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Localized regions of egg cytoplasm that promote expression of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi*

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SUMMARY

Embryogenesis in ascidians is known to be of the mosaic type, a property that suggests the presence of cytoplasmic factors in the egg which are responsible for specification of the developmental fates of early blastomeres. Endoderm cells are present in the trunk region of tadpole larvae, and these cells specifically express alkaline phosphatase (AP). Endoderm cells originate exclusively from blastomeres of the vegetal hemisphere of early embryos. To obtain direct evidence for cytoplasmic determinants of endoderm specification, we carried out cytoplasmic-transfer experiments by fusing blastomeres and cytoplasmic fragments from various regions. Initially, presumptive-epidermis blastomeres (blastomeres from the animal hemisphere) were fused to cytoplasmic fragments from various regions of blastomeres of 8-cell embryos of *Halocynthia roretzi*, and development of endoderm cells was monitored by histochemical staining for AP. AP activity was observed only when presumptive-epidermis blastomeres were fused with cytoplasmic fragments from the presumptive-endoderm blastomeres. The results suggest that cytoplasmic factors that promote the initial event of endoderm differentiation (endoderm determinants) are present in endoderm-lineage blastomeres. Next, to examine the presence and localization of endoderm determinants in the egg, cytoplasmic fragments from various regions of unfertilized and fertilized eggs were fused with the presumptive-epidermis blastomeres. The results suggest that endoderm determinants are already present in unfertilized eggs, and that they are segregated by movements of the ooplasm after fertilization. Initially, these determinants move to the vegetal pole of the egg. Then, prior to the first cleavage, their distribution extends in the equatorial direction, namely, to the entire vegetal hemisphere from which future endoderm-lineage blastomeres are formed.

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

INTRODUCTION

It has been suggested that maternal information localized in particular regions of the egg cytoplasm plays important roles in the determination of developmental fates during the early development of animals. One hypothesis is that the origin of cell-type heterogeneity can be traced back to the heterogeneity in the egg cytoplasm. The study of ascidian embryogenesis has provided various pieces of evidence for autonomous development, whereby blastomeres isolated from early embryos differentiate into tissues according to their normal fates. This phenomenon can be taken as an indication that prelocalized ooplasmic factors specify tissue precursor cells during embryogenesis (Conklin, 1905a). Indeed, experiments involving cytoplasmic redistribution and cytoplasmic transfer have revealed the presence of cytoplasmic determinants that confer on muscle-lineage cells the ability to become muscle cells (Whittaker, 1980 and 1982; Deno and Satoh, 1984; Nishida, 1992b).

Endoderm cells are present in the central part of the trunk region of tadpole larvae. These cells are homogeneous in appearance and rich in yolk granules. They may serve as a source of nutrients, and they do not differentiate to their terminal state during larval development in *Halocynthia* since tadpoles do not feed before metamorphosis. However, during embryogenesis, endoderm cells initiate some processes associated with differentiation and they begin to produce a tissue-specific enzyme, alkaline phosphatase (AP), at gastrulation (Minganti, 1954; Whittaker, 1977; Fig. 1). The role of AP in the endoderm is not yet known. Strictly speaking, the enzyme may be present in much smaller amounts in other tissues, but AP has often been used as a molecular marker of endoderm differentiation (e.g. Whittaker, 1977).

Inhibitors of transcription do not prevent the appearance

of AP activity even if the treatment is started before fertilization (Whittaker, 1977; Bates and Jeffery, 1987a). Such drugs can suppress the appearance of markers of differentiation of other tissues. This result suggests that maternal mRNA for AP is present in the egg cytoplasm. However, there is also evidence against this possibility. Non-nucleate fragments of fertilized eggs do not develop AP activity, an indication of a requirement for a nucleus (Bates and Jeffery, 1987a).

