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Regionality of egg cytoplasm that promotes muscle differentiation in embryo of the ascidian, *Halocynthia roretzi*

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Summary

Development of ascidians occurs in typical mosaic fashion: blastomeres isolated from early embryos differentiate into tissues according to their normal fates, an indication that cytoplasmic determinants exist in early blastomeres. To provide direct evidence for such cytoplasmic determinants, we have devised methods for fusing blastomeres and cytoplasmic fragments from various regions. (1) Presumptive-epidermis blastomeres were fused to cytoplasmic fragments from various regions of blastomeres of 8-cell embryos of *Halocynthia roretzi* and development of muscle cells was monitored by an antibody to ascidian myosin. Muscle differentiation was observed only when presumptive-epidermis blastomeres were fused with fragments from the posterior region of B4.1 (posterior-vegetal) blastomeres, the normal progenitor of muscle cells. The results indicate that muscle determinants are present and localized in the cytoplasm that enters muscle-lineage cells. (2) To investigate the presence and localization of muscle determinants in the egg, cytoplasmic fragments from various regions of unfertilized and fertilized eggs were fused with the presumptive-epidermis blastomeres, and formation of muscle cells was assessed by monitoring myosin, actin and acetylcholinesterase expression. These proteins were expressed only when cytoplasm from a restricted region of the eggs, i.e. the vegetal region, after the first phase of ooplasmic segregation, and posterior region, after the second phase of segregation, were fused. Based on these experiments, it is suggested that muscle determinants are segregated by ooplasmic movements after fertilization. They move initially to the vegetal pole of the egg and, prior to first cleavage, to the posterior region from whence future muscle-lineage blastomeres are formed. The inferred movements of muscle determinants correspond to those of the myoplasm, a microscopically visible portion of the egg cytoplasm.

Key words: ascidian embryogenesis, fate determination, muscle differentiation, cell fusion, cytoplasmic transfer, cytoplasmic determinants.

Introduction

The study of ascidian early embryology has provided conclusive evidence for determinate and cell-autonomous development. In this group of animals, developmental fate is coupled to the lineal descent of a cell and specification of cell fate is achieved cell-autonomously, since blastomeres show a mosaic behaviour after isolation from embryos. The latter phenomenon is taken as evidence that prelocalized ooplasmic factors specify tissue precursor cells during embryogenesis (Conklin, 1905a).

The developmental fate of each blastomere of an 8-cell ascidian embryo is shown in Fig. 1. The larval muscle cells have three origins, B, A and b-line blastomeres. Each cell of the B4.1 (posterior-vegetal) cell pair gives rise to 14 of 21 muscle cells, which are located along the anterior-mid region of one side of the tail in *Halocynthia*. Each A4.1 (anterior-vegetal) cell contributes two muscle cells in the posterior region of the tail, and each b4.2 (posterior-animal) cell contributes five muscle cells located along the caudal tip of the tail (Nishida and Satoh, 1983; Nishida, 1987). Muscle cells that arise from B-line cells are designated primary muscle cells; those that arise from the A and b-line cells are designated secondary muscle cells.

Development of muscle during ascidian embryogenesis has been intensively investigated (for reviews see Satoh et al., 1990; Nishida, 1992). The hypothesis that prelocalized cytoplasmic determinants play a crucial role in the determination of muscle fate is derived from observations demonstrating that (1) visible pigment granules are inherited by muscle-lineage cells through invariant cleavage (Conklin, 1905b); (2) isolated muscle-precursor blastomeres exhibit mosaic behaviour (e.g., Reverberi and Minganti, 1964) and (3) muscle differentiation occurs in cleavage-arrested embryos (e.g., Whittaker, 1973). These observations, however, only consider differentiation of primary muscle cells. Secondary muscle cell development is non-autonomous and is thought to require cellular interactions with cells of other tissues (Meedel et al., 1987; Nishida, 1990). The above observations are consistent with the existence of cytoplasmic determinants that direct the differentiation of primary muscle cells. However, direct proof of this requires that after the transfer of cytoplasm to a heterotopic position there is a change in the fate of the

recipient. Cytoplasmic redistribution has been achieved by changing the position of the third cleavage furrow by embryo compression (Whittaker, 1980) or by microsurgery (Whittaker, 1982). In these studies, myoplasm (containing yellow pigment) was partitioned not only to B4.1 cells but also to b4.2 cells. The expression of ectopic muscle-specific acetylcholinesterase (AchE) was observed in about 19% and 35% of operated embryos, respectively. Transfer of cytoplasm from B4.1 cells to A4.1 cells by microinjection has also been carried out. Muscle formation was determined by the expression of AchE (Deno and Satoh, 1984). In such experiments, only about 2% of operations yielded positive results. Such observations suggest the presence of muscle determinants in muscle-progenitor cells but, as mentioned above, recent cell lineage studies (Nishida and Satoh, 1983) indicate that the recipient blastomeres used in these studies normally form secondary muscle. Experiments employing cytoplasmic transfer would provide more meaningful results if a4.2 cells of the 8-cell embryos were employed as recipients since they normally do not give rise to muscle cells.

To investigate the presence in the primary muscle-lineage cells of cytoplasmic factors for specification of muscle fate of the blastomeres, and to reveal the presence and localization of muscle determinants in the uncleaved egg, partial embryos that were derived from the fusion of a4.2 non-muscle precursor cells with cytoplasmic fragments, obtained from various regions of embryos and eggs, were examined for their ability to express muscle-specific proteins.

