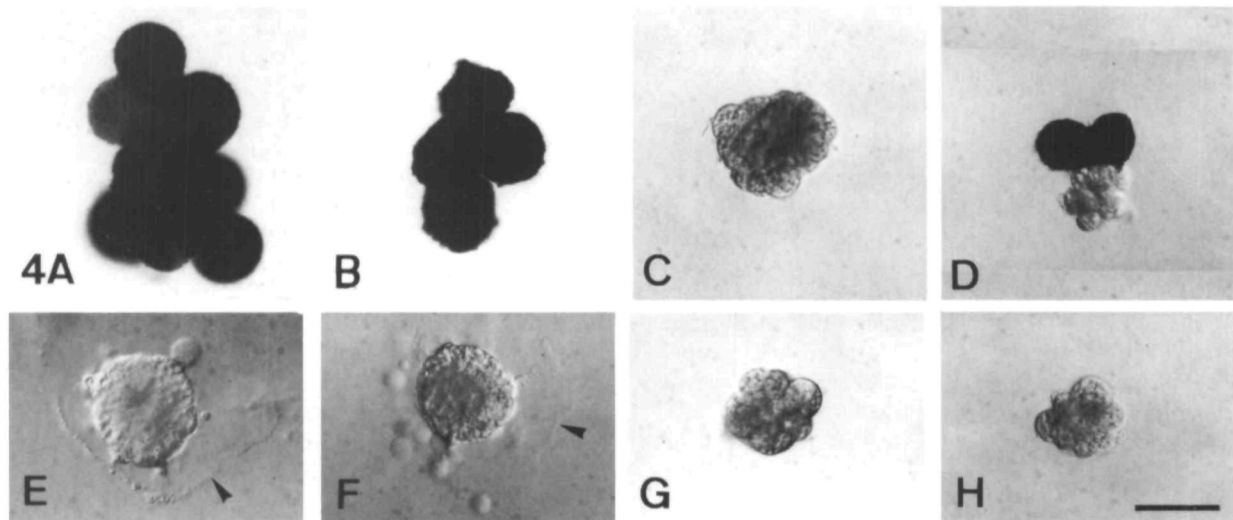

Fig. 4. Expression of AchE in partial embryos. (A) B7.4 partial embryo derived from an isolated B7.4 muscle-lineage blastomere from a 64-cell embryo. The partial embryo consists of eight AchE-positive cells. (B) B7.8 partial embryo. The embryo consists of four positive cells. (C) A7.8 partial embryo showing no staining for AchE. (D) A8.16 partial embryo originating from the A8.16 cell of the 110-cell embryo. The embryo consists of two large positive cells and a few small negative cells. (E) b7.9 partial embryo. Larval tunic materials are indicated by the arrowhead. (F) b7.10 partial embryo. (G) b8.17 partial embryo. (H) b8.19 partial embryo. Photographs C through H were taken with Nomarsky optics in order to show the multicellular composition of the partial embryos and presence of larval tunic materials (E,F). Bar, 50 μm.
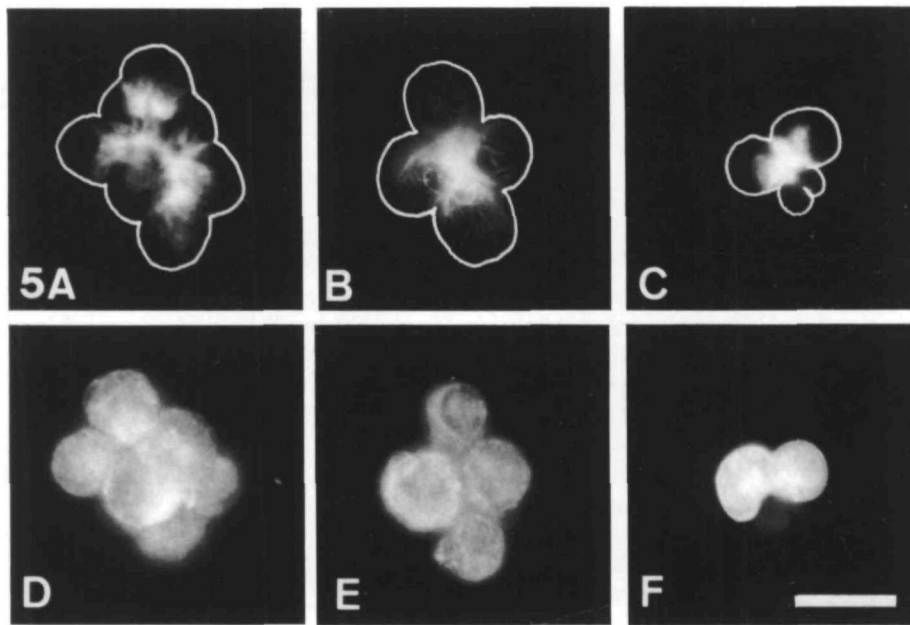
Fig. 5. Expression of filamentous actin and Mu-2 antigen in partial embryos. (A) B7.4, (B) B7.8, and (C) A8.16 partial embryos were stained with NBD-phallacidin. Outlines of embryos are indicated by white lines. Filamentous structures are visible. In B, the focus has been adjusted to the surface of the cells. (D) B7.4, (E) B7.8, and (F) A8.16 partial embryos were stained with Mu-2 antibody. In C and F, A8.16 partial embryos consist of two large cells positive for staining and a few small negative cells. Bar, 50 μm.