Expression of AP occurs in partial embryos that are derived from isolated presumptive-endoderm blastomeres (Whittaker, 1990, Nishida, 1992a). The expression of AP can be observed in continuously dissociated embryos (Nishida, 1992a). In embryos in which cell division is arrested after cleavage stages, AP activity appears only in endoderm-lineage cells (Whittaker, 1977; Whittaker and Meedel, 1989). These observations demonstrate the cell autonomy of endoderm differentiation and suggest the existence of cytoplasmic determinants that direct the differentiation of endoderm cells. However, direct proof of this possibility requires the transfer of cytoplasm to a heterotopic position and a resultant change in the fate of recipient cells. In the present study, in order to investigate the presence and localization of cytoplasmic factors that specify endoderm fate in eggs and embryos, non-endoderm precursor cells and cytoplasmic fragments from various regions of eggs and embryos were fused by a previously devised method (Nishida, 1992b).

MATERIALS AND METHODS

Embryos

Naturally spawned eggs of *Halocynthia roretzi* were artificially fertilized and reared in Millipore-filtered sea water that contained 50 μg/ml streptomycin sulfate (MFSW) at 9-13°C. At 13°C, tadpole larvae hatched about 35 hours after fertilization.

Isolation of blastomeres and preparation of fragments of blastomeres and eggs

Manually dechorionated eggs were reared in MFSW in 0.9% agar-coated plastic dishes. At the 8-cell stage, a4.2 (presumptive-epidermis) blastomeres, for subsequent fusion with cytoplasmic fragments, were isolated with a fine glass needle. Fragments of blastomeres were made by bisection with a glass needle. Fragments were severed from eggs such that the volume of each fragment was approximately equal to that of an a4.2 blastomere. After waiting for cell division, only non-divided fragments whose counterparts divided were used as non-nucleate, cytoplasmic fragments. Identification of nucleate and non-nucleate fragments by this method was verified by staining some fixed specimens with a fluorescent dye that is specific for DNA, namely, DAPI. Isolated blastomeres and cytoplasmic fragments were prepared from eggs fertilized at different times so that their cell cycles were synchronized at the time of fusion.

Fusion of blastomeres and cytoplasmic fragments

A method involving polyethylene glycol and electric field-mediated fusion (PGEF-mediated fusion) was used to fuse blastomeres and cytoplasmic fragments one by one under a stereomicroscope. The success rate of such fusion was about 90%. The methods for fusion were described previously (Nishida, 1992b). Briefly, an a4.2 blastomere and a cytoplasmic fragment were allowed to adhere firmly to one another as a result of treatment with 40% (w/v) polyethylene glycol in water. Then a single rectangular electrical pulse of 110 V/cm was applied to the adhering cells for 10-20 μseconds in fusion medium (0.77 M D-mannitol in 0.25% Ca^{2+}- and Mg^{2+}-free artificial sea water). Then cells were immediately transferred to sea water. Fused blastomeres divided with a cell cycle of normal duration and were allowed to develop until unoperated larvae hatched.

Histochemical staining for alkaline phosphatase (AP)

Larvae and embryos were fixed in 5% formaldehyde in sea water for 10 minutes at room temperature, or they were fixed in cold 70% ethanol for 30 seconds. In general, fixation with formaldehyde was used. Histochemical staining for AP activity was performed by the method described by Whittaker and Meedel (1989), with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate. This reaction results in the formation of brownish-purple deposits.

RESULTS

A *Halocynthia* larva that was histochemically stained for AP is shown in Fig. 1. The staining is restricted to endoderm cells. The endodermal strand, which is located ventrally in the tail of the larva, is not stained. Whittaker (1977) and Bates and Jeffery (1987a) described AP activity in posterior notochord cells of the larval tail in *Ciona intestinalis* and *Styela plicata*. However, no staining was observed in notochord cells in *Halocynthia*.