Materials and methods

Embryos

Adult ascidians, *Halocynthia roretzi* (Drasche), were collected in the vicinity of Asamushi Marine Biological Station, Aomori, Japan. Naturally spawned, yellow-translucent eggs (280 μm in diameter) were fertilized and reared in Millipore-filtered sea water that contained 50 μg ml^{-1} streptomycin sulfate (MFSW) at 9-13°C. At 13°C tadpole larvae hatched about 35 hours after fertilization.

Isolation of blastomeres and preparation of blastomere and eggs fragments (see Figs 2, 6)

Fertilized eggs were manually dechorionated with sharpened tungsten needles and reared in 0.9% agar-coated plastic dishes containing MFSW. At the 8-cell stage, a4.2 blastomeres, which would be subsequently fused with blastomere and egg fragments, were isolated from embryos with a fine glass needle under a stereomicroscope (Olympus SZH-121). Fragments were made of blastomeres by bisection with a glass needle. Fragments were severed from eggs with a glass needle such that their volume was approximately equal to that of an a4.2 blastomere. Enucleated, cytoplasmic fragments from eggs and blastomeres were used in fusion experiments with a4.2 cells. Isolated blastomeres and cytoplasmic fragments were prepared such that their cell cycles were synchronized at the time of fusion.

Fusion of blastomeres and cytoplasmic fragments (see Fig. 3)

a4.2 blastomeres and cytoplasmic fragments were transferred to plastic dishes filled with MFSW. The bottom of the dishes was coated with 0.9% agar containing small holes. A blastomere and a fragment were placed into each hole and allowed to establish close contact. 40% (w/v) polyethylene glycerol (PEG 6000; Wako Ltd, Osaka) in water, which has higher specific gravity than MFSW, was placed into the hole and, after a 30 seconds incubation, the blastomere and fragment, which were firmly adherent, were transferred to MFSW. Fusion never occurred after PEG treatment only. Consequently, an electric pulse was employed to trigger fusion. [The PEG treatment was necessary to assure adhesion of cells and fragments during the application of the electrical pulse.] Adhering cells were transferred to the fusion medium (0.77 M D-mannitol in 0.25% Ca^{2+}- and Mg^{2+}-free artificial sea water) and aligned between electrodes with the plane of adhesion perpendicular to the electric field. Electrodes were carbon rods with a bare area (1×5 mm^2) exposed to the fusion medium, the remainder being covered with acrylic resin. The distance between electrodes was 5 mm, and their resistance was 20 kohms in the fusion medium. A single rectangular pulse of 110 V/cm was applied for 10-20 μseconds, the cells were then immediately transferred to sea water. Embryos were usually reared at 9-13°C, but fused cells were always maintained at 9°C from fusion to first division in order to ensure sufficient time for the division apparatus to develop. This initial low temperature incubation was essential because rearing fused blastomeres at 13°C resulted in partial cleavage furrows which appeared only in the region that had been the a4.2 blastomere. At 9°C, *Halocynthia* embryos develop normally. Fused blastomeres divided with a normal cell cycle duration and were allowed to develop until unoperated larvae hatched.

Markers for muscle differentiation

Differentiation of muscle in embryos that were derived from fused cells was evaluated by expression of three muscle-specific products, myosin, actin and acetylcholinesterase (AchE). The monoclonal antibody, Mu-2, specifically recognizes the myosin heavy chain of ascidians (Nishikata et al., 1987b; Makabe and Satoh, 1989). Immunohistochemical staining was carried out by the standard methods (e.g., Nishida, 1990). Embryos were stained for indirect immunofluorescence with Mu-2 antibody and FITC-conjugated secondary antibody.

In some experiments, muscle formation was monitored by the presence of actin and AchE. 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin (Wako Chemicals Ltd. Osaka), a drug that binds specifically to filamentous actin and emits yellow fluorescence, was employed; embryo staining was as described previously (Nishida, 1990).

Histochemical detection of AchE was carried out according to Karnovsky and Roots (1964) after embryos were fixed for 10 minutes in 5% formalin in seawater at room temperature. The reaction results in the formation of brown deposits. A characteristic of the three staining methods is the generation of an intense signal, restricted to differentiated muscle cells of the tail (see Nishida, 1990; Nishida, 1992a for staining of whole-mount specimens of *Halocynthia* larvae).

Results

Fusion of presumptive-epidermis blastomeres and cytoplasmic fragments of blastomeres from 8-cell embryos

To identify cytoplasmic regions capable of promoting muscle differentiation in 8-cell embryos, initial experiments were carried out in which presumptive-epidermis blastomeres (a4.2; Fig. 1) were fused with cytoplasmic fragments from various regions of blastomeres derived from 8-cell embryos. The a4.2 blastomere was used as a recipient in this case as it normally gives rise to the epidermis and

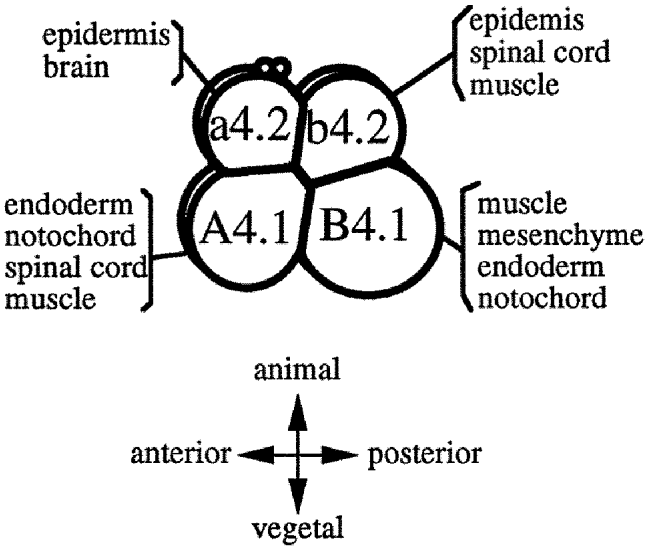

Fig. 1. Lateral view of the bilaterally symmetrical 8-cell embryo of *Halocynthia roretzi* demonstrating the orientation of its blastomeres and major descendant tissues (Nishida and Satoh, 1983).

