Localization of determinants for formation of the anterior-posterior axis in eggs of the ascidian *Halocynthia roretzi*

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**SUMMARY**

Unfertilized eggs of the ascidian *Halocynthia roretzi* are radially symmetrical along the animal-vegetal axis. After fertilization, ooplasmic segregation results in formation of an anterior-posterior axis horizontally, and eggs become bilaterally symmetrical. When 8-15% of the cytoplasm of the posterior-vegetal region of the egg was removed after the second phase of ooplasmic segregation, most of the embryos completed gastrulation but developed into radialized larvae along the animal-vegetal axis with no apparent anterior-posterior axis. Removal of cytoplasm from other regions did not affect formation of this latter axis. The cleavage pattern of the embryos that were deficient in posterior-vegetal cytoplasm (PVC) exhibited radial symmetry instead of the complicated bilateral symmetry of normal embryos. Detailed comparisons of cleavage patterns revealed the duplication of the anterior cleavage pattern in the originally posterior halves of the PVC-deficient embryos. The PVC-deficient larvae lacked muscle cells, which are normally derived from the posterior blastomeres. Examination of the developmental fates of the early blastomeres of the PVC-deficient embryos revealed that all of the vegetal blastomeres had assumed anterior fates. These results suggest that the PVC-deficient embryos are totally anteriorized.

When posterior-vegetal cytoplasm was transplanted to the anterior-vegetal position of PVC-deficient eggs, the axial deficiency was overcome, and reversal of the anterior-posterior axis was observed. The results of transplantation of posterior-vegetal cytoplasm to the anterior-vegetal position in normal eggs demonstrated that formation of the anterior structure is suppressed by posterior-vegetal cytoplasm. These results suggest that posterior fate is specified by the presence of posterior-vegetal cytoplasm, while anterior fate is specified by the absence of posterior-vegetal cytoplasm. Thus, posterior-vegetal cytoplasm determines the anterior-posterior axis by generating the posterior cleavage pattern and conferring posterior fates on cells, as well as by inhibiting anterior fates that would otherwise occur by default.

Key words: ascidian embryogenesis, axis determination, cytoplasmic determinants, cleavage pattern, cytoplasmic transfer

**INTRODUCTION**

In the unfertilized eggs of many kinds of animal, there is a single axis, namely, the animal-vegetal axis, and eggs exhibit radial symmetry along the axis. The second axis is established just after fertilization or during early embryogenesis, and embryos become bilaterally symmetrical. In the ascidian egg, the second axis, which corresponds to the future anterior-posterior axis, is generated during ooplasmic segregation. Movements of the ooplasm progress in two phases between fertilization and the first cleavage (Conklin, 1905; Hirai, 1941; Sawada and Osanai, 1981; Jeffery and Meier, 1983; Sardet et al., 1989). During the second phase of ooplasmic segregation, vegetally located cytoplasm moves towards the future posterior pole (Fig. 1). This movement is the first observable evidence of an anterior-posterior axis during ascidian embryogenesis.

The study of ascidian embryogenesis has provided various types of evidence for the presence of localized maternal factors, in specific regions of the egg cytoplasm, that play important roles in the determination of developmental fates during early embryogenesis (reviewed by Venuti and Jeffery, 1986; Meedel, 1992; Nishida, 1992b; Satoh, 1993). Indeed, experiments involving redistribution of cytoplasm and transfer of cytoplasm have revealed the presence of cytoplasmic determinants that confer on muscle-, endoderm- and epidermis-lineage cells the ability to become muscle, endoderm, and epidermis, respectively (Whittaker, 1980, 1982; Deno and Satoh, 1984; Nishida, 1992a, 1993, 1994). These three kinds of cytoplasmic determinant move in different directions during ooplasmic segregation. Prior to the onset of the first cleavage, three kinds of determinant settle at sites corresponding to specific regions of the future bilateral fate map (Nishida, 1994).

In addition to determinants of cell type, cytoplasmic determinants for gastrulation movements are also present in the ooplasm. Ortolani (1958) bisected fertilized eggs near the equator and showed that the animal fragments developed into permanent blastulae. Bates and Jeffery (1987) showed that when small region of vegetal-pole cytoplasm was removed from the zygote between the first and second phase of segregation, the embryos fail to gastrulate. The removal of cytoplasm at the vegetal pole could be mimicked by UV irradiation of the fertilized egg near the vegetal pole (Jeffery,
MATERIALS AND METHODS

Embryos
Naturally spawned eggs of *Halocynthia roretzi* were artificially fertilized and then manually dechorionated with sharpened tungsten needles. To facilitate normal development, dechorionated embryos were reared in the supernatant of a homogenate of cleaving embryos that contained 50 µg/ml streptomycin sulfate and 50 µg/ml kanamycin sulfate (Nishida and Satoh, 1985). At 13°C, tadpole larvae hatched about 35 hours after fertilization. The temperature was lowered to 9°C to lengthen the duration of the stage required for microsurgery.

