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Vegetal egg cytoplasm promotes gastrulation and is responsible for specification of vegetal blastomeres in embryos of the ascidian *Halocynthia roretzi*

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SUMMARY

An animal-vegetal axis exists in the unfertilized eggs of the ascidian *Halocynthia roretzi*. The first phase of ooplasmic segregation brings the egg cortex to the vegetal pole very soon after fertilization. In the present study, when 5-8% of the egg cytoplasm in the vegetal pole region was removed between the first and second phase of segregation, most embryos exhibited failure of gastrulation, as reported previously in *Styela* by Bates and Jeffery (*Dev. Biol.* 124, 65-76, 1987). The embryos that were deficient in vegetal pole cytoplasm (VC-deficient embryos) developed into permanent blastulae. They consisted for the most part of epidermal cells and most lacked the derivatives of vegetal blastomeres, such as endoderm, muscle and notochord. Removal of cytoplasm from other regions did not affect embryogenesis. The cleavage of the VC-deficient embryos not only exhibited radial symmetry along the animal-vegetal axis but the pattern of the cleavage was also identical in the animal and vegetal hemispheres. Examination of the developmental fates of early blastomeres of VC-deficient embryos revealed that the vegetal blastomeres had assumed the fate of animal cells. These results suggested that the VC-deficient embryos had been totally animalized.

When vegetal pole cytoplasm was transplanted to the animal pole or equatorial position of VC-deficient eggs, gastrulation occurred, starting at the site of the transplantation and tissues derived from vegetal blastomeres formed. Therefore, it appears that vegetal pole cytoplasm specifies the site of gastrulation and the cytoplasm is responsible for the specification of vegetal blastomeres. It is suggested that, during the second phase of ooplasmic segregation, cytoplasmic factors responsible for gastrulation spread throughout the entire vegetal hemisphere.

Key words: ascidian embryogenesis, gastrulation, cytoplasmic determinants, cleavage pattern, cytoplasmic transfer

INTRODUCTION

An animal-vegetal axis can be recognized in the eggs of many kinds of animal. In most cases, the animal pole region develops into ectoderm while the vegetal pole region gastrulates and gives rise to endoderm. It has been assumed that localized factors in the egg cytoplasm are responsible for the differences in developmental potential of embryonic cells along the animal-vegetal axis. In echinoderms, only the vegetal fragments of bisected eggs gastrulate (Hörstadius, 1937; Maruyama et al., 1985; Zhang et al., 1990). It was shown recently that removal of a portion of the vegetal pole cytoplasm equal to approximately 8% of the total volume of the egg suppresses archenteron formation in starfish (Kuraishi and Osanai, 1994). When the vegetal pole cytoplasm is transplanted to the vegetal position of animal fragments of eggs, the fragments gastrulate (Kiyomoto and Shirai, 1993). These results demonstrate that the cytoplasmic determinants that are required for archenteron formation are localized in the vegetal pole region in echinoderm eggs. In *Drosophila*, ventral cells invaginate to form mesoderm cells and at least the mesoderm of the central part invaginates autonomously (Leptin and Roth, 1994). This process is under the control of several transcription factors, identified from mutations known as *dorsal*, *twist* and *snail*.

In ascidians, evidence has been accumulating to indicate that cytoplasmic factors in the egg play important roles in the determination of developmental fates during early embryogenesis (for reviews, see Venuti and Jeffery, 1989; Meedel, 1992; Nishida, 1992b; Satoh, 1993). Experiments involving the transplantation of cytoplasm have revealed the presence of muscle, endoderm and epidermis determinants (Nishida, 1992a, 1993, 1994a). These three kinds of cytoplasmic determinants move in different directions during ooplasmic segregation. Prior to the onset of the first cleavage, three kinds of determinant settle at sites that correspond to specific regions of the future bilateral fate map. In addition to determinants of cell type, cytoplasmic determinants of the movements associated with gastrulation are also present in the ooplasm of ascidians. 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 a small region of vegetal pole cytoplasm was removed from the zygote between the first and second phase of ooplasmic segregation, embryos failed to

gastrulate. The removal of cytoplasm at the vegetal pole is mimicked by the effects of UV irradiation of the fertilized egg near the vegetal pole (Jeffery, 1990a). These results suggest that factors that are required for gastrulation are localized in the vegetal pole region after the first phase of segregation.

In the present study, vegetal pole cytoplasm was transplanted to heterotopic positions. It appeared that the vegetal pole cytoplasm was required for and was sufficient to promote gastrulation, as well as the specification of vegetal blastomeres in embryos of *Halocynthia roretzi*.

MATERIALS AND METHODS

Embryos

Naturally spawned eggs of *Halocynthia roretzi* were artificially fertilized and then manually devitellinated with sharpened tungsten needles. Devitellinated embryos were reared in Millipore-filtered (pore size, 0.45 μm) seawater that contained 50 μg ml^{-1} streptomycin sulfate and 50 μg ml^{-1} kanamycin sulfate. When embryos were reared to later stages of embryogenesis, in order to facilitate normal formation of a neural tube, devitellinated embryos were cultured in the supernatant of a homogenate of cleaving embryos (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 a desired stage for microsurgery.

Microsurgery

Egg fragments were removed as follows. Fertilized eggs were oriented by reference to the positions of the polar bodies and the transparent myoplasm. 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.

Blastomeres were isolated with a fine glass needle at the 8-cell stage. Blastomeres were cultured separately in agar-coated plastic dishes until controls hatched.

Transplantation of egg cytoplasm by fusion of fragments

Egg fragments that had been severed from eggs were fused to the animal pole region of egg cells by polyethylene glycol- and electric field-mediated fusion (PGEF-mediated fusion), as described previously (Nishida, 1994a). In brief, an egg fragment was allowed to adhere firmly to an egg cell at the desired position during treatment with 30% (w/v) polyethylene glycol dissolved 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 μsec in fusion medium (0.77 M D-mannitol in 0.25% Ca^{2+}-free artificial seawater). Then the specimen was immediately transferred to seawater. Fused egg cells divided with the normal schedule of cleavage. In this study, egg fragments were stained with 0.025% Nile Blue B in seawater for 2 minutes. After fusion, the fate of the region that originated from the fused fragment was traced by monitoring the vital blue staining.