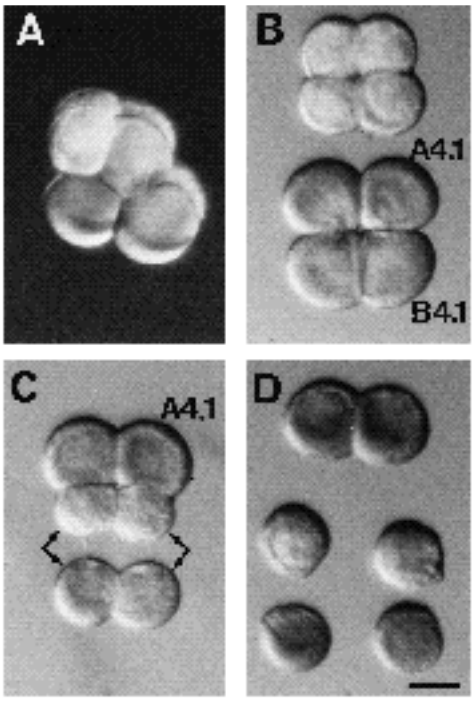

Fig. 2. Preparation of anterior and posterior fragments of muscle lineage (B4.1) cells of the 8-cell embryo. (A) Lateral view of a 8-cell embryo. The orientation is same as in Fig. 1. (B) Animal (upper in this photograph) and vegetal (lower) quartets were separated by a fine glass needle. Anterior is at the top of the photograph. (C) The B4.1 blastomere pair was bisected into anterior and posterior halves (double-headed arrows), using the A4.1 blastomere pair as an indicator of anterior pole. (D) All the B4.1 fragments were separated from each other and from A4.1. Scale bar, 100 μm.

brain of tadpole larvae. Additionally, isolated a4.2 blastomeres autonomously develop into epidermis (Nishikata et al., 1987a) but never express myosin as detected by Mu-2 antibody (Nishikata et al., 1987b).

To prepare cytoplasmic fragments of muscle-lineage cells derived from 8-cell embryos (Fig. 2A), vegetal quartets were isolated from embryos with a fine glass needle (Fig. 2B). Using the A4.1 blastomere pair as an indicator of the anterior pole, B4.1 blastomere pairs (primary muscle precursors) were bisected into anterior and posterior halves (Fig. 2C). Finally all the B4.1 fragments were separated from each other and from A4.1 blastomeres (Fig. 2D). The anterior and posterior fragments were cultured and, after 45 minutes, one of them divided. Non-divided fragments were used for fusions as enucleated cytoplasmic fragments, the divided fragments were discarded. The ability to distinguish nucleated and enucleated fragments by this method was verified by staining fragments produced in the same way with the fluorescent dye for DNA, DAPI. None of the 14 non-divided fragments had a nucleus, while all of the 14 divided fragments had nuclei. a4.2 blastomeres were isolated from a second group of 8-cell embryos which were fertilized 45 minutes (one cell cycle at 13°C) after the insemination of the first group. Thus, cell cycles of blastomeres and cytoplasmic fragments were synchronized.

Using polyethylene glycol and electric field-mediated fusion (PGEF-mediated fusion; Fig. 3), blastomeres and cytoplasmic fragments were fused with a high success rate (average 89%; Table 1). This is an important consideration as the number of blastomeres and cytoplasmic fragments capable of being prepared within the period employed here was limited. When one pole of a fused cell was injured by a tungsten needle, the whole fused cell cytolized as if it was a single cell, confirming that fusion had occurred. Fused blastomeres continued to divide and developed into multicellular embryos (average 85% of cases).

Table 1. *Myosin expression of fused blastomeres*

Cytoplasmic fragments	Operated specimens	Successfully fused	Successfully divided	Myosin expression
(a) 8-cell stage				
B4.1 posterior	69	55	42	41 (98%)
B4.1 anterior	54	41	33	0 (0%)
a4.2	46	44	41	0 (0%)
(b) Before fertilization				
unfertilized egg	62	58	40	7 (18%)
(c) First phase of ooplasmic segregation				
vegetal	58	50	41	41 (100%)
animal	61	59	58	0 (0%)
equatorial	56	50	43	0 (0%)
(d) Second phase of ooplasmic segregation				
posterior	71	62	47	45 (96%)
anterior	61	53	40	0 (0%)
animal	63	57	55	0 (0%)
vegetal	66	57	54	3 (6%)
lateral	54	49	44	0 (0%)

Myosin heavy chain was detected by immunofluorescence with a monoclonal antibody. No distinction was made between anterior and posterior origin of cytoplasmic fragments of a4.2 blastomeres. Unfertilized eggs were cut without orientation. In d, results of fusion of both of the left and right fragments are shown together as lateral fragments. The three positive specimens obtained by vegetal fragment fusion in d may be the result of a cutting plane that was inclined in the posterior direction.