Histology
Eggs undergoing ooplasmic segregation were fixed in Bouin’s fluid. The fixed specimens were dehydrated through an ethanol series and cleared with xylene. The cleared specimens were embedded in paraffin, sectioned at 8 µm and attached to glass slides. Milligan’s trichrome staining was performed essentially as described by Jeffery (1989). After treatment with xylene to remove paraffin, sections were rinsed successively in 100% and 95% ethanol, allowed to mordant in potassium dichromate-HCl for 5 minutes, and stained with acid fuchsin for 8 minutes. The stain was fixed in phosphomolybdic acid for 3 minutes, and then slides were counterstained with orange G for 10 minutes and with fast green for 15 minutes. They were then washed with 1% acetic acid for 3 minutes. The stained sections were dehydrated in 95% and 100% ethanol, cleared in xylene and mounted in Parmount (Fisher Scientific, New Jersey).

Removal of egg fragments
Fertilized eggs after the second phase of ooplasmic segregation were oriented for microsurgery using the positions of the polar bodies and the transparent myoplasm as markers (Fig. 1; Nishida, 1992a). Then, fragments of various regions were severed from eggs with a glass needle under a stereomicroscope (SZH-10; Olympus). The volumes of the egg fragments that had been removed were calculated from their diameters, which were measured with an ocular micrometer.

Markers of the differentiation of specific tissues
Differentiation of epidermis was evaluated by monitoring the expression of the Epi-2 antigen, which is specifically recognized by a monoclonal antibody (Nishikata and Satoh, 1990). All the monoclonal antibodies were generously provided by Dr T. Nishikata (Konan University, Kobe, Japan). Indirect immunofluorescence staining with monoclonal antibodies was carried out by standard methods using FITC-conjugated secondary antibody.

Scanning electron microscopy (SEM)
Embryos were fixed in 2.5% glutaraldehyde in seawater for 1 hour at room temperature, and then they were dehydrated through a graded ethanol series. Critical-point drying was performed with CO2 in a model HCP-1 (Hitachi) apparatus. The dried embryos were oriented on aluminum stubs and coated with a thin layer of gold using a ion coater (model IB-2; Eiko Engineering). Samples were observed and photographed with a MINI-SEM (Hitachi-Akashi).

Isolation of blastomeres and inhibition of cell division
Each blastomere was isolated with a fine glass needle at the 8-cell stage. Blastomeres were cultured separately in agar-coated plastic dishes until controls hatched. To inhibit cell division, 110-cell embryos were cultured in seawater that contained cytochalasin B (Aldrich Chemical Co.) at a final concentration of 2.5 µg/ml. Arrested embryos were reared until control embryos reached the middle-tailbud stage and then they were processed for immunostaining with a notochord-specific monoclonal antibody.

Transplantation of egg cytoplasm by cell fusion
Egg fragments that had been severed from eggs were fused to anterior or posterior regions of egg cells by polyethylene glycol- and electric field-mediated fusion (PGEF-mediated fusion), as described previously (Nishida, 1994). In brief, an egg fragment was allowed to adhere firmly to an egg cell at the desired position as a result of treatment with 30% (w/v) polyethylene glycol in water. Then a single rectangular electrical pulse of 800 V/cm was applied to the adhering egg fragment and egg cell for 10-20 useconds in fusion medium (0.77 M D-mannitol in 0.25% CaSO4). At 13°C, tadpole larvae hatched about 35 hours after fertilization. The temperature was lowered to 9°C to lengthen the duration of the stage required for microsurgery.

RESULTS

Ooplasmic segregation and the embryonic axis in *H. roretzi*
The ooplasm of ascidian eggs moves extensively after fertilization, and this process is known as ooplasmic segregation. Fig. 1 shows ooplasmic segregation in eggs of *H. roretzi*. Movements of the ooplasm occur in two phases between fertilization and the first cleavage. In unfertilized eggs, a first miotic spindle is located at the animal pole. Myoplasm, which is segregated to muscle-lineage cells during embryogenesis, is located in the cortical region of the egg. It can be recognized as clear cytoplasm in living eggs and as a deep red, yolkless region in sections stained with Milligan’s trichrome method (Fig. 1A,D), as is also the case in *Styela* (Jeffery, 1989). The first phase (0-10 minutes after insemination at 9°C) involves contraction of the plasma membrane and cortex in the direction of the vegetal pole and results in segregation of the myoplasm to the vegetal region. Eggs show radial symmetry along the animal-vegetal axis. During the second phase (85-110 minutes), the myoplasm moves towards the future posterior pole together with a sperm aster and forms a myoplasmic...
Ascidian A-P axis determinants

Ascidian A-P axis determinants

Fig. 1. Ooplasmic segregation in the eggs of Halocynthia roretzi. (A-C) Living eggs. (D-F) Paraffin sections of eggs stained by Milligan’s trichrome method. The animal pole is up and the vegetal pole is down. Yolk granules are stained green. (A,D) Unfertilized eggs. A first meiotic spindle is visible at the animal pole in the section. (B,E) Fertilized eggs after the first phase of ooplasmic segregation. Myoplasm, which is transparent in the living egg and is stained deep red in the section, has concentrated in the vegetal-pole region. (C, F) Eggs after the second phase of ooplasmic segregation. The anterior pole is to the left. The second polar body is present at the animal pole. The myoplasm has moved towards the future posterior pole. Arrow indicates the trail of the pronuclei. Solid line indicates the cutting plane for removal of a posterior-vegetal fragment of the egg. In the sectioned specimen, an aster surrounding the pronuclei is visible. Scale bar, 100 µm.

domain just vegetal of the equator. After the male pronucleus has encountered the female pronucleus at the posterior pole, they move together to the center of egg. The trail of the pronuclei is visible as a clear region (Fig. 1C, arrow) in living eggs. The myoplasm and the trail of the pronuclei were used as posterior markers for the orientation of eggs. During the second phase of ooplasmic segregation, the bilateral symmetry of the egg becomes established. The first cleavage occurs 160 minutes after fertilization.