Table 1. *Expression of muscle-specific features in partial embryos*

	No. of embryos that showed muscle feature / No. of embryos examined (%)								
Origin of partial embryos	B7.4	B7.8	B7.5	A7.8	b7.9	b7.10	A8.16	b8.17	b8.19
Stage of isolation	64-cell	64-cell	64-cell	64-cell	64-cell	64-cell	110-cell	110-cell	110-cell
AchE	$\frac{29}{29}$ (100%)	$\frac{24}{24}$ (100%)	N.D.	$\frac{0}{56}$ (0%)	$\frac{0}{18}$ (0%)	$\frac{0}{23}$ (0%)	$\frac{20}{42}$ (48%)	$\frac{0}{31}$ (0%)	$\frac{0}{22}$ (0%)
Filamentous actin	$\frac{52}{58}$ (90%)	$\frac{28}{32}$ (88%)	N.D.	$\frac{1}{33}$ (3%)[a]	$\frac{0}{37}$ (0%)	$\frac{0}{29}$ (0%)	$\frac{16}{42}$ (38%)	$\frac{1}{30}$ (3%)[a]	$\frac{0}{26}$ (0%)
Mu-2 antigen	$\frac{30}{30}$ (100%)	$\frac{53}{53}$ (100%)	$\frac{31}{31}$ (100%)	$\frac{0}{50}$ (0%)	$\frac{0}{38}$ (0%)	$\frac{0}{39}$ (0%)	$\frac{24}{83}$ (28%)	$\frac{0}{48}$ (0%)	$\frac{0}{62}$ (0%)

N.D., Not determined.

[a] One embryo had a bright fluorescent spot, but the spot showed no filamentous structures and, therefore, may not represent a true positive result.

each of which expressed AchE (Fig. 4B), filamentous actin (Fig. 5B) and Mu-2 antigen (Fig. 5E). The development of B7.8 partial embryos coincided with the developmental fate of this cell in normal embryogenesis.

In present series of experiments, the B7.5 isolates were examined only for the expression of Mu-2 antigen (Table 1). All of the isolates developed the antigen. The developmental fate of this blastomere is two muscle cells and two endodermal cells.

(ii) A-line partial embryos

The fate of the A7.8 cell of the 64-cell embryo includes two muscle cells and several spinal cord cells. None of the partial embryos derived from this blastomere developed muscle-specific features (Fig. 4C).

The developmental fate of the A8.16 cell of the 110-cell embryo is two muscle cells and several spinal cord cells. In a previous study, incorporating the intracellular injection of horseradish peroxidase, the fate of the

A8.16 cell in *Halocynthia* was shown to be only two muscle cells (Nishida, 1987). However, recent analysis using FITC–dextran as a tracer molecule indicates that the fate of each A8.16 cell is not just two muscle cells but also a small number of spinal cord cells (Nishida, unpublished observation). Moreover, tracing of the neural cell lineage in *Ciona intestinalis* in serial optical sections had led to the same conclusion (Nicol and Meinertzhagen, 1988). These two lines of evidence strongly suggest that the fate of the A8.16 should be considered as two muscle cells plus a few spinal cord cells.