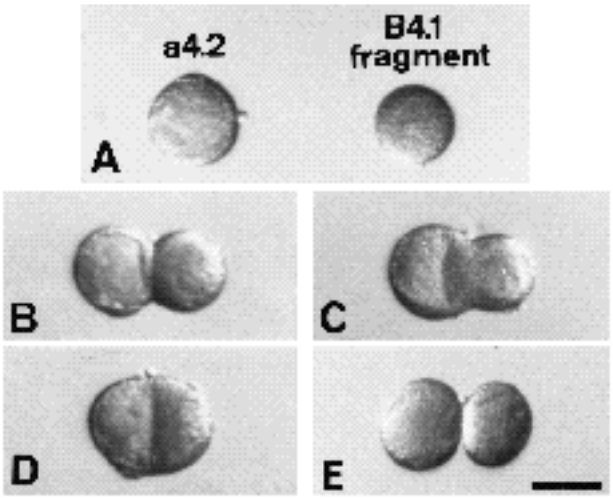

Fig. 3. Fusion of an a4.2 blastomere and a cytoplasmic fragment of a B4.1 blastomere. (A) Isolated a4.2 blastomere and B4.1 cytoplasmic fragment before fusion. (B) Specimens adhere after treatment with polyethylene glycol. (C) 3 minutes after an electric pulse was applied; initiation of fusion. In this specimen, cytoplasm of the B4.1 fragments is flowing into the a4.2 blastomere. (D) 8 minutes after the electrical pulse, fusion is completed and the fused cells are coalescing to form a single spherical mass. The boundary between cytoplasm,which originated from the a4.2 cell, and the B4.1 fragment is visible. After a while it could not be recognized and yolk granules were distributed evenly. Then a clear region appeared in the center, probably due to the mitotic spindle. (E) 53 minutes after the electrical pulse, the resultant fused cell divides at the same time as isolated but unfused a4.2 blastomeres divide. Such fusion products continued to divide and developed into multicellular embryos. Scale bar, 100 μm.

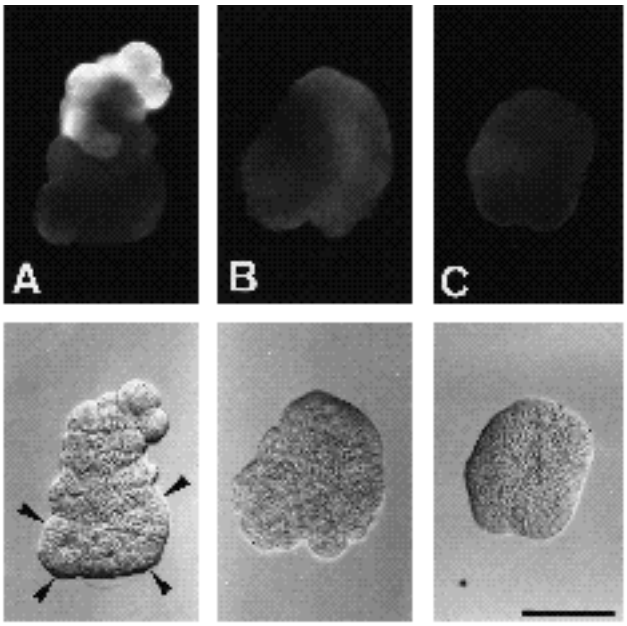

Fig. 4. Expression of myosin heavy chains in embryos derived from fused blastomeres, as detected by immunofluorescence with a monoclonal antibody. Isolated a4.2 blastomeres were fused with cytoplasmic fragments derived from (A) the posterior region of B4.1 blastomeres, (B) the anterior region of B4.1 and (C) a4.2 from 8-cell embryos. Upper photographs are fluorescent images; lower photographs are corresponding Nomarski images. The epidermal region is indicated by arrowheads. Scale bar, 100 μm.

Isolated a4.2 blastomeres that were fused with posterior cytoplasmic fragments of B4.1 cells continued to divide with normal cell cycle duration and were allowed to develop until unoperated embryos hatched. Overt muscle cell differentiation was observed in 98% of cases when muscle cells were identified with a monoclonal antibody against ascidian myosin (Table 1a). In such cases, cells expressing myosin lined one pole of the embryonic surface and were in a small cluster (Fig. 4A). Such myosin-expressing cells were larger than neighboring non-muscle cells, which was the case for muscle cells in unoperated larvae. Differentiation of epidermis always occurred in other parts of the embryos (Fig. 4, arrowheads). Epidermal areas possessed a smooth surface and secreted transparent larval tunic materials. Epidermis differentiation in these cases is noteworthy as it confirms that the recipient of cytoplasm was an epidermis-precursor cell. In contrast, fusion of anterior cytoplasmic fragments of B4.1 never resulted in muscle differentiation (Fig. 4B). In control experiments, a4.2 blastomeres were fused with cytoplasmic fragments of other a4.2 blastomeres. No muscle differentiation was observed (Fig. 4C). The fused blastomeres developed into permanent blastulae, balls of epidermis, which were morphologically indistinguishable from embryos derived from isolated a4.2 cells. These results suggest that (1) the ability to promote muscle differentiation is associated with the cytoplasm of muscle-precursor blastomeres and not epidermal precursors, and (2) the active agent is localized in the posterior region of B4.1, where primary muscle-lineage cells are formed after successive cleavages. The anterior region of B4.1 primarily contains components involved with the formation of endoderm.