Markers of the differentiation of specific tissues

Differentiation of epidermis was evaluated by monitoring the expression of the Epi-2 antigen using a previously described monoclonal antibody (Nishikata et al., 1987b). Formation of endoderm was monitored by histochemical detection of alkaline phosphatase (AP) activity by the method of Whittaker and Meedel (1989), with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate. The reaction results in the formation of brownish-purple deposits. Differentiation of muscle was monitored by following the expression of myosin heavy chains and acetylcholinesterase (AchE). Myosin was detected with a monoclonal antibody (Nishikata et al., 1987a; Makabe and Satoh, 1989). AchE activity was detected histochemically, as described by Karnovsky and Roots (1964), with acetylcholine iodide as the substrate, resulting in deposition of a brown precipitate. Differentiation of notochord was evaluated by monitoring the expression of the Not-1 antigen with a previously described monoclonal antibody (Nishikata and Satoh, 1990). All the monoclonal antibodies mentioned above were generously provided by Dr T. Nishikata (Konan University, Kobe, Japan). Indirect immunofluorescence staining with monoclonal antibodies was carried out by standard methods with FITC-conjugated second antibodies.

RESULTS

Removal of cytoplasmic fragments after the first phase of ooplasmic segregation

The ooplasm of ascidian eggs moves after fertilization and this process is known as ooplasmic segregation (Conklin, 1905; Hirai, 1941; Sawada and Osanai, 1981; Jeffery and Meier, 1983; Sardet et al., 1989; for *Halocynthia* see figure 1 in Nishida, 1994b). The movement of the ooplasm progresses in two phases between fertilization and the first cleavage. In unfertilized eggs, a first meiotic 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. 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. The distribution of egg cytoplasm shows 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 domain in a region just vegetal of the equator. During the second phase of ooplasmic segregation, the bilateral symmetry of the egg becomes established. The first cleavage occurs 160 minutes after fertilization.

Cytoplasmic fragments with volumes equal to 8-15% of that of an entire egg were removed from various regions of fertilized eggs with a fine glass needle between the first and second phase of ooplasmic segregation. Removal of animal fragments (n=38) and equatorial fragments (n=35) had no effect on normal development. By contrast, as reported in *Styela* (Bates and Jeffery, 1987), when vegetal cytoplasm was removed, gastrulation did not occur and eggs developed into malformed larvae that resembled permanent blastulae (Fig. 1B,C) in 67% of cases (n=46). Removal of a region of vegetal cytoplasm equal to 5-8% of an egg volume resulted in the malformations at a similar frequency (67%, n=96). When the volume of cytoplasm that was removed was reduced to 2.5-5%, the proportion of the malformed larvae fell to 28% (n=61). During the first phase of ooplasmic segregation, contraction of the egg surface brings sperm towards the vegetal pole region (Sardet et al., 1989). Thus, the larger were the vegetal fragments that were removed, the more frequently were sperm nuclei removed from eggs. When sperm had been removed, eggs did not cleave and such cases were not included in the numbers of specimens cited above. In all subsequent experiments, only 5-8% of the cytoplasm was removed. Under these conditions, sperm nuclei were removed in approximately 25% of cases.

For the generation of reproducible malformations, a series

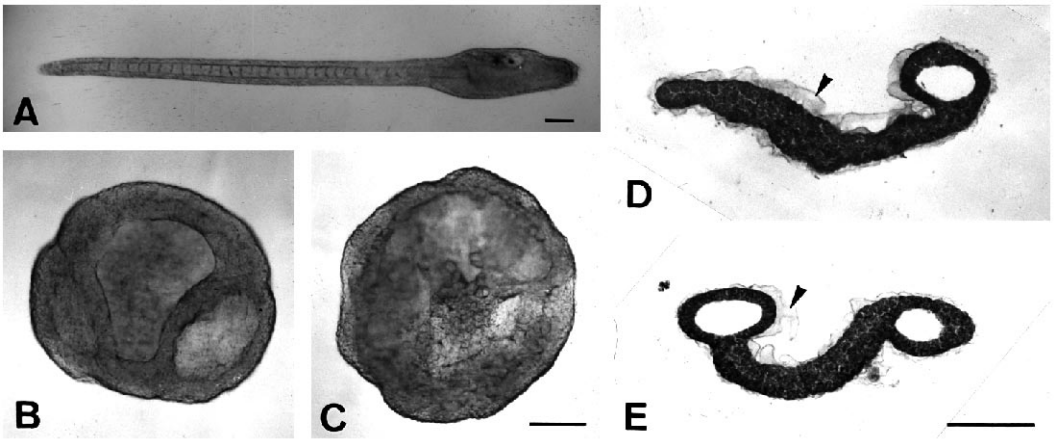

Fig. 1. (A) Normal larva of *Halocynthia roretzi*. (B,C) Larvae derived from eggs from which vegetal pole cytoplasm had been removed (VC-deficient eggs). No head or tail is visible. Larvae resemble permanent blastulae although they are flattened, as shown in D and E. The larval tunic that surrounds the embryos is transparent and is invisible in these photographs. (D,E) Vertical sections of VC-deficient larvae, stained with hematoxylin-eosin. There are some cavities and the larvae are flattened and consist mainly of epithelial cells. Arrowheads indicate the larval tunic. Scale bar, 100 μm.

of experiments was performed to determine the most effective timing of removal of cytoplasm. The frequency of the malformations was maximum and constant, being around 80%, with removal of cytoplasm between 20 and 60 minutes after fertilization at 9°C. After 60 minutes, the frequency gradually decreased, falling to 0% at 90 minutes, which corresponds to the time just after the start of the second phase of segregation. Therefore, in the experiments described below, vegetal pole cytoplasm equal to 5-8% of an egg volume was removed 20-50 minutes after fertilization. The resultant eggs were designated eggs deficient in vegetal pole cytoplasm or VC-deficient eggs.