When the A8.16 cells were isolated at the 110-cell stage, 48%, 38% and 28% of the isolates developed AchE (Fig. 4D), filamentous actin (Fig. 5C), and Mu-2 antigen (Fig. 5F), respectively. Although the proportion of positive specimens with each kind of staining varied among batches of eggs, a significant fraction always stained positively in each case. Typical A8.16 embryos that were positive for staining (68% of the total number of positive specimens) had two larger

positive cells and a few smaller negative cells, supporting the recent above-mentioned assertion of the fate of A8.16.

(iii) *b-line partial embryos*

The fate of the b7.9 cell of the 64-cell embryo is three muscle cells, one cell of the endodermal strand or spinal cord, and a lot of epidermis cells. The fate of the b7.10 cell is two muscle cells, spinal cord cells and epidermis cells. In accordance with their developmental fates, the b7.9 and b7.10 partial embryos generated larval tunic materials, which are normally secreted by epidermal cells (Fig. 4E and F, arrowheads). However, no expression of any of the three kinds of marker of muscle differentiation was ever observed.

The fate of the b8.17 cell of the 110-cell embryo is mostly restricted to muscle, i.e., its fate is only a single cell of the endodermal strand or spinal cord in addition to three muscle cells. Nonetheless, the b8.17 partial embryos never showed any muscle-specific features (Fig. 4G). Likewise, although the fate of the b8.19 cell is two muscle cells and a few spinal cord cells, the b8.19 partial embryos also failed to express any muscle-specific features (Fig. 4H). The b8.17 and b8.19 partial embryos did not secrete larval tunic materials, in accordance with the fact that the epidermal fate areas have segregated to their sibling cells.

Timing of determination in A-line muscle cells

The A-line progenitors of muscle cells isolated from the 64-cell embryos never developed muscle-specific features, while some of those isolated from the 110-cell embryos did so autonomously. The time at which isolates of A-line cells acquire the autonomous ability to develop muscle-specific features, i.e., the timing of determination, was therefore investigated in greater detail. My main aim was to assess whether the seventh cell division, which occurs between the 64-cell and the 110-cell stages, is the critical point in the determination.

The precise times of isolation adopted in this experiment are shown in Fig. 6. The relative time of zero minutes indicates the beginning of division of the A7.8 cell, which yields the A8.15 cell (spinal cord progenitor) and A8.16 cell (muscle and spinal cord progenitor). The compaction of the A8.16 cell is accomplished at approximately 30 min. The next division of the A8.16 cell occurs at 95 min, but it is not shown in Fig. 6. In the above-mentioned experiments, the isolation at the 64-
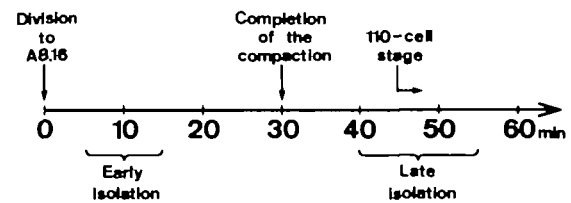

Fig. 6. Time sequence of the events during the eighth generation of an A-line muscle-lineage cell (A8.16), with the timing of early and late isolations indicated. Relative time zero corresponds to the beginning of the cell division that yields the A8.16 cell. The division of the A8.16 cell occurs at relative time 95 min, but it is not indicated in this figure.

cell stage was performed between about −20 and −5 min relative to zero time, and the isolation at the 110-cell stage was performed between about 45 and 55 min. In this experiment, the A8.16 cells were isolated just after they were formed and at around the 110-cell stage. The isolation between relative times 5 and 15 min is referred to as early isolation, and that between 40 and 55 min, as late isolation. The A8.16 cell does not divide in the period between early and late isolation. The nuclei of the A8.16 cells were not yet visible at the time of early isolation, but they were clearly visible at the time of late isolation. The partial embryos that were derived from the isolates were examined for the expression of muscle-specific features.