In ascidians, the anterior-posterior axis (A-P axis) of eggs and embryos does not correspond precisely to the A-P axis of larvae because cells do not remain stationary during morphogenetic movements. In the animal hemisphere, the A-P axis of eggs and early embryos corresponds precisely to the A-P axis of larvae because positional rearrangement does not take place in the animal hemisphere. Anterior-animal blastomeres give rise to head epidermis of larvae and posterior-animal blastomeres develop into tail epidermis. In the vegetal hemisphere, anterior blastomeres, which are designated A-line cells (Conklin, 1905), mainly give rise to anterior endoderm, notochord, and spinal cord. Notochord and spinal cord cells are located in the larval tail. Posterior blastomeres, which are designated B-line cells, mainly develop into posterior endoderm, mesenchyme, and muscle. Muscle cells are found in the tail, while posterior endoderm and mesenchyme cells are found in the trunk region (Conklin, 1905; Nishida, 1987). In the present paper, ‘anterior’ and ‘posterior’ are used in the way that these adjectives have been traditionally applied to eggs and early embryos. Thus, in the present context, ‘anterior fate’ does not mean the fate that gives rise to the anterior structures of larvae.

Removal of cytoplasmic fragments after the second phase of ooplasmic segregation

Cytoplasmic fragments with volume equal to 8-15% of that of an entire egg were removed from various regions of fertilized eggs with a fine glass needle and the development of the manipulated eggs was examined. Between the first and second phase of ooplasmic segregation, removal of vegetal cytoplasm resulted in failure of gastrulation, as previously reported in Styela (Bates and Jeffery, 1987). Removal of cytoplasm from other regions did not affect normal development. After the second phase of ooplasmic segregation, removal of animal \( (n=59) \), anterior \( (n=66) \), lateral \( (n=56) \), and vegetal \( (n=49) \) cytoplasm had no effect on normal development (Fig. 2A). By
contrast, when posterior cytoplasm was removed, the eggs developed into malformed larvae in 43% (33 out of 76) of cases (see Fig. 2B,F). The malformed larvae did not have a distinct head and tail, and they seem to be radially symmetrical along one axis.

To achieve reproducibility of malformation, we carried out three series of experiments to determine the most effective timing of removal, as well as the position and volume of cytoplasm that was removed. Removal of the posterior-vegetal cytoplasm (Fig. 1C) was more effective than removal of the posteriormost cytoplasm. Removal of the posterior-animal cytoplasm did not result in malformation. The occurrence of the malformation was achieved with gradually increasing frequency when removal of posterior-vegetal cytoplasm was carried out at 95-125 minutes after fertilization at 9°C, and the frequency was constant for removal between 125 and 145 minutes. After 145 minutes, it was difficult to cut eggs, probably because of formation of the mitotic apparatus. When the posterior-vegetal cytoplasm was removed at 125-145 minutes, deletion of 8-15% of the total volume of the egg resulted in malformation in approximately 80% of cases, while removal of 5-8% of the egg volume resulted in malformation in about 35% of cases. Removal of ooplasm equivalent to more than 15% of the egg volume was associated with the possible removal of pronuclei. Therefore, in the experiments described hereafter, posterior-vegetal cytoplasm equivalent to 8-15% of the egg volume was removed 125-145 minutes after fertilization. We designated such eggs as eggs deficient in posterior-vegetal cytoplasm or PVC-deficient eggs.

The possibility that malformation was caused merely by disturbance of posterior cytoplasmic architecture during the microsurgery, was examined. When the cytoplasmic bridge between the egg and the fragment was extended until the egg and its fragment were connected by only a thin string, then the glass needle was withdrawn without actual cutting of the bridge, the fragment was retracted into the egg and the egg became spherical once again before cleavage occurred. In 81% of such cases, normal larvae developed. In the remaining 19% of cases, larvae showed some abnormalities. However, they all had a distinct head and tail. These results suggest that posterior-vegetal cytoplasm must, indeed, be removed from the egg to obtain a radialized larva.