The possibility that the malformations were caused merely by disturbance of vegetal cytoplasmic architecture during microsurgery was examined. The bridge of cytoplasm and plasma membrane between the egg and the fragment was extended until the egg and its fragment were connected by only a thread and 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 all such cases (n=27), normal larvae developed. Therefore, the possibility mentioned above is unlikely and vegetal cytoplasm must, indeed, be removed from the egg to obtain a malformed larva.

Morphology of and cell differentiation in VC-deficient larvae

VC-deficient eggs developed into larvae with astonishing morphology, as shown in Fig. 1B,C. They had neither a distinct head nor a tail and looked like so-called permanent blastulae, although they were somewhat flattened (Fig. 1D,E). The entire surface of each larva consisted of epithelium. There were

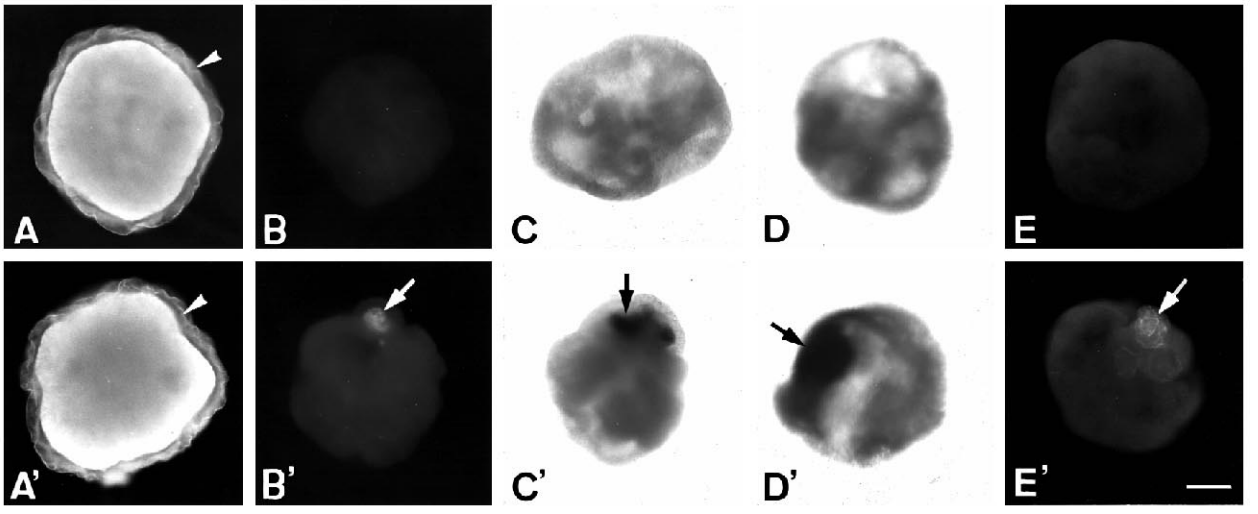

Fig. 2. Tissue differentiation of VC-deficient larvae, as examined by monitoring expression of markers of various cell types. (A,A′) The Epi-2 antigen (marker of epidermis) was expressed over the entire surface of the two specimens of VC-deficient larvae. Arrowheads indicate the larval tunic, which is secreted by epidermal cells. (B,B′) Negative (B) and positive (B′) specimens for the expression of myosin (muscle marker), respectively. (C,C′) Negative and positive specimens for the expression of acetylcholinesterase (muscle marker), respectively. (D,D′) Negative and positive specimens for the expression of alkaline phosphatase (endoderm marker), respectively. (E,E′) Negative and positive specimens for the expression of the Not-1 antigen (notochord marker), respectively. Arrows in B′,C′,D′,E′ indicate areas with positive staining. Scale bar, 100 μm.

several cavities surrounded by a single layer of epithelial cells. Apart from the surface epithelial cells, few cells were seen within the larvae (Fig. 1D,E). The VC-deficient larvae were surrounded by a larval tunic, which is normally secreted by epidermal cells. Therefore, it is likely that the epithelial cells of the VC-deficient larvae differentiated into epidermal cells.

Tissue differentiation was examined in the malformed larvae by monitoring the expression of various molecular markers. Expression of the Epi-2 antigen, which is a marker of differentiation to epidermis, was observed over the entire surface of the larvae in all of 48 cases (Fig. 2A,A′). This result confirms that the larvae consisted mainly of epidermis cells. Muscle cells were scarcely detectable when the expression of myosin (2% of 48 cases) and of acetylcholinesterase (9% of 43 cases) was examined (Fig. 2B,B′, C,C′, respectively). Alkaline phosphatase (AP) activity, which is a marker of endoderm, was detected in 17% (n=46) of the malformed larvae (Fig. 2D,D′). The Not-1 antigen, which is a marker of notochord, was detected in 10% (n=49) of the larvae (Fig. 2E,E′). Only small numbers of muscle, endoderm and notochord cells were present even in the cases where evidence of their differentiation was detected (Fig. 2B′-E′, arrows). Thus, the VC-deficient larvae consisted of large numbers of epidermal cells, and most of the larvae lacked mesodermal and endodermal tissues. In normal embryogenesis, the epidermis is derived exclusively from animal blastomeres of early embryos, while