Fusion of presumptive-epidermis blastomeres and various cytoplasmic egg fragments

The ooplasm of ascidian eggs undergoes dramatic movements after fertilization, namely ooplasmic segregation (Conklin, 1905b). Movement of ooplasm that is inherited by muscle-lineage blastomeres during cleavage and ultimately by muscle cells of larva, has been especially well described in several ascidians (Conklin, 1905b; Sawada and Osanal, 1981; Jeffery and Meier, 1983; Sardet et al., 1989). This ooplasm is referred to as the myoplasm (Conklin, 1905a). In some species, pigment granules (e.g., the yellow pigment in *Styela*) are concentrated in myoplasm, so that this portion of the embryonic cytoplasm is easily distinguished from other embryonic regions. In *Halocynthia* eggs, the myoplasm is a more transparent region of the egg cytoplasm (Hirai, 1941) and located in the cortex of unfertilized eggs (Fig. 5A). The first phase of ooplasmic segregation (0-10 minutes after insemination at 9°C) is accompanied by a rapid contraction of the egg cortex, resulting in a segregation of myoplasm to the vegetal pole (Fig. 5B). During the second phase of segregation (85-110 minutes), the myoplasm moves towards the future posterior pole,

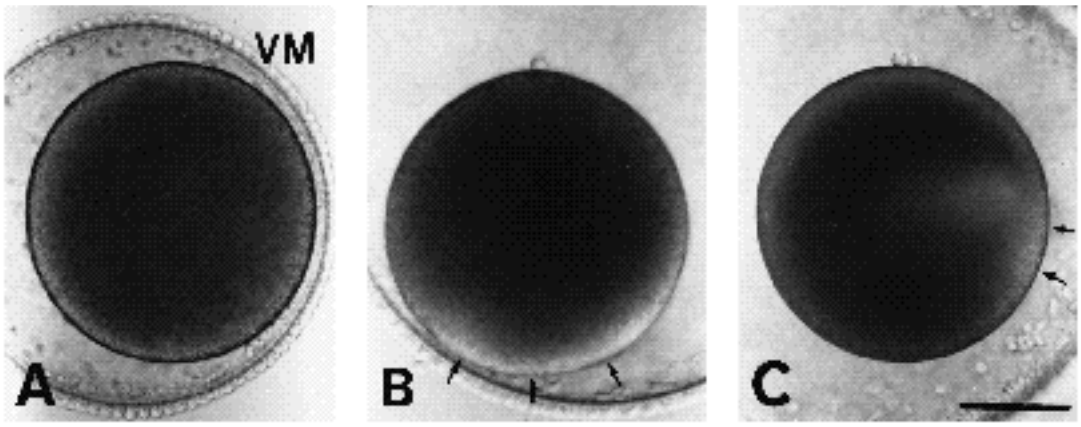

Fig. 5. Ooplamic segregation in *Halocynthia* eggs. (A) Unfertilized egg. Note the lack of visible clues as to the polarity of the unfertilized egg. VM, vitelline membrane (chorion). (B) A fertilized egg after the first phase of segregation. 45 minutes after fertilization at 9°C. The transparent myoplasm (arrows) has moved towards the vegetal pole. The first polar body is visible at the animal pole. (C) An egg after the second phase of segregation, 120 minutes after fertilization. The myoplasm (arrows) have moved towards the future posterior pole. The second polar body is present at the animal pole. Scale bar, 100 μm.

together with the sperm aster, and forms a crescent-shaped domain (Fig. 5C). First cleavage occurs at 160 minutes at 9°C.

The presence and localization of cytoplasm having muscle-promoting ability was investigated in uncleaved eggs. Fragments of eggs, approximately equal in volume to that of an a4.2 blastomere, were prepared before fertilization, after the first phase of segregation (20-50 minutes after fertilization), and after the second phase of segregation (110-130 minutes; Fig. 6). Except for fragments of unfertilized eggs, a4.2 blastomeres and egg fragments were prepared from eggs fertilized at different times so that cell cycles of a4.2 cells and egg fragments were synchronized at the time of fusion.

In unfertilized eggs, the first meiotic spindle is situated at the animal pole and is difficult to discern with stereomicroscopy. Consequently, fragments were made without orientating and were found to contain female nuclei in 3 out of 21 cases when examined by DAPI staining of fixed specimens. Because of the inability to discern female nuclei in unfixed unstained fragments, all fragments were used for fusion. The electrical pulse used here activated unfertilized eggs. Similarly, the electrical pulse activated unfertilized egg fragments during fusion with a4.2 cells. Cortical contractions were observed in the area that had been egg fragments following application of the electrical pulse. The fused cells divided, although the first division was delayed when compared to the duration of the normal cell cycle. Eventually, fused cells developed into multicellular embryos, which expressed myosin in 18% of the cases (Table 1b, Fig. 7A).

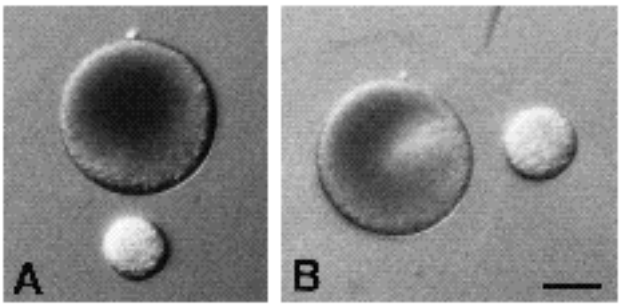

Fig. 6. Preparation of egg fragments. (A) Vegetal fragment, containing myoplasm and severed from an egg after the first phase of segregation, is depicted. (B) Posterior fragment, containing myoplasm and severed from an egg after the second phase of segregation, is shown to the right. Scale bar, 100 μm.

After preparation of fragments from fertilized eggs, only fragments whose egg counterparts divided were fused with isolated a4.2 blastomeres (Table 1c and d). Although animal fragments derived from eggs after the first phase of segregation never divided, they always contained female nuclei and were used for fusion. Normally, sperm nuclei are present near the vegetal cortex before the second phase of segregation; consequently, vegetal fragments made before this event occasionally divided. Fragments that divided were discarded. Because male and female pronuclei move to the center of egg during the second phase of segregation of myoplasm, none of the fragments derived from eggs after this period divided. Other than fragments derived from the animal pole of eggs after first phase of segregation, the absence of nuclei in the fragments was confirmed by nuclear staining with DAPI in more than ten fixed specimens of each kind of the fragments. Out of a total of 108 specimens, no fragment had a nucleus.