PVC-deficient eggs developed into two morphologically distinct types of radialized larva. The larvae with the morphology shown in Fig. 2B were designated type A (70% of radialized larvae) and those shown in Fig. 2F were designated type B (30% of radialized larvae). Tissue differentiation was examined in these radialized larvae by monitoring the expression of various molecular markers. Expression of the Epi-2 antigen, which is a marker of epidermis, was observed in all type A and type B larvae. The Epi-2 antigen was localized in the outer epithelium (Fig. 2C,G). Alkaline phosphatase (AP) activity, which is a marker of endoderm, was observed within all of the type A and type B larvae (Fig. 2D,H). The Not-1 antigen, which is a marker of notochord, was also detected in all radialized larvae. In type A larvae, the innermost cell mass expressed the Not-1 antigen; in type B larvae, some notochord cells protruded from the larva (Fig. 2E,I). Such protrusion of cells was the main difference between type A and type B larvae. Muscle cell was scarcely observed when the expression of myosin (4% of 72 cases) and of acetycholinesterase (0% of 21 cases) was examined. Thus, the radialized larvae consisted of an outer epidermal layer, an intermediate endodermal layer, and an inner mass of notochord cells. They lacked muscle cells. Such organization of the three kinds of tissue was also recognized in specimens observed with Nomarsky optics (Fig. 2B,F). There were some unidentified cells in the larvae because markers for all cell types are not yet available. However, it is likely that these unidentified cells were cells of the spinal cord for the reason given in a later section.

Cleavage pattern and the development of radialized embryos

The cleavage pattern of ascidian embryos is unique and invariant (Conklin, 1905; Satoh, 1979). Cleavages progress in a bilaterally symmetrical manner. The cleavage pattern differs significantly between the anterior half and the posterior half of the embryo, especially in the vegetal hemisphere (Fig. 3). At the 8-cell stage, the cells of the posterior-vegetal blastomere pair, the B4.1 pair, protrude posteriorly from the embryos (Fig. 3B). Successively, only the cells of the most posterior blastomere pair at each stage undergo unequal cleavage. These cleavages occur three times prior to the 64-cell stage, producing smaller cells posteriorly (Fig. 3E,G).

In most of the PVC-deficient embryos, the cleavage pattern was totally changed and cleavages were radially symmetrical. At the 8-cell stage, there were no protruded blastomeres that resembled the B4.1 cells (Fig. 3C,D). At the 16- and 32-cell stages, the blastomeres were arranged radially. Therefore, no anterior-posterior axis was recognized in either the animal or the vegetal hemisphere (Fig. 3F,H), and no unequal cleavage was observed. Detailed comparisons of SEM images of embryos at the 76-cell stage (initial gastrula; Fig. 3L) and the 118-cell stage (middle gastrula; Fig. 3K,L,L′) revealed that the cleavage pattern had been anteriorized in the PVC-deficient embryos. In normal 76- and 118-cell embryos, the arrangement of anterior blastomeres is somewhat radial, while that of vegetal blastomeres is irregular (Conklin, 1905; Nishida, 1986). There is a row of eight spinal cord precursor blastomeres in the anterior margin of the vegetal hemisphere (arrowheads in Fig. 3L,K). Inside, there is a row of eight notochord precursors (arrows). In the center, the cells are endodermal precursors (open circle) (Nishida, 1987). In the PVC-deficient radial embryos, the arrangement of all of the vegetal blastomeres resembled that of the anterior part of a normal embryo (Fig. 3L,L'). It appeared that a row of spinal cord precursors (arrowheads) and a row of notochord precursors (arrows) encircled the central endodermal precursors (open circle).

The PVC-deficient embryos gastrulated (Fig. 3L,L′), but formation of a neural tube was not observed. When all of the vegetal cells had gastrulated, type A embryos were formed (Fig. 3N). By contrast, when vegetal cells gastrulated only partially, some notochord cells differentiated outside the embryos (Fig. 3O), with resultant formation of type B embryos.

Developmental fates in the radialized embryos

The cleavage pattern of the PVC-deficient embryos was anteriorized. In the next set of experiments, we investigated whether or not the developmental fate of each blastomere of
the PVC-deficient embryo was also anteriorized. In the first experiment, cells of 8-cell embryos were manually dissociated with a fine glass needle, and all eight blastomeres from a single embryo were cultured together as a single group of eight partial embryos. Then the number of partial embryos in each group that contained notochord cells was determined. Notochord cells were identified by their morphological features. They elongated and protruded from the partial embryos and a vacuole formed on one side of each notochord cell (Fig. 4A, arrow; Nishida 1991). During normal embryogenesis, 32 of a total of 40 notochord cells originate from the anterior-vegetal A4.1 blastomere pair of 8-cell embryos. Only the 8 notochord cells of the tip of the larval tail originate from the posterior-vegetal B4.1 blastomere pair (Nishida, 1987). Recent experiments involving isolation of blastomeres showed that A4.1 cells autonomously give rise to notochord cells in isolation, while B4.1 cell do not (Nakatani and Nishida, 1994). Fifteen normal, 8-cell embryos were dissociated into single cells and cultured (Fig. 4A). One of eight partial embryos in a group developed notochord cells in 7 cases. Two of eight partial embryos contained notochord cells in 8 cases. In no case did three or more of the eight partial embryos in a group develop notochord cells. The partial embryos that contained notochord cells were always derived from the isolated A4.1 cells. Thus, the maximal number of notochord-containing embryos in a group was two of eight. Twenty PVC-deficient, radial 8-cell embryos were processed similarly (Fig. 4B). The numbers of cases in which 1, 2, 3, and 4 of 8 partial embryos developed notochord cells were 0, 7, 7, and 6, respectively. The notochord-containing partial embryos were derived exclusively from the vegetal isolates. In no case did five or more partial embryos develop notochord cells. The maximum