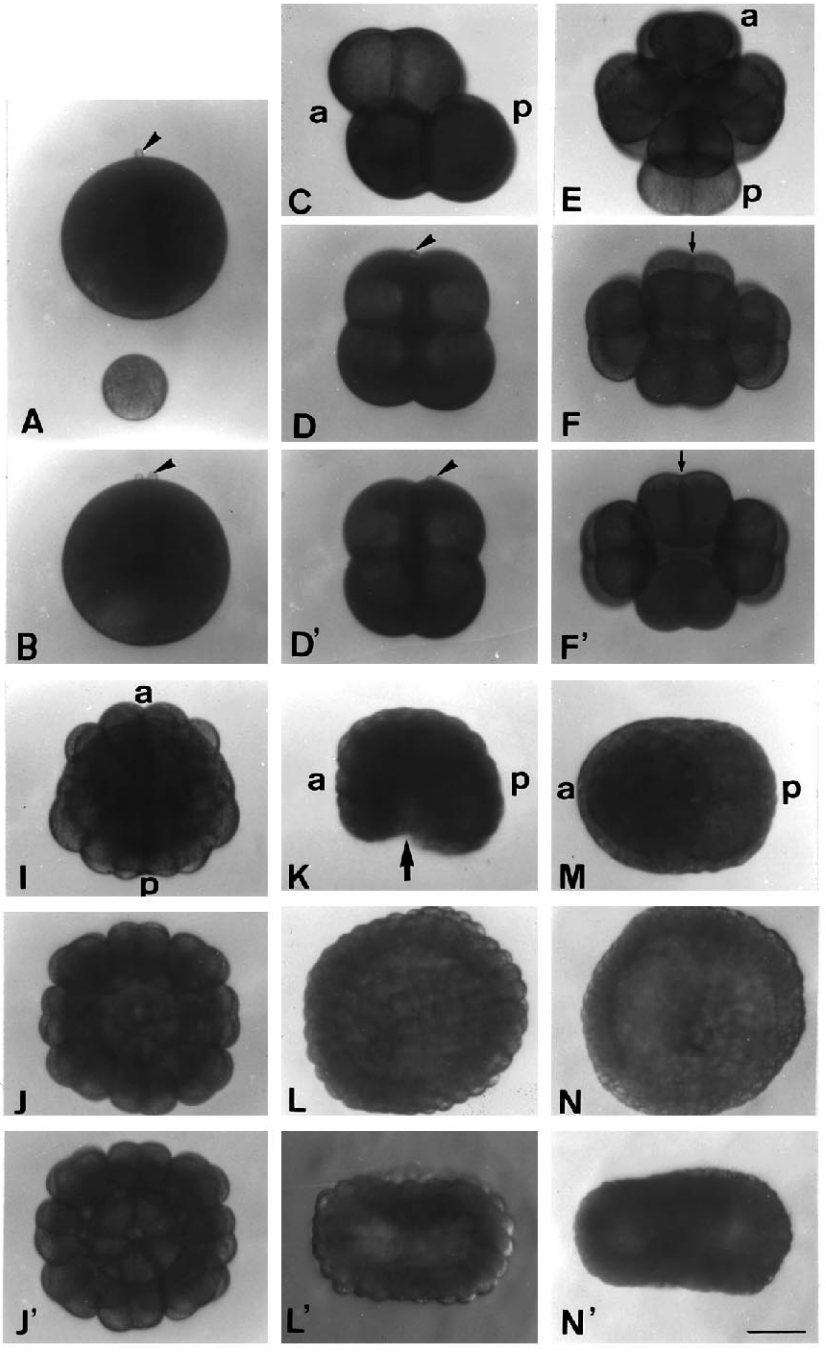

Fig. 3. Cleavage patterns and the development of normal and VC-deficient embryos. (A) A vegetal pole fragment was removed from an egg between the first and second phase of ooplasmic segregation. Arrowhead indicates the first polar body. (B) A clear cytoplasmic region that corresponds to sperm aster is located near the vegetal pole in a VC-deficient egg. The arrowhead indicates the first and second polar bodies. (C) Lateral view of a normal 8-cell embryo. The animal pole is up; anterior (a) is to the left and posterior (p) is to the right. The posterior-vegetal B4.1 blastomere pair protrudes from the embryo in the posterior direction. (D,D′) Two orthogonal lateral views of the same 8-cell VC-deficient embryo. The embryo has no apparent anterior-posterior axis. Arrowheads indicate polar bodies. (E) Animal view of a normal 16-cell embryo with the anterior pole up. (F,F′) Animal and vegetal views of a VC-deficient embryo at the 16-cell stage. Arrows indicate the first cleavage plane. (G) A normal 32-cell embryo. (H,H′) Animal and vegetal views of a VC-deficient embryo at the 32-cell stage. (I) A normal 64-cell embryo. (J,J′) Animal and vegetal views of a VC-deficient embryo at the 64-cell stage. Radial symmetry is apparent along the animal-vegetal axis. (K) Lateral view of a normal middle gastrula, with anterior to the left. The arrow indicates the direction of invagination. (L,L′) Polar and lateral views of VC-deficient embryos, respectively, at the stage corresponding to the middle gastrula. A blastocoel is apparent in the embryo. No gastrulation has occurred. The embryo has become flattened along the animal-vegetal axis. (M) Normal neurula. Dorsal view. (N,N′) Polar and lateral views, respectively, of VC-deficient embryos, at the stage corresponding to the neurula. No formation of a neural tube is visible. Scale bar, 100 μm.

muscle, notochord and endoderm originate from vegetal blastomeres. Thus, the VC-deficient larvae had only the component that is normally derived from the animal half of early embryos.

Ooplasmic segregation, cleavage patterns and the development of VC-deficient embryos

In normal development, the sperm aster moves with the myoplasm from the position near the vegetal pole towards the posterior pole during the second phase of ooplasmic segregation (Conklin, 1905; Sardet et al., 1989). In *Halocynthia*, as the aster grows, a clear cytoplasmic region becomes visible just posterior to the center of the eggs after the second phase of segregation, since yolk granules are excluded by the aster (Nishida, 1994b). When vegetal pole cytoplasm was removed after the first phase of ooplasmic segregation (Fig. 3A), the clear region appeared near the vegetal pole in most cases (Fig. 3B). It was suggested that the sperm aster remained around the vegetal pole instead of moving to the posterior region. Although there is no direct evidence, this observation might suggest that some processes were stalled during the second phase of segregation.

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. At the 8-cell stage, the cells of the posterior-vegetal blastomere pair, the B4.1 pair, protrude posteriorly from the embryo (Fig. 3C). Then only the cells of the most posterior pair of blastomeres at each stage undergo unequal cleavage (Fig. 3E,G,I).