Four independent experiments, with different batches of eggs, were carried out (Table 2). In two of these experiments, the embryos were examined for expression of AchE, and, in the other two experiments, for expression of the Mu-2 antigen. After early isolation, no expression of the muscle-specific features was detected. The results after late isolation were consistent with my previous results, with some (24 %) of the partial embryos expressing muscle-specific features. The difference in results between times of isolation was shown to be statistically significant (Table 2). These results indicate that A8.16 cells isolated soon after they have been formed are not yet determined to give rise to muscle, while some portion of the cells isolated later in the cell cycle are determined to give rise to muscle.

Comparison with mesodermal induction in amphibian embryos

During amphibian embryogenesis, mesodermal induc-

Table 2. *Expression of muscle-specific features in A8.16 partial embryos*

| | | No. of embryos that showed muscle feature | | |
| | | No. of embryos examined | | |
		Early isolation	Late isolation	Probability*
Exp. 1	AchE	$\frac{0}{39}$	$\frac{6}{13}$	$P<0.01$
Exp. 2.	AchE	$\frac{0}{41}$	$\frac{13}{52}$	$P<0.001$
Exp. 3	Mu-2	$\frac{0}{25}$	$\frac{7}{42}$	$P<0.05$
Exp. 4	Mu-2	$\frac{0}{34}$	$\frac{9}{45}$	$P<0.001$

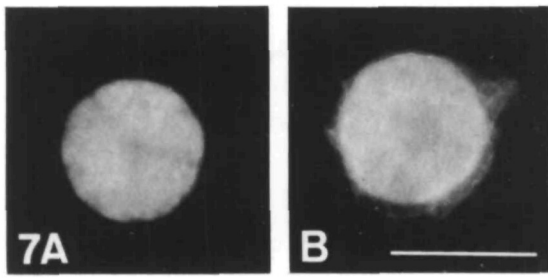* Probability was calculated by Fisher's exact probability test.

Fig. 7. (A) Expression of the Epi-2 antigen in a partial embryo that originated from an isolated epidermis-lineage cell from a 110-cell embryo. (B) A partial embryo originating from an isolated b7.9 cell, which is developmentally fated to become both epidermis and muscle. Epi-2 antigen is expressed in this partial embryo. Bar, 50 μm.

tion is involved in the formation of muscle (Nieuwkoop, 1973). In order to compare the properties of amphibian mesodermal induction and those of the process required for determination of muscle in the secondary lineage, differentiation of epidermis in partial embryos derived from secondary muscle-lineage cells (mainly b-line) was examined (see also Discussion).

As mentioned earlier, most of the b7.8 and b7.9 cells, which are fated to give rise to both muscle and epidermis, generated a larval tunic, a feature of the epidermis, in isolation. Conversely, none of the b8.17 and b8.19 muscle-lineage cells examined, which have separated from their sibling cells with respect to their fate (i.e., from b8.18 and b8.20, which generate epidermis), developed larval tunic in isolation. However, even when present, the larval tunic of partial embryos sometimes slipped off during the transfer of embryos between dishes, and it was also difficult to observe tunic material because of its transparency. Therefore, differentiation of epidermis was examined by immunohistochemical detection of the Epi-2 antigen (Fig. 2D).

As a positive control, epidermis precursor cells were isolated from the 110-cell embryos. Each of these isolates developed into a small permanent blastula, and in all cases the Epi-2 antigen was expressed (Fig. 7A). Another positive control was provided by the partial embryos that originated from isolated b7.9 and b7.10 cells, which are fated to become both epidermis and muscle. All partial embryos showed expression of Epi-2 (Fig. 7B). However, the b8.17 cells (43 specimens) and b8.19 cells (22 specimens) which are b-line cells of the secondary muscle lineage, never developed the Epi-2 antigen. The expression of Epi-2 was also examined in the A8.16 partial embryos (14 specimens). No embryo expressed the antigen. In summary, differentiation to epidermis does not occur in partial embryos derived from the secondary muscle-lineage cells isolated before they acquire the capacity for autonomous development into muscle.

Discussion

A detailed analysis of cell lineages during ascidian embryogenesis has revealed that tail muscle cells of larvae are derived from progenitor cells that belong to three different lines: the B-line (primary); and A- and b-lines (secondary) (Nishida and Satoh, 1983). When B4.1 blastomeres were isolated from 8-cell embryos and cultured until control larvae hatched, most of them autonomously developed muscle-specific features (AchE, Mu-2 antigen and myofilament). In contrast, A4.1 and b4.2 quarter embryos seldom expressed such features (Deno *et al.* 1985; Nishikata *et al.* 1987*a*; Meedel *et al.* 1987). Results such as these led Meedel *et al.* (1987) to suggest for the first time that different mechanisms may control the development of muscle cells in the primary and secondary muscle lineages.