Fig. 2. (A) Normal larva derived from an egg from which anterior cytoplasm had been removed. (B-E) Type A larvae that were derived from eggs from which posterior-vegetal cytoplasm had been removed (PVC-deficient eggs). (F-I) Type B larvae that originated from PVC-deficient eggs. Type A and type B larvae show radial symmetry along the vertical axis with no apparent anterior-posterior axis. There is no distinct head and tail. These larvae consist of the outer epidermal layer, which was stained immunohistochemically for the Epi-2 antigen (C,G); an intermediate endodermal layer, which was stained histochemically for alkaline phosphatase (D,H); and an inner mass of notochord cells, which was stained immunohistochemically for the Not-1 antigen (E,I). Scale bar, 100 µm.
number was four. These vegetal partial embryos never contained muscle cells (n=70), as determined with a monoclonal antibody against myosin. These results suggest that, in extreme cases, all four vegetal cells in radialized embryos assumed the anterior A4.1 fate.

In the next experiments, cleavage was arrested by treatment with cytochalasin B after the 110-cell stage, and then notochord differentiation was monitored by following the expression of the Not-1 antigen. Nishikata and Satoh (1990) reported that, when normal 110-cell embryos are arrested, some of the notochord precursor cells express the Not-1 antigen. In their experiments, the arrangement and maximum number of Not-1-positive cells coincided with those of the notochord-lineage cells at the 110-cell stage. These earlier results were confirmed in this investigation. In the anterior-vegetal quarter of the 110-cell embryo there is a row of 8 A-line notochord precursors (A8.5, A8.6, A8.13, and A8.14 pairs), and in the posterior-vegetal quarter there are two smaller B-line precursors (B8.6 pair). This arrangement was frequently observed in the cleavage-arrested 110-cell embryos, as shown in Fig. 4C. The numbers of Not-1-positive cells are shown in Fig. 5. The maximum number was ten. The radialized 110-cell embryos were cleavage-arrested and analyzed in a similar manner. In most cases, the number of Not-1-positive cells exceeded ten and reached as many as 17 in some cases (Fig. 5). The distribution of Not-1-positive cells in the arrested embryos was different from that in the control, and Not-1-positive cells encircled the embryos (Fig. 4D). When normal and radialized embryos were cleavage-arrested and stained for alkaline phosphatase, the activity was detected in several vegetal-pole cells. These observations suggest that the notochord-precursor-like cells of the radicalized embryos observed by SEM, as described in the previous section, were indeed notochord-precursor cells. They support the hypothesis that the PVC-deficient embryos are anteriorized and consist of duplicated anterior parts of the normal embryo.

**Axis reversal by transplantation of posterior cytoplasm to an anterior position**

The posterior-vegetal cytoplasm is necessary for formation of posterior structures of embryos. In our next experiments, we asked whether posterior-vegetal cytoplasm is sufficient for the formation of posterior structures. Egg fragments containing posterior-vegetal cytoplasm were transplanted to heterotopic positions by fusing the fragments to the anterior-vegetal region of PVC-deficient eggs by PGEF-mediated fusion. The results are shown in Table 1. A total of 82% of the PVC-deficient eggs developed into radialized larvae. When the posterior-vegetal cytoplasm was transplanted to the anterior-vegetal position, 68% of specimens developed into tail larvae with a distinct head and tail (Fig. 6A), while 32% of the specimens gave rise to radialized larvae. The tail larvae were always found to contain muscle cells in the tail region when examined by specific staining for myosin (Fig. 6B). Thus, the deficiency in the formation of the A-P axis was overcome to a significant extent. In control experiments, anterior-vegetal cytoplasm was transplanted in the same way, but anterior-vegetal cytoplasm had no analogous activity (Fig. 6C).

In 32 cases, posterior-vegetal fragments were vitally stained with Nile Blue and then fused to anterior-vegetal regions in order to examine whether the original A-P axis was restored.
or reversed. In 22 out of 32 cases (68%), tailed larvae developed. Among the 22 tailed larvae, 18 specimens (82%) had the blue staining in their tail and mesenchyme, which is derived from the posterior (B-line) blastomeres. Fig. 6D-H shows the cleavage pattern in a typical case. At the 8-cell stage, one blastomere pair protruded in the original anterior direction, resembling the B4.1 blastomere pair (compare Fig. 6G with Fig. 3B). These B4.1-like cells divided unequally to yield larger B5.1-like cells and smaller B5.2-like cells at the 16-cell stage. These cells inherited labelled posterior-vegetal cytoplasm. These results together indicate that the original A-P axis was reversed by transplantation of posterior-vegetal cytoplasm to an anterior-vegetal position in PVC-deficient eggs. It is suggested that posterior-vegetal cytoplasm is sufficient for ectopic formation of posterior structures.