In approximately 80% of the VC-deficient embryos, the cleavage pattern was totally changed and cleavages were radially symmetrical. At the 8-cell stage, there were no protruding blastomeres that resembled the B4.1 cells (Fig. 3D,D′). The partitioning of yolk became equal in animal and vegetal blastomeres. Differences in size between animal and vegetal blastomeres were less marked than in normal 8-cell embryos. At the 16-, 32- and 64-cell stages, the blastomeres were arranged radially along the animal-vegetal axis. No unequal cleavage was observed. Moreover, the patterns of cleavages in the animal and vegetal hemispheres were identical. Thus, the cleavage pattern showed not only radial symmetry but also animal-vegetal symmetry (Fig. 3F-J, F′-J′).

In normal ascidian embryos, gastrulation starts at the vegetal pole (Fig. 3K). There is no blastocoel. A single layer of endoderm and mesoderm precursor cells invaginates to push against a single layer of ectoderm precursor cells. The VC-deficient embryos failed to invaginate, but they did become flattened along the animal-vegetal axis (Fig. 3L,L′). An unusual blastocoel was observed within the embryos. At the stage that corresponds normally to the neurula, no evidence of neural tube formation was observed. Embryos became still flatter such that, in many cases, the animal and vegetal walls of the embryos were in close contact. The shape of embryos resembled that of red blood cells (Fig. 3N,N′).

Developmental fates in the VC-deficient embryos

The features of tissue differentiation, the cleavage pattern and the development of the VC-deficient embryos suggested that all parts of the embryos had been animalized. In the next set of experiments, we investigated whether or not the developmental fate of each blastomere of the VC-deficient embryo had already been animalized at an early stage. In these experiments, cells of 8-cell embryos were manually dissociated with a fine glass needle. Each animal and vegetal blastomere was cultured separately and allowed to develop into a partial embryo. Then the morphology and formation of epidermis of animal and vegetal partial embryos were compared.

Animal partial embryos that were derived from single isolated blastomeres of the animal hemisphere of normal 8-cell embryos, resembled permanent blastulae in terms of morphology and all of them expressed the Epi-2 antigen (104 cases; Fig. 4A,B). By contrast, vegetal partial embryos were irregular in shape and never expressed the Epi-2 antigen (125 cases; Fig. 4C,D). These results are identical to those reported previously by Reverberi and Minganti (1946) and Nishikata et al. (1987b). Only animal blastomeres developed into epidermis, faithfully following their developmental fate.

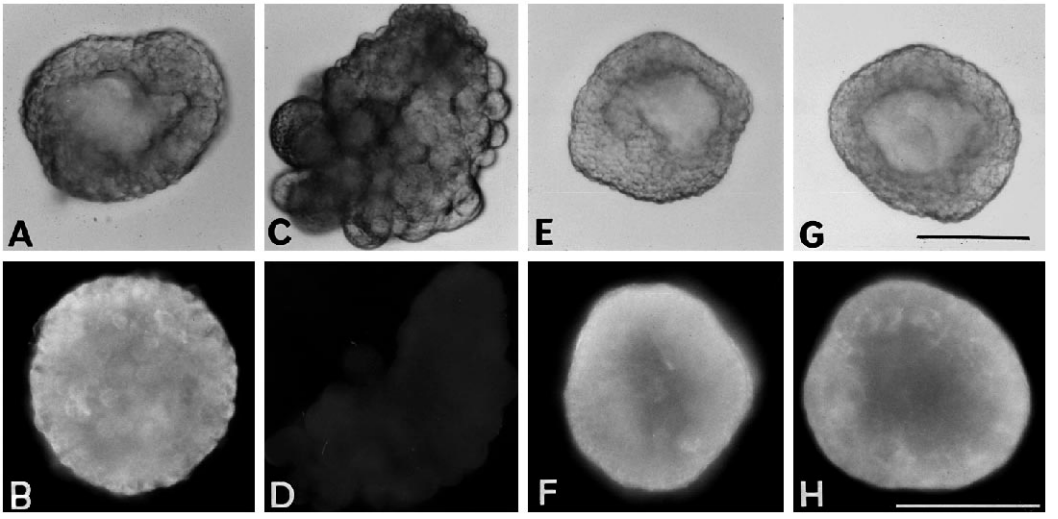

Fig. 4. Developmental fates in VC-deficient embryos. (A) A partial embryo derived from an isolated animal blastomere of a normal 8-cell embryo. It has developed into a permanent blastula. (B) The Epi-2 antigen was expressed in a partial embryo resembling that in A. (C) A vegetal partial embryo that was derived from a normal 8-cell embryo. It is an irregularly shaped mass of cells. (D) Expression of the Epi-2 antigen was not observed in embryos such as that in C. (E,G) Animal and vegetal partial embryos derived from VC-deficient 8-cell embryos, respectively. Both embryos are permanent blastulae. (F,H) Animal and vegetal partial embryos derived from VC-deficient 8-cell embryos, respectively, both expressing the Epi-2 antigen. Scale bar, 100 μm.

The VC-deficient embryos were treated similarly. Both animal partial (Fig. 4E,F) and vegetal partial (Fig. 4G,H) embryos had the morphology of permanent blastulae. All of the animal partial embryos expressed the Epi-2 antigen (51 cases). By contrast to normal embryos, all of the vegetal-partial embryos expressed the antigen over their entire surface (57 cases). These results indicate that the developmental fate of vegetal blastomeres is converted to that of animal blastomeres in VC-deficient embryos.

Transplantation of vegetal pole cytoplasm to heterotopic positions

Vegetal pole cytoplasm is necessary for gastrulation and for the specification of the vegetal blastomeres. In the next experiments, an attempt was made to determine whether vegetal pole cytoplasm is sufficient for determination of the site of gastrulation and for the formation of vegetal blastomeres. Egg fragments containing vegetal pole cytoplasm were labeled with Nile Blue and then transplanted to heterotopic positions by fusion to the animal pole region or the equatorial region of VC-deficient eggs by PGEF-mediated fusion.