After the first phase of segregation, eggs were radially symmetrical along the animal-vegetal axis, and possessed a first polar body at the animal pole and myoplasm at the vegetal pole. From such specimens, animal, equatorial and vegetal fragments were prepared for fusion (Table 1c, Fig. 7B-D). Resultant fused cells divided with a normal cell cycle duration. Embryos that were derived from the fusion of vegetal fragments with a4.2 cells expressed myosin in all cases. In contrast, fusion of animal or equatorial fragments with a4.2 cells never resulted in myosin expression.

After the second phase of segregation, myoplasm was situated in the future posterior region of the embryo and

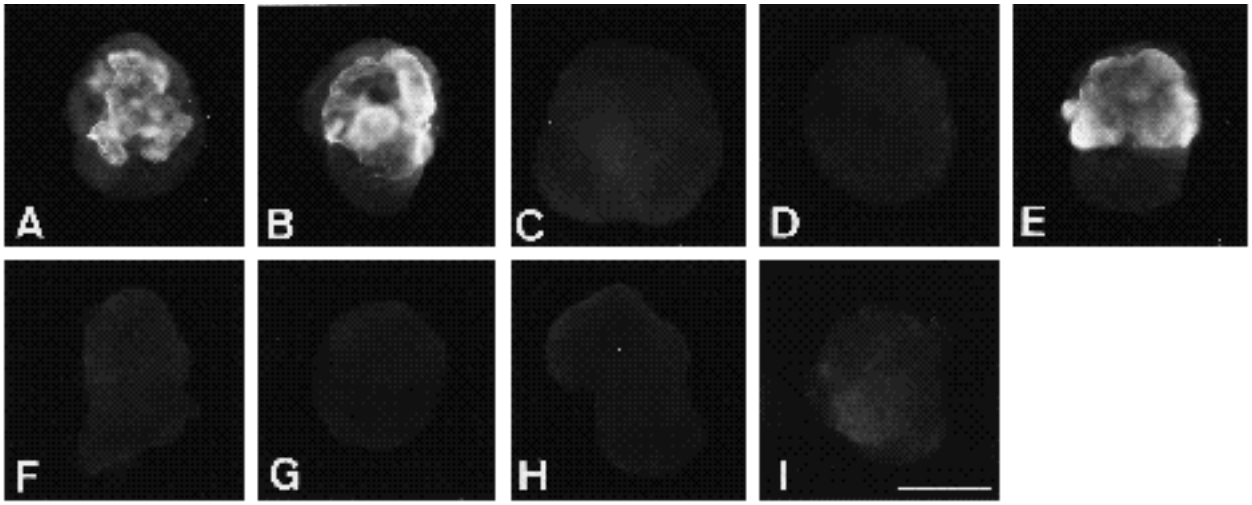

Fig. 7. Expression of myosin heavy chains as detected by immunofluorescence with a monoclonal antibody in embryos derived from fused blastomeres. Isolated a4.2 blastomeres were fused with various cytoplasmic fragments derived from (A) unfertilized eggs; (B) the vegetal region, (C) the animal region, and (D) the equatorial region of fertilized eggs at the end of the first phase of ooplasmic segregation; (E) the posterior region, (F) the anterior region, (G) the animal region, (H) the vegetal region, and (I) the lateral region of fertilized eggs at end of the second phase of segregation. Evidence of muscle cell differentiation is observed in A, B and E. Scale bar, 100 μm.

eggs were bilaterally symmetrical. The results of experiments employing animal, vegetal, anterior, posterior and lateral fragments are shown in Table 1d, and Fig. 7E-I. Among them, only fusion of posterior fragments resulted in expression of myosin at a high frequency (96%). When a4.2 cells were fused with fragments of unfertilized eggs, vegetal fragments after the first phase of segregation and posterior fragments after the second phase of segregation, large myosin-expressing cells developed as a single cluster. Differentiation of epidermis also occurred in these cases. In over one-half of the embryos, myosin-expressing cells were located within the embryos and were surrounded by an epidermis (Fig. 7A,B,E).

Expression of additional markers of muscle differentiation in fused cells

To examine the expression of other markers of muscle differentiation, the presence of filamentous actin (F-actin) and acetylcholinesterase (AchE) were monitored in the embryos derived from the fusion of the a4.2 cells with posterior and anterior fragments after the second phase of segregation (Table 2, Fig. 8). When the formation of F-actin was monitored using NBD-phallacidin, intensive staining was observed in 95% of cases in which a4.2 cells were fused with posterior fragments. F-actin-containing cells were formed as a single cluster. F-actin was found in the periphery of each cell (Fig. 8A). The peripheral localization of F-actin and myofibrils is a characteristic feature of muscle cells of normal larvae (Pucci-Minafra, 1965; Cavey and Cloney, 1972; Terakado, 1972; Nishida, 1990). In contrast, none of the embryos formed F-actin when a4.2 cells were fused with the anterior fragments (Fig. 8B).