Development of muscle-specific features in isolated muscle-lineage blastomeres

As clearly shown in the present study, B-line muscle-lineage cells, isolated from 64-cell embryos, autonomously developed muscle-specific features (AchE, filamentous actin, Mu-2 antigen). In addition, some A-line isolates acquired the capacity for autonomous development around the 110-cell stage. If the isolation were performed at even later stages, we would expect that the proportion of the A-line partial embryos that showed muscle-specific features would increase. However, the isolation at stages later than the 110-cell stage is difficult because inaccuracies arise in identification of blastomeres under the stereomicroscope. Conversely, in the b-line isolates, none of the partial embryos showed any muscle-specific development even when cells were isolated as late as the 110-cell stage, at which time their developmental fates are predominantly restricted to muscle. For example, the fate of the b8.17 cell is three muscle cells and one small cell of the spinal cord or endodermal strand (see Fig. 6i in Nishida (1987)). Nevertheless, isolated b8.17 cells failed to show any muscle-specific development. Present results from isolation of blastomeres reconfirm that the consequences of isolation differ remarkably between primary and secondary lineages, and they provide strong evidence in support of the hypothesis that different mechanisms control the development of muscle in the two kinds of lineage, as advocated initially by Meedel *et al.* (1987).

Failure of muscle development in isolated cell of the secondary lineage is unlikely to be due to nonspecific damage caused by isolation procedures. The b7.9 and b7.10 cells, which are fated to generate both muscle and epidermis, did not express any muscle-specific features when isolated, but they did show autonomous development of epidermis-specific features. Thus, the failure to differentiate is specific to features of muscle cells in these cases. Moreover, the requirement for association between cells is not a nonspecific cell-mass effect because the B7.4 and B7.8 partial embryos expressed muscle-specific features, even though they consisted of only 8 and 4 cells. In contrast, A-line and b-line muscle-progenitor blastomeres that were isolated from 8-cell embryos developed into partial embryos which con-

sisted of a lot of cells. Nevertheless, these embryos rarely developed muscle-specific features.

The hypothesis that different mechanisms control the development of muscle in the primary and secondary lineages is also supported indirectly by the following two lines of evidence. 1. *Expression of muscle-specific features in cleavage-arrested embryos*: If the cleavage of cleavage-stage embryos is permanently arrested by treatment with cytochalasin B, an inhibitor of cytokinesis, the arrested embryos produce AchE and Mu-2 antigen only in blastomeres of the primary (B-line) muscle lineage. Blastomeres of the secondary (A-line and b-line) muscle lineage do not express the enzyme or the antigen (Whittaker, 1973; Satoh, 1979; Nishikata *et al.* 1987*a*). However, in the case of *Ascidia ceratodes*, Meedel *et al.* (1987) reported that cleavage-arrested 8-cell embryos express AchE in the A-line blastomeres as well as in the B-line blastomeres. They also observed that A-line muscle-lineage blastomeres isolated from the 8-cell and 16-cell embryos develop AchE at high frequency, but they do not develop myofilaments. There are differences between species with respect to responses to isolation and cleavage-arrest of A-line cells. 2. *Time of expression of differentiation-specific phenotypes*: The expression of AchE in primary postmitotic precursors of muscle cells precedes that in secondary precursors of muscle cells (Nishida and Satoh, 1983; Deno *et al.* 1985; Meedel *et al.* 1987). In *Halocynthia* embryos, AchE activity is histochemically detectable first in B-line cells by the 14th hour of development (neurula), then in A-line cells by the 20th hour (early tailbud), and finally in b-line cells by the 25th hour (middle tailbud). It has also been reported that expression of mRNA for muscle actin in primary lineage cells precedes that in secondary lineage cells (Tomlinson *et al.* 1987).