In the first series of trials, vegetal egg fragments equivalent to 5-8% of an egg volume were fused to the animal pole region just beside the first polar body of VC-deficient eggs immediately after the first phase of ooplasmic segregation. In these cases, blue-stained transplanted cytoplasm always protruded from each egg together with the second polar body and a large blue second polar body was extruded. Therefore, transplantation of vegetal egg fragments was performed after formation of the second polar body (approximately 110 minutes after fertilization) (Fig. 5A). This stage corresponds to the completion of the second phase of segregation. As recipients of cytoplasm, only VC-deficient eggs with the clear region near the vegetal pole such as the egg shown in Fig. 3B, were used. Such eggs always failed to gastrulate.

Table 1. Results of transplantation of egg fragments to VC-deficient eggs

Cytoplasm	Position of transplant	Gastrulation (no. examined)	Tail formation	ALP	AchE
vegetal	animal	94% (31)	66% (38)	100% (29)	100% (7)
vegetal	equatorial	71% (14)	51% (13)	100% (13)	ND
animal	animal	0% (25)	0% (22)	23% (13)	0% (5)

Numbers of specimens examined are indicated in parentheses.
ALP, Expression of alkaline phosphatase; AchE, expression of acetylcholinesterase; ND, not determined.

The results of cytoplasmic transfer are shown in Table 1. When vegetal fragments of eggs were fused to the animal-pole region (Fig. 5A), transplanted cytoplasm stayed at the site of fusion during subsequent cleavage stages (Fig. 5B). The normal cleavage pattern was not restored. At the gastrula stage (approximately 10 hours after fertilization at 13°C), gastrulation movement was observed in 94% of cases. In these specimens, gastrulation always started in the animal pole region that had blue staining (Fig. 5C). As gastrulation proceeded, most of the blastomeres of the animal hemisphere, including non-labeled cells, invaginated. In 66% of cases, embryos developed into tailed larvae with labeled cells within their bodies (Fig. 5D) and notochord in their tails. Expression of

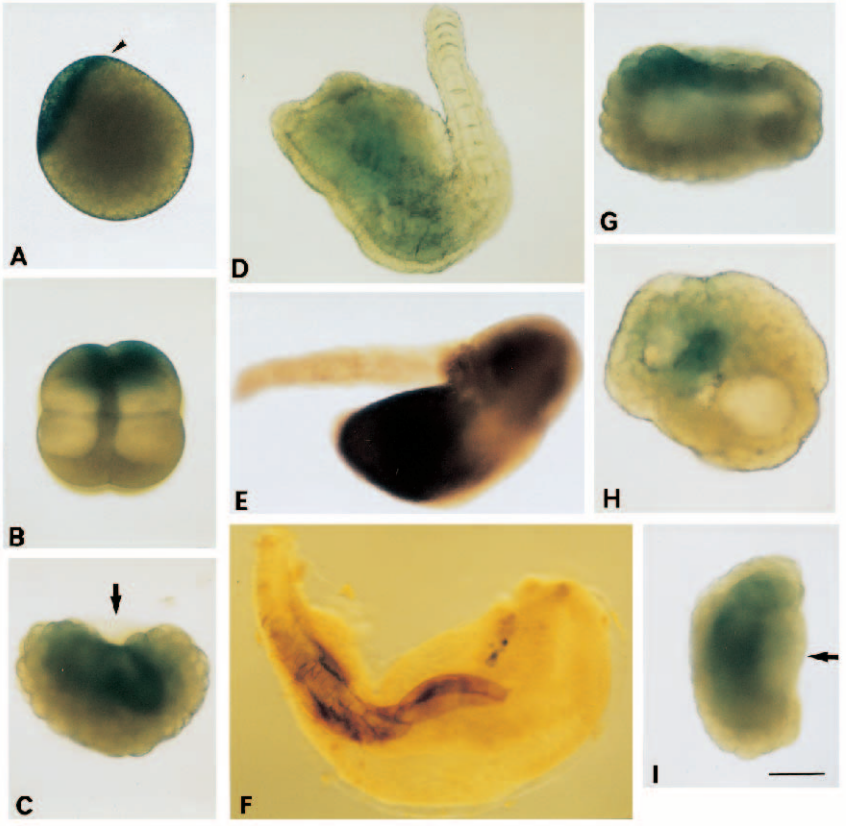

Fig. 5. Transplantation of vegetal pole cytoplasm to VC-deficient eggs. (A-F) Vegetal pole cytoplasm was stained with Nile Blue and then fused to the animal pole position of a VC-deficient egg. (A) Just after fusion. Vegetal pole cytoplasm was introduced just beside the polar bodies (arrowhead). (B) 8-cell stage. Lateral view. (C) Gastrula stage. Cells with blue staining have gastrulated from the animal pole region. The arrow indicates the direction of invagination. (D) The resultant larva has blue label within its body. A tail with notochord cells is visible. (E) Alkaline phosphatase activity was detected in the head region, revealing the presence of endoderm. (F) Acetylcholinesterase activity was also detected in the muscle cells. (G,H) In control experiments, animal pole cytoplasm was transplanted to the animal pole region of VC-deficient embryos. (G) Gastrula stage. Gastrulation did not take place. (H) The resultant larva has the morphology of a permanent blastula. (I) Vegetal pole cytoplasm was fused at an equatorial position of a VC-deficient egg. At the gastrula stage, cells with blue staining gastrulated from the equatorial region. An arrow indicates the direction of invagination. Scale bar, 100 μm.

alkaline phosphatase (a marker of endoderm) and acetylcholinesterase (a marker of muscle) was detected in all cases (Fig. 5E,F, respectively). In control experiments, animal pole cytoplasm was transplanted in the same way, but animal pole cytoplasm had no analogous activity (Fig. 5G,H).