The presence of AchE was evaluated histochemically in embryos derived from fused cells. Deno et al. (1985) reported that the isolated a4.2 cells of *Halocynthia* some-

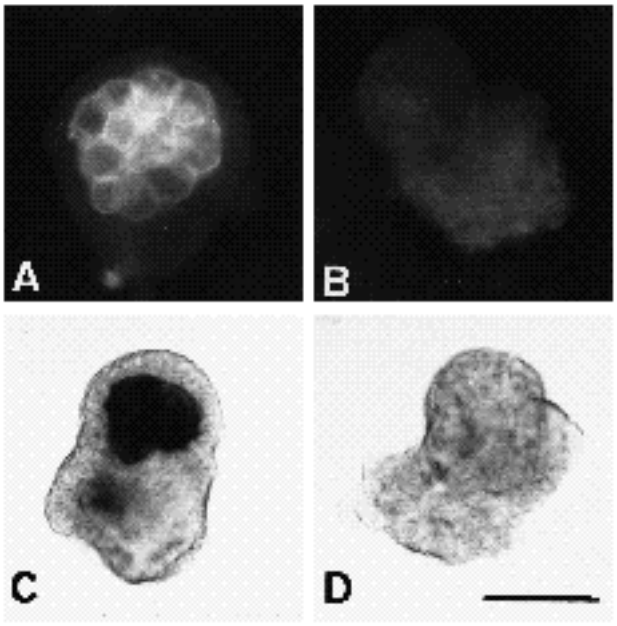

Fig. 8. Expression of markers of muscle differentiation. (A, B) Embryos were stained by NBD-phallacidin to demonstrate F-actin. (A) Isolated a4.2 cell fused with the posterior fragment of a egg after the second phase of segregation. Within the embryo, some cells show a fluorescence typical of NBD-phallacidin. (B) Embryo derived from the fusion of an isolated a4.2 cell and the anterior fragment of an egg after the second phase of segregation. (C, D) Embryos histochemically stained for AchE. (C) Isolate a4.2 cell fused with the posterior fragment of an egg after the second phase of segregation. Within the embryo, cells possess AchE reaction product. (D) Embryo derived from the fusion of an isolated a4.2 cell and the anterior fragment of egg after the second phase of segregation. Scale bar, 100 μm.

Table 2. *Actin and AchE expression of fused blastomeres*

Cytoplasmic fragments	Operated specimens	Successfully fused	Successfully divided	Expression of markers
(a) F-actin				
2nd phase posterior	25	25	22	21 (95%)
2nd phase anterior	30	28	23	0 (0%)
(b) AchE				
2nd phase posterior	24	24	21	20 (95%)
2nd phase anterior	28	24	21	0 (0%)

Filamentous actin was detected with NBD-phallacidin. AchE was detected histochemically.

times (4% of cases) expressed AchE when partial embryos were prepared for histochemistry at a time corresponding to the late tailbud stage. This expression of AchE in partial embryos may correspond to the staining of some cells in the head region of normal larvae that originate from a4.2 blastomeres. However, when fixation and staining were carried out by the middle tailbud stage, only muscle cells were stained in normal embryos, and only partial embryos that were derived from muscle lineage blastomeres developed AchE; those from the a4.2 cells did not. Therefore, in this study embryos were stained at a period corresponding to the middle tailbud stage. Embryos that were derived from the fusion of a4.2 cells and posterior fragments expressed AchE activity in 95% of cases (Fig. 8C). In contrast, those that were derived from the fusion of anterior fragments never expressed AchE activity (Fig. 8D).

Discussion

Results of experiments in which muscle differentiation was evaluated by expression of myosin heavy chain are summarized in Fig. 9. Cytoplasm that has the ability to promote myosin expression in presumptive-epidermis blastomeres was localized to the posterior region of B4.1 cells, a region from which primary muscle are normally formed (Conklin, 1905b; Ortolani, 1955; Nishida, 1987). A regional difference was apparent in the cytoplasm's ability to promote myosin expression when fragments from various regions of eggs were fused to presumptive-epidermis cells (Fig. 9B-D). Fusion of fragments of unfertilized eggs with a4.2 cells resulted in myosin expression in only 18% of the cases examined. This low rate may reflect: (1) cytoplasm with muscle-promoting ability is not concentrated in unfertilized eggs, or (2) because unfertilized eggs were cut without knowledge of their polarity, factors necessary for myosin expression may have been missed in many cases as a result of the randomness of the microsurgery. Reververi and Ortolani (1962) suggest that determinants are uniformly distributed in unfertilized eggs, because twin larvae develop from halves of the same unfertilized egg regardless of the direction of bisection of the egg.

When fertilized eggs were bisected horizontally, the animal fragments did not develop normally, suggesting that ooplasmic determinants become asymmetrically distributed after fertilization (Ortolani, 1958). In this study, it is shown that after the first phase of segregation, only vegetal fragments have the ability to induce myosin expression. After the second phase, only the posterior fragments have this ability. The results obtained are almost all or none manner, and unambiguously demonstrate regional differences in the egg cytoplasm. It is suggested that muscle determinants are already present and localized in uncleaved egg, and that their movements correspond to the movement of myoplasm. Following the completion of ooplasmic segregation, and before first cleavage, the determinants moved to the site of primary muscle-lineage cell formation. Posterior fragments of eggs, after the second phase of segregation, have the ability to promote development not only of myosin but also of F-actin and AchE. Therefore, several kinds of marker of