**Inhibition of anterior fates by posterior cytoplasm**

Because the A-P axis was reversed by transplantation of posterior-vegetal cytoplasm, one might suppose that generation of anterior structures from the original anterior part of the egg must be inhibited and the original anterior fate must be changed to posterior fate by the introduced posterior cytoplasm. This possibility was examined by transplanting posterior-vegetal cytoplasm to the anterior-vegetal position of normal eggs with posterior-vegetal cytoplasm in the original position. Thus, the operated eggs had posterior-vegetal cytoplasm on both sides of the egg. Most of them developed into malformed larvae, in which discrimination of head and tail was difficult. Formation of anterior structures was monitored by examining for the presence of notochord cells (Table 2). In approximately half of the cases, the number of cells that expressed the Not-1 antigen was significantly reduced (Fig. 7A) and, in 7% of cases, no expression of the Not-1 antigen was observed at all. By contrast, the number of muscle cells that expressed myosin increased in 60% of cases (n=20; Fig. 7B). These results suggest that posterior-vegetal cytoplasm suppresses the expression of anterior fate.

In control experiments, anterior-vegetal cytoplasm was transplanted to the anterior-vegetal position of normal eggs. There was no significant reduction in number of notochord cells. Approximately 70% of larvae had a distinct head and tail and, in 20% of cases, a morphologically normal tailbud developed (Fig. 7C). In another control experiment, anterior-vegetal cytoplasm was transplanted to the posterior-vegetal position of normal eggs. Of these eggs, 81% (n=47) had a distinct head and tail (Fig. 7D).

**DISCUSSION**

The results of this investigation suggest that the posterior-vegetal cytoplasm, after the second phase of ooplasmic segregation, is required for formation of posterior components of the ascidian embryo. The PVC-deficient embryos showed a radialized pattern of cleavage, which was equivalent to a duplication of the pattern of cleavage in the anterior half of the normal embryo. The developmental fates of early blastomeres were also anteriorized. Thus, removal of posterior-vegetal cytoplasm caused total duplication of the anterior half. When posterior-vegetal cytoplasm was transplanted to the anterior-vegetal region, the anterior-posterior axis of the embryos was reversed. This result suggests that the posterior-vegetal cytoplasm is sufficient for formation of posterior components even when transplanted to a non-standard location. The posterior-vegetal cytoplasm contains most of the myoplasm (Fig. 1C,F), which is known to play an important role in the determination of muscle fate. Jeffery and Swalla (1990) pointed out that myoplasm, after the first phase of ooplasmic segregation, has multiple roles. The results of the present investigation suggest that myoplasm, after the second phase of ooplasmic segregation, is important not only for the determination of muscle, but also for specification of the embryonic axis by directing posterior fate.

**The roles of the posterior-vegetal cytoplasm**

The posterior-vegetal cytoplasm appears to include factors involved in four distinct processes, as follows. (1) **Muscle formation.** The PVC-deficient larvae lacked muscle cells. It was shown previously by cytoplasmic-transfer experiments that muscle determinants are localized in the posterior region after the second phase of ooplasmic segregation (Nishida, 1992a). (2) **Suppression of anterior fate.** When posterior-vegetal cytoplasm was transplanted to the anterior position of PVC-deficient eggs, reversal of the embryonic axis occurred. When posterior-vegetal cytoplasm was transplanted to the anterior position of normal eggs, formation of the anterior

![Fig. 5. Histogram showing the distribution of numbers of Not-1-positive blastomeres in cleavage-arrested normal 110-cell embryos (upper) and radialized 110-cell embryos that were derived from the PVC-deficient eggs (lower). In normal arrested embryos the number never exceeded ten (broken vertical line).](image)

![Table 1. Results of transplantation of egg fragments to PVC-deficient eggs](table)

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Position of transplant</th>
<th>n</th>
<th>Tailed</th>
<th>Radialized</th>
<th>Significance</th>
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<tr>
<td>–</td>
<td>–</td>
<td>65</td>
<td>18%</td>
<td>82%</td>
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<tr>
<td>Posterior-vegetal</td>
<td>Anterior-vegetal</td>
<td>50</td>
<td>68%</td>
<td>32%</td>
<td>P&lt;0.001</td>
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<tr>
<td>Anterior-vegetal</td>
<td>Anterior-vegetal</td>
<td>55</td>
<td>15%</td>
<td>85%</td>
<td>0.77  P&gt;0.5</td>
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</table>

The resultant embryos were divided into two categories based on their morphology, namely, tailed larvae and radialized larvae. Probability was calculated by the $\chi^2$ test when results were compared to the results in the first row, namely, results when no transplantation was carried out.
### Table 2. Results of transplantation of egg fragments to normal eggs

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Position of transplant</th>
<th>n</th>
<th>Normal amount of notochord</th>
<th>Reduced amount of notochord</th>
<th>No notochord</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior-vegetal</td>
<td>Anterior-vegetal</td>
<td>46</td>
<td>41%</td>
<td>52%</td>
<td>7%</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Anterior-vegetal</td>
<td>Anterior-vegetal</td>
<td>49</td>
<td>84%</td>
<td>16%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Anterior-vegetal</td>
<td>Posterior-vegetal</td>
<td>47</td>
<td>81% of embryos developed into tailed larvae.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers of notochord cells were estimated from the expression of the Not-1 antigen. The embryos were tentatively divided into three categories on the basis of the number of notochord cells. Probability was calculated by the $\chi^2$ test when the results in the first and second rows were compared.