In the next experiments, vegetal pole cytoplasm was transplanted to the equatorial region of VC-deficient eggs. In these specimens, gastrulation also started at the site of blue staining and, in half of such cases, tailed larvae developed. These results showed that vegetal pole cytoplasm can specify the site of gastrulation even when it is in unusual positions and, moreover, that formation of tissues derived from vegetal blastomeres can be achieved by transplantation of vegetal pole cytoplasm.

In the last set of experiments, vegetal pole cytoplasm was transferred to the animal pole region of normal fertilized eggs from which vegetal pole cytoplasm had not been removed. Thus, the operated eggs had vegetal pole cytoplasm at both poles. These embryos did not exhibit any gastrulation-related movement. The most plausible explanation is that, because ascidian embryos have no blastocoel, it is difficult to gastrulate from both sides of an embryo.

Removal of vegetal cytoplasm after the second phase of ooplasmic segregation

Bates and Jeffery (1987) and Nishida (1994b) reported that removal of a small region of vegetal pole cytoplasm after the second phase of segregation had no effect on embryogenesis. This observation was reconfirmed in the present study, as mentioned in the first section of the Results. Removal just after the second phase had started was without any effect. Bates and Jeffery (1987) suggested that factors required for gastrulation might spread throughout the entire vegetal hemisphere during the second phase. This suggestion was examined by removing cytoplasmic fragments of gradually larger volume from the vegetal pole after the second phase of segregation. During the second phase, male and female pronuclei move to the center of the egg. Therefore, cytoplasmic fragments with volumes of up to 50% could be removed from the vegetal pole region without removal of nuclei.

The results are summarized in Fig. 6. In order completely to suppress gastrulation and the expression of alkaline phosphatase (the marker of endoderm), the entire vegetal hemisphere had to be removed. These results support the hypothesis that factors required for gastrulation are distributed throughout the vegetal hemisphere at this stage.

DISCUSSION

Removal of vegetal pole cytoplasm between the first and second phases of segregation resulted in animalization of embryos. Therefore, vegetal pole cytoplasm is required for gastrulation and for specification of vegetal blastomeres. When vegetal pole cytoplasm was transplanted to the animal pole position, the animal-vegetal axis was reversed. This result indicates that vegetal pole cytoplasm is sufficient to promote gastrulation and to specify vegetal fates.

The role of the vegetal pole cytoplasm

The vegetal pole cytoplasm after the first phase of segregation had multiple roles, as indicated previously by Jeffery and Swalla (1990). In *Halocynthia*, the cytoplasm appears to contain factors that are involved in three distinct processes, as described below.

(1) Specification of the developmental fates of vegetal blastomeres

Removal of cytoplasm equivalent to only 5-8% of an egg volume prevented the formation of all the components derived from blastomeres of the vegetal hemisphere, such as endoderm, muscle and notochord. In *Halocynthia*, the presence and localization in zygotes of several kinds of tissue determinant has been reported (Nishida, 1992a, 1993, 1994a). The localization of muscle and endoderm determinants is restricted to the vegetal pole region between the first and second phase of segregation (Fig. 7). Therefore, it is likely that these determinants were removed in the VC-deficient eggs. In contrast to muscle and endoderm determinants, epidermis determinant is broadly distributed within the egg cytoplasm. This broad distribution may explain preferential formation of epidermis that occurred in the VC-deficient embryos.

Removal of vegetal pole cytoplasm also suppressed notochord formation. It is known that notochord is induced during ascidian embryogenesis (Nakatani and Nishida, 1994). Therefore, inducer cells or competent cells or both might be absent from the VC-deficient embryos. Thus, removal of

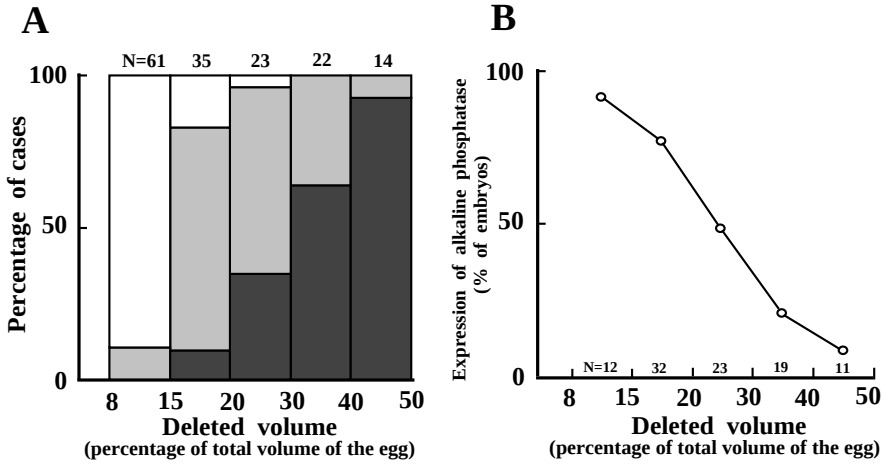

Fig. 6. Various amounts of vegetal cytoplasm were removed after the completion of the second phase of ooplasmic segregation. The volume removed as a percentage of the total egg volume is indicated on the abscissa. Removal of 50% indicates removal of the entire vegetal half. (A) Larval morphology. Unshaded areas represent the proportion of larvae with normal morphology. Lightly shaded areas represent the proportion of deformed larvae that still had a distinct head and tail. Heavily shaded areas represent the proportion of permanent blastulae. (B) The percentages of larvae that expressed alkaline phosphatase activity were scored and plotted against the volume of cytoplasm that was removed.