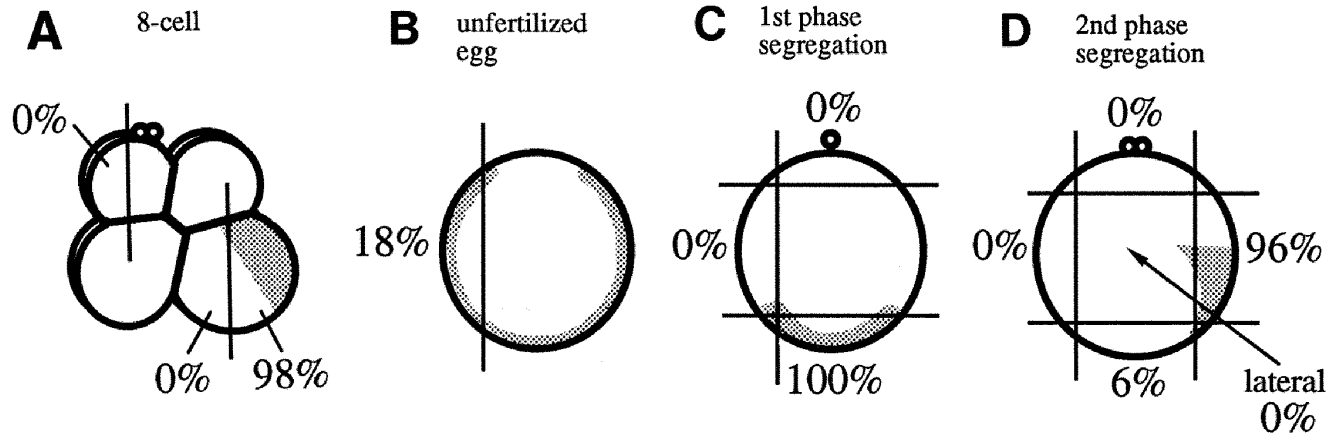

Fig. 9. Schematic representation of the myoplasm localization (shaded) during ooplasmic segregation and at the 8-cell stage. Planes of section are shown and results of the fusion described herein are presented as percentages of specimens that showed myosin expression. Small circles at the animal pole of eggs and embryo represent polar bodies. (A) Lateral view of 8-cell embryo. (B) Unfertilized egg. (C) Egg after the first phase of segregation; it is radially symmetrical along the animal-vegetal axis. (D) Lateral view of an egg after the second phase of segregation in which it has become bilaterally symmetrical. Results of fusion of both the left and right fragments are shown together in lateral fragments. In A and D, the anterior pole is at the left of the embryo.

muscle differentiation were promoted by the introduction of cytoplasm from the posterior egg fragment.

Primary muscle lineage cells in isolation develop multiple markers of muscle differentiation. Even when dissociated embryonic cells of ascidians are cultured from first cleavage, differentiation of primary muscle cells occurs (Nishida, 1992b). Results of experiments with isolated and dissociated blastomeres indicate that fate determination and initial events of muscle cell differentiation are autonomous. That is, these processes do not require cellular interaction or communication with cells from the other lineages. Therefore, muscle determinants, which are segregated into muscle-lineage cells, appear to be sufficient for determination of the fate and expression of characteristics of the primary lineage.

On the other hand, in normal development, secondary muscle lineage cells are generated in the lateral region of embryos. Embryos that were derived from the fusion of lateral fragments of eggs after the second phase of segregation did not develop features characteristic of muscle cells. This observation supports the idea that development of secondary muscle cells are mediated by interactions with cells from other lineages (Meedel et al., 1987; Nishida, 1990).

Because a4.2 blastomeres are epidermis-precursor cells, it is not unexpected that differentiation of epidermis always occurred in embryos derived from the fusion between an a4.2 blastomere and a cytoplasmic fragment. Cytoplasm introduced by fusion neither suppressed epidermal fate nor totally changed the fate of recipient cells. Differentiated muscle cells were formed as a single cluster in the embryo. Therefore, introduced muscle determinants may not diffuse in fused cells, and they may only be inherited by some descendant cells. As suggested by results of other studies (Conklin, 1931; Jeffery and Meier, 1983), they may also bind to the cytoplasmic skeleton. If correct, this possibility may have led to difficulties in transfer of muscle determinants with micropipettes (Deno and Satoh, 1984).

As yet, elucidation of the identity, mode of action and manner of localization of cytoplasmic determinants awaits their isolation and characterization. Visible pigment granules and mitochondria are concentrated in the myoplasm. Normal larvae develop from bisected and myoplasm-enriched egg fragments (Bates, 1988). Despite the redistribution of yellow pigment into endoderm cells in these cases, expression of myosin and AchE is restricted to muscle cells of the tail. Zygotes of *Molgula arenata*, an anural ascidian, do not display mitochondrial localization, but still express AchE in the muscle progenitor cells of a vestigial tail (Whittaker, 1979). These observations suggest that pigment granules and mitochondria are not muscle determinants. Myosin heavy chains (Makabe et al., 1990), muscle actin (Tomlinson et al., 1987; Kusakabe et al., 1991), and AchE (Perry and Melton, 1983; Meedel and Whittaker, 1983) genes are expressed at the gastrula stage, suggesting that muscle determinants may be regulatory molecules, responsible for activation of a cascade of specific genes that ultimately results in synthesis of muscle-specific proteins. Determinants might be localized molecules or localized active forms of widely distributed molecules.

In insects, cytoplasmic determinants for the formation of anteroposterior and dorsoventral axes (Ingham, 1988) and determinants for germ cells (Illmensee and Mahowald, 1974) are present in the ooplasm. The results of this study provide further support for the existence of cytoplasmic determinants that directly specify somatic tissue types in ascidian eggs. By our novel fusion method, blastomeres and cytoplasmic fragments can be fused in a fashion involving specific numbers and types of cells and fragments. Advantages of the present method are: (1) donors and recipients of cytoplasm can be chosen from desired parts of embryos and eggs, and (2) a large volume of cytoplasm can be transferred compared to microinjection methods. The fusion method will facilitate investigations of cytoplasmic determinants of tissues other than muscle in ascidians, as well as the search for cytoplasmic determinants in eggs of other animals.

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