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**Fig. 6.** Transplantation of posterior-vegetal cytoplasm to PVC-deficient eggs. (A) Posterior-vegetal cytoplasm was transplanted to the anterior-vegetal position of a PVC-deficient egg. A larva with a distinct head and tail developed. (B) The same larva in which myosin was visualized by immunostaining. Muscle cells can be seen in the tail. (C) Radialized larva in which anterior-vegetal cytoplasm had been transplanted to an anterior-vegetal position. (D-I) Posterior-vegetal cytoplasm was labeled with Nile Blue and then fused to an anterior-vegetal position of a PVC-deficient egg. The original anterior pole is to the left. (D) Just after fusion. Lateral view. The large arrow shows the position of the polar body. The small arrow indicates the trail of pronuclei, which is a marker of the posterior pole of the egg. (E) 2-cell stage. Animal view. (F) 4-cell stage. (G) 8-cell stage. Lateral view. A blastomere pair that has blue labelling protrudes anteriorly from the embryo. (H) The labelled cells have divided unequally at the 16-cell stage. Vegetal view. The embryo resembles a normal 16-cell embryo but with its axis reversed. (I) The resultant larva has blue label in its tail and mesenchyme cells (arrowhead). Scale bar, 100 µm.
components of each embryo was inhibited. Therefore, transplanted posterior-vegetal cytoplasm suppressed anterior fate. (3) Generation of a posterior cleavage pattern. The PVC-deficient embryos showed a radialized cleavage pattern that was a duplication of the anterior pattern. Transplantation of posterior-vegetal cytoplasm to an anterior-vegetal position caused reversal of the anterior-posterior polarity of the cleavage pattern. Therefore, posterior-vegetal cytoplasm is necessary and sufficient for generation of a posterior pattern of cleavage. (4) Morphogenesis for tail formation. This property is less well defined than the above-described three properties. However, the PVC-deficient larvae did lack a distinct tail. There may be factors that control morphogenetic movements for formation of the tail. With respect to similar morphogenetic factors, Bates and Jeffery (1987) and Jeffery (1990) suggested the existence of an ooplasmic factor that is responsible for gastrulation movements in ascidian embryos. When a small region of vegetal-pole cytoplasm is removed between the first and second phase of ooplasmic segregation, or the vegetal pole is irradiated with UV light, the embryos do not gastrulate. In spite of the deficiency in morphogenesis, such embryos retain their normal mechanisms for cell-type specification and a normal cleavage pattern.

It is likely that the PVC-deficient larvae lacked mesenchyme cells. This absence was not directly proved because no good markers are available for differentiation to mesenchyme. However, our observations of the cleavage patterns of PVC-deficient embryos at the 76-cell stage and examination of developmental fates at the 110-cell stage with cleavage-arrested PVC-deficient embryos suggest that the PVC-deficient embryos were totally anteriorized and, therefore, they lacked mesenchyme-lineage cells. Thus, the posterior-vegetal cytoplasm may also be involved in formation of mesenchyme.

It is not clear whether different factors are responsible for the distinct roles described above, or whether multiple roles should be attributed to a single factor. It is possible that all the properties are attributable to one single factor. In *Drosophila*, a graded signal that is provided by a concentration gradient of bicoid protein, exerts total control over the development of the anterior portion of the fly embryo (Driever and Nusslein-Volhard, 1988). In *C. elegans*, the activity of the product of the *skn-1* gene determines the identity of the EMS blastomere of the 4-cell embryo. The EMS cell gives rise to various types of cell and generates an invariant cleavage pattern after the 4-cell stage (Bowerman et al., 1992, 1993). In the ascidian, we are only just starting to define the way in which the posterior half of the embryos is specified by localized maternal factors.

**Posterior dominance of developmental fate**

Removal of posterior cytoplasm caused posterior blastomeres to adopt the fate of anterior blastomeres. When posterior cytoplasm was transplanted to an anterior position, posterior fate overcame anterior fate. By contrast, transplantation of anterior cytoplasm to a posterior position did not affect development. Therefore, posterior fate is dominant over anterior fate. In other words, anterior fate may occur by default. The presence of posterior cytoplasm specifies posterior fate, while anterior fate is directed by the absence of posterior cytoplasm.

It was shown recently that inductive interactions at the 32-cell stage are involved in formation of notochord during ascidian embryogenesis (Nakatani and Nishida, 1994). Inductive influences are generated from endoderm precursors and adjacent notochord precursors. Anterior to the notochord precursors, there are spinal cord precursors (Fig. 3I). We do not yet know whether spinal cord is induced by notochord. Anterior to the spinal cord precursors, there are precursors of brain and sensory pigment cells. These cells are induced during gastrulation (Rose, 1939; Reverberi and Minganti, 1946; Reverberi et al., 1960; Okado and Takahashi, 1988). In *H. roretzi*, sensory pigment cells are induced by spinal cord precursors at the 180-cell stage (Nishida, 1991). As the notochord and brain are induced, it is likely that anterior components of ascidian embryos are induced sequentially during embryogenesis. In contrast, endoderm precursors, which are in the vegetal pole region, and muscle precursors, which reside in the posterior region are determined by cytoplasmic factors in the egg (Nishida, 1992a, 1993). Provided that posterior fate is determined by cytoplasmic factors present in the egg and, in addition, that anterior fate is determined by sequential inductions during embryogenesis, it is easy to explain the posterior dominance that was observed in the present study. Without posterior cytoplasm, sequential inductions also proceed in the original posterior direction, with formation of anterior components. When posterior cytoplasm is present, sequential inductions cannot occur because the developmental fate of cells has already been determined by maternal cytoplasmic factors. There may be no maternal determinants unique to the anterior part of the zygote.