It is noteworthy that, in *Ciona*, the three-quarter embryo that results from ablation of the pair of B4.1 blastomeres develops muscle cells in its caudal tip (Deno *et al.* 1984; Meedel *et al.* 1987). In *Halocynthia*, the three-quarter embryo also develops muscle-specific antigen in the caudal tip (our unpublished observation). These results indicate that cells of the primary muscle lineage are not required for the development of secondary muscle cells.

The precise timing of determination in the A-line muscle lineage appears to occur part of the way through the eighth generation. It should be noted that, irrespective of the stage of isolation (early isolation and late isolation during the eighth generation), the A8.16 cells have the same fate and contain the same cytoplasmic components, because no division occurs between the times of early and late isolations. Nevertheless, a clear difference is evident between the results of early and late isolations. Our experiment confirms that the low degree of restriction of the cell to a muscle-specific fate and/or the low degree of segregation of myoplasm are unlikely to be the only reasons for the observation that A-line muscle-lineage blastomeres do not show muscle-specific development if isolated at early stages.

This result together with the results of isolation of the

b-line muscle-lineage cells indicate that expression of muscle-specific features in the secondary lineages requires an association with cells from other lineages. Determination of muscle in the secondary lineages may be a process that is dependent on intercellular interactions. To test directly the role of such interactions in the determination of muscle in secondary lineages, experiments should be performed in which presumptive muscle cells are combined with other types of cell and the resultant effects on cell fates are followed.

Comparison with mesodermal induction in amphibia

During amphibian embryogenesis, the induction of mesoderm is involved in the formation of muscle (Nieuwkoop, 1973). It is known that the animal cap isolated from the blastula develops into epidermis when it is cultured as an explant. In contrast, when the animal cap is combined with vegetal cells, the cells of the animal cap give rise to muscle in addition to other mesodermal tissues and, of course, ectodermal tissues (Nieuwkoop, 1969). In ascidian embryos, b-line muscle-progenitor cells are cells of the animal hemisphere (Fig. 1). Moreover, all of the b-line cells other than four muscle-lineage cells of the 110-cell embryos are epidermis progenitors. By analogy with amphibian development, we might suppose that differentiation of the epidermis might have occurred in those b-line muscle-lineage partial embryos that did not show any evidence of muscle-specific differentiation. Epidermis fate is segregated and separated from muscle fate at the eighth generation (the 110-cell stage) in the b-line, facilitating the isolation of muscle progenitor cells that are not associated with epidermis fate, and allowing the possibility of differentiation to epidermis by b-line presumptive muscle cells to be tested.

Unlike the other cells of the b-line, isolated b8.17 and b8.19 cells do not develop into epidermis and, thus, b-line muscle-lineage cells may already differ substantially from the other b-line epidermis-lineage cells even before the stage at which b-line muscle-lineage cells develop the capacity for autonomous development into muscle. It seems that, in the secondary muscle lineages, intercellular interactions may be required for differentiation of muscle phenotype, rather than the choice of a muscle pathway among the repertoire of developmental pathways. In this regard, the interactions between cells that are responsible for muscle development in secondary lineages may be considered to represent permissive induction (Gurdon, 1987). These results suggest that, although intercellular interactions are required for the process of muscle differentiation in the secondary lineage, the properties of the process differ from those of mesodermal induction in amphibian embryos.

However, we must not ignore another possible explanation for our results. All of the secondary muscle-lineage cells, such as the b8.17, b8.19, and A8.16 cells, are fated to give rise to spinal cord cells in addition to muscle cells. Therefore, the presumptive muscle cells that fail to develop into muscle cells in isolation may develop into spinal cord cells. Unfortunately, there is no way to examine this possibility, because we have no

marker specific for the differentiation of spinal cord cells at present.

Evidence has accumulated to indicate the important roles of intercellular interactions in the development of certain kinds of tissue, even in animals with determinate and invariant cell lineages. In ascidian embryogenesis, the development of brain and pigment cells depends on such interactions (Rose, 1939; Reverberi *et al.* 1960; Nishida and Satoh, 1989). Recent results have demonstrated that such interactions are important in embryogenesis of *C. elegans*, even at the early cleavage stage (Schierenberg, 1987; reviewed by Emmons, 1987). Muscle development from the AB blastomere (minor muscle lineage) of *C. elegans* embryos depends on the presence of cells of other lineages (Priess and Thomson, 1987), as has also been shown to be the case in ascidian embryos, in this study.

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