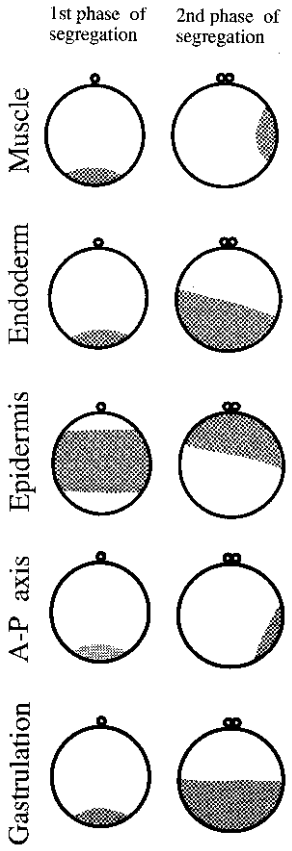

Fig. 7. Schematic comparison of the movements of various cytoplasmic factors in ascidian zygotes. The animal pole is at the top. An egg after the first phase of segregation is rotationally symmetrical, while an egg after the second phase is bilaterally symmetrical. In these diagrams, anterior is to the left and posterior is to the right. In the upper three rows, localization of the tissue determinants for muscle, endoderm and epidermis after the first and second phases of ooplasmic segregation are represented schematically (Nishida, 1992a; 1993; 1994a). Shaded areas represent locations of cytoplasmic determinants. In the fourth row, the movement of the determinant for formation of the anterior-posterior (A-P) axis is shown (Nishida, 1994b). The bottom row indicates the distribution of the determinant for gastrulation, as inferred from the results of the present experiments.

vegetal pole cytoplasm might suppress the formation of vegetally derived tissues both directly and indirectly.

(2) Specification of the site of gastrulation

By removing vegetal egg fragments, Bates and Jeffery (1987) showed that vegetal pole cytoplasm is required for the movements associated with gastrulation. The effect of removal of vegetal pole cytoplasm can be mimicked by the effect of UV irradiation near the vegetal pole (Jeffery, 1990a). Jeffery (1990b) suggested that a UV-sensitive maternal mRNA that encodes a cytoskeletal protein (P30) might be involved in gastrulation. The present study demonstrates that vegetal pole cytoplasm is sufficient for determination of the site of gastrulation even when transplanted to a non-standard location. Factors required for gastrulation must spread throughout the entire vegetal hemisphere during the second phase of segregation (Fig. 7)

(3) Generation of a unique cleavage pattern

The cleavage pattern of ascidian embryos is complicated but invariant. Cleavages progress in a bilaterally symmetrical manner. The cleavage pattern differs significantly between the anterior and posterior halves and between the animal and vegetal halves of the embryo. In earlier experiments (Nishida, 1994b), posterior-vegetal cytoplasm was removed after the completion of the second phase of segregation. In the resultant embryos, cleavages were radially symmetrical along the animal-vegetal axis. However, such embryos still exhibited differences in cleavage patterns between the animal and vegetal hemispheres. In the present study, the cells of VC-deficient embryos cleaved with radial symmetry. Moreover, the cleavage patterns in the animal and vegetal hemispheres were identical.

The various observations suggested that two distinct factors are involved in generation of the unique cleavage pattern of ascidian embryos. One is required for generation of the different patterns in the animal and vegetal hemispheres. This factor is localized at the vegetal pole after the first phase of segregation. Another factor is required to generate the difference between anterior and posterior halves. This factor is also localized at the vegetal pole temporarily and moves to the posterior pole during the second phase of segregation.

In *Styela*, when vegetal pole cytoplasm equivalent to 5-15% of the egg volume is removed between the first and second phase of segregation, or when the vegetal pole is irradiated with UV light, embryos do not gastrulate. However, in spite of the deficiency in morphogenesis, such embryos have a normal cleavage pattern, and they express both alkaline phosphatase and acetylcholinesterase, the markers of endoderm and muscle, respectively (Bates and Jeffery, 1987; Jeffery, 1990b). The results obtained in *Styela* indicate that the factor that promotes gastrulation is separable from muscle and endoderm determinants and from factors that control the cleavage pattern. In the experiments with *Styela*, egg fragments were removed by extrusion of a portion of the egg through a hole in the vitelline membrane and then severing it from the remaining ooplasm. A few experiments were done in *Halocynthia* using the method of Bates and Jeffery (1987), and we observed the subsequent radialization of the cleavage pattern and the absence of endoderm and muscle. There are differences between *Styela* and *Halocynthia*. *Halocynthia* eggs have twice the diameter and hence eighth the volume of *Styela* eggs. Therefore, it is possible that cytoplasmic determinants are localized in a relatively restricted region. It might be possible to remove most of the relevant factors together in *Halocynthia* while, in *Styela*, the determinants of muscle, endoderm, and of the cleavage pattern, could be distributed over a broader vegetal region than that involved in gastrulation.

Movements of various factors during ooplasmic segregation

In unfertilized eggs of ascidians, unlike those of echinoderm, it seems likely that various cytoplasmic determinants are uniformly distributed along the animal-vegetal axis since twin larvae develop from halves of the same unfertilized egg regardless of the plane of bisection of the egg (Reverberi and Ortolani, 1962). The ooplasm moves after fertilization, bringing various kinds of cytoplasmic factor to specific positions.

Fig. 7 summarizes the postulated movements of known cytoplasmic factors. Most of the factors, apart from the determinant of epidermis, are temporarily segregated to the vegetal pole region during the first phase of segregation. Then, the second phase of segregation starts. The various factors move in two ways. The muscle determinant and antero-posterior axis determinant (Nishida, 1994b) move to the future posterior pole. This movement coincides closely with visible movements of the cytoplasm. The vegetal pole cytoplasm moves together with the sperm aster and this process is mediated by microtubules (Sawada and Schatten, 1988). The second type of movement is that of the endoderm determinant and the deter-

minant for gastrulation. Their distribution extends over the entire vegetal half during the second phase of segregation. This movement does not coincide with the visible movement of the vegetal cytoplasm. These determinants might bind to the cytoskeleton (Jeffery and Meier, 1983) during the first phase of segregation. Then they might be released from the cytoskeleton and diffuse to the vegetal hemisphere. Alternatively, mRNAs encoding these factors might be segregated to the vegetal pole. Then translation of these mRNAs would occur and the protein products might diffuse to the vegetal hemisphere, as observed in the case of the *bicoid* mRNA and protein in *Drosophila* eggs (Driever and Nüsslein-Volhard, 1988).

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