It is not clear whether a fixed anterior-posterior axis exists in the animal hemisphere at early stages because the major fate of both anterior and posterior animal cells is epidermis, in addition to brain, which is induced at the anterior margin.

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**Fig. 7.** Transplantation of egg fragments to a normal egg. (A,B) Posterior-vegetal cytoplasm was transplanted to an anterior-vegetal position of normal eggs. The resultant embryos had reduced numbers of notochord cells that expressed the Not-1 antigen (A) and increased numbers of muscle cells that expressed myosin (B). (C,D) Anterior-vegetal cytoplasm was transplanted to an anterior-vegetal (C) and to a posterior-vegetal (D) position of normal eggs. In both cases, normal larvae developed. Scale bar, 100 µm.
Mechanisms for generation of a unique cleavage pattern

Ascidian embryos exhibit a complicated but invariant cleavage pattern (see, for example, Fig. 3I,K). The pattern of cleavage in the animal hemisphere and anterior-vegetal region is rather regular. By contrast, that in the posterior-vegetal region is extremely irregular, mainly because of successive unequal cleavages. To gain some insight into the mechanisms, the present author proposes the hypothesis that the posterior pole of the vegetal hemisphere attracts a spindle pole.

The third cleavage occurs horizontally, but the cleavage plane is slightly inclined and, consequently, the B4.1 cell pair protrudes posteriorly from the embryo (Fig. 3B). This phenomenon may be explained if, within B3 cells (posterior pair at the 4-cell stage), the vegetal pole of the third spindle is drawn towards the posterior, with resultant inclination of the spindle axis. After the 8-cell stage, three successive, unequal cleavages occur, such that only the cells of the most posterior blastomere pair at each stage undergo unequal cleavage, always producing smaller cells posteriorly. All the other cleavages are equal cleavages in terms of cell size (for details of the cleavage pattern of ascidians, see Conklin, 1905, and Satoh, 1979). The smallest blastomere of the 64-cell embryo, B7.6, never divides during embryogenesis. It gives rise to a single cell in the endodermal strand (Nishida, 1987). During these unequal cleavages, the posterior pole may attract the spindle pole, with resultant production of smaller cells at the posterior pole. The present investigation suggests that factors responsible for the attraction of the spindle pole are transferable together with the posterior-vegetal fragments of eggs. These hypotheses must be examined by observations of the axis and the position of each spindle during normal embryogenesis, as well as in experimentally manipulated embryo.

Similarity to the dorsoventral axis formation in amphibian eggs

In unfertilized eggs of many kinds of animal, there is an animal-vegetal axis. The second axis is specified just after fertilization or during early embryogenesis. The two axes are the basis for the bilateral body plan. As pointed out by Bates and Jeffery (1987), there are remarkable similarities between ascidians and amphibians in the process that is involved in establishment of the second axis of the egg, which is the anterior-posterior axis in ascidians and the dorsal-ventral axis in amphibians. In Xenopus, the specification of the dorsal-ventral axis begins after fertilization with a shift of vegetal cytoplasm to the future dorsal side (Scharf and Gerhart, 1980). This rearrangement of ooplasm may correspond to ooplasmic segregation in ascidian eggs. In Xenopus, it was shown recently that transplantation of dorsal cytoplasm to a ventral position causes formation of a secondary axis (Yuge et al., 1990). Dominance of dorsal fate is observed. Hainski and Moody (1992) reported that mRNA from dorsal blastomeres can alter ventral blastomeres and yield a secondary axis, an indication that dorsal determinants are maternal mRNAs.

In Ciona and Ascidia, the present author proposes the hypothesis that the posterior pole of the vegetal hemisphere attracts a spindle pole. Thus, the third cleavage occurs horizontally, but the cleavage plane is slightly inclined and, consequently, the B4.1 cell pair protrudes posteriorly from the embryo (Fig. 3B). This phenomenon may be explained if, within B3 cells (posterior pair at the 4-cell stage), the vegetal pole of the third spindle is drawn towards the posterior, with resultant inclination of the spindle axis. After the 8-cell stage, three successive, unequal cleavages occur, such that only the cells of the most posterior blastomere pair at each stage undergo unequal cleavage, always producing smaller cells posteriorly. All the other cleavages are equal cleavages in terms of cell size (for details of the cleavage pattern of ascidians, see Conklin, 1905, and Satoh, 1979). The smallest blastomere of the 64-cell embryo, B7.6, never divides during embryogenesis. It gives rise to a single cell in the endodermal strand (Nishida, 1987). During these unequal cleavages, the posterior pole may attract the spindle pole, with resultant production of smaller cells at the posterior pole. The present investigation suggests that factors responsible for the attraction of the spindle pole are transferable together with the posterior-vegetal fragments of eggs. These hypotheses must be examined by observations of the axis and the position of each spindle during normal embryogenesis, as well as in experimentally manipulated embryo.

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(accepted 1 August 1994)