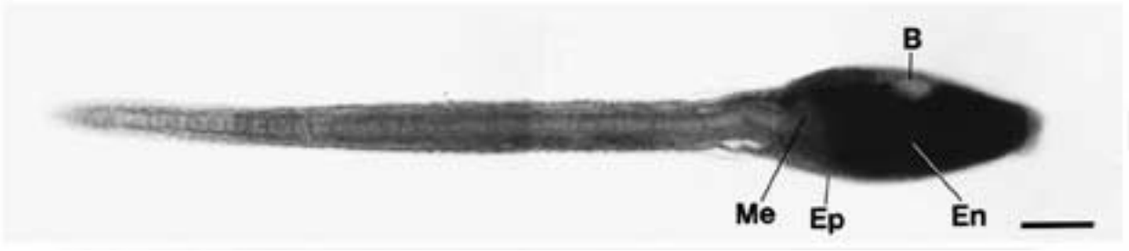

Fig. 1. A *Halocynthia* larva that was histochemically stained for alkaline phosphatase (AP). Staining is restricted to endoderm cells (En), which are located in the central part of the trunk region of the tadpole larva. Epidermis (Ep), brain (B), and mesenchyme (Me) are not stained. There is no AP activity in the tail. Scale bar, 100 μm.

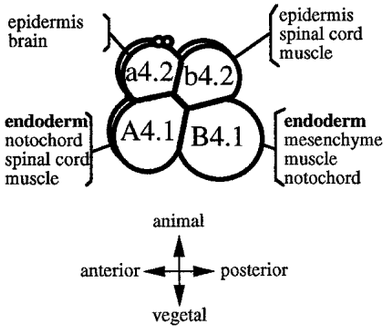

Fig. 2. Lateral view of the bilaterally symmetrical 8-cell embryo, demonstrating the orientation of its blastomeres and major descendant tissues.

In ascidian embryos, the pattern of cleavage and the developmental fates of blastomeres are invariant (Conklin, 1905b; Ortolani, 1955; Nishida, 1986, 1987). The developmental fate of each blastomere of the 8-cell embryo is shown in Fig. 2 (Nishida and Satoh, 1983). All of the endoderm cells of a larva are derived from the vegetal blastomeres of an 8-cell embryo, namely, the anterior A4.1 cell pair and the posterior B4.1 cell pair. The A4.1 cells give rise to anterior endoderm and the B4.1 cells develop into posterior endoderm. By contrast, blastomeres of the animal hemisphere (a4.2 and b4.2 cell pairs) have no progeny in the endoderm. Whittaker (1990) reported that isolated vegetal blastomeres of *Ciona intestinalis* develop AP activity autonomously. We confirmed this result in *Halocynthia* embryos. The A4.1 and B4.1 cells were isolated from the 8-cell embryos, and then they were cultured as partial embryos until unoperated larvae hatched. Eventually the A4.1 partial embryos developed AP activity in 90 out of 90 cases (Fig. 3A), and the B4.1 partial embryos expressed AP in 102 out of 110 cases (Fig. 3B). By contrast, none of 198 partial embryos that were derived from isolated animal blastomeres expressed AP (Fig. 3C). These results demonstrate the mosaic behavior of each blastomere of the 8-cell embryo with respect to endoderm specification. The a4.2 (anterior-animal) blastomeres were used as a recipient of cytoplasm in this study because these cells never develop AP activity in isolation. The a4.2 blastomeres normally give rise to the epidermis and brain of tadpole larvae (Fig. 2).

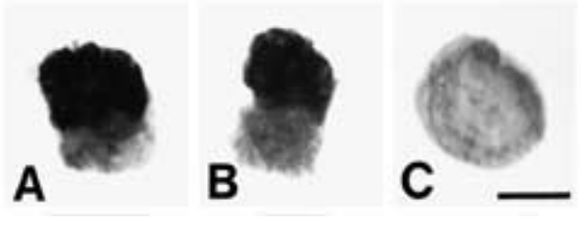

Fig. 3. The expression of AP in partial embryos that were derived from isolated blastomeres of 8-cell embryos. (A) An A4.1 partial embryo shows AP activity. (B) A B4.1 partial embryo shows AP activity. (C) A partial embryo, derived from an animal blastomere, does not show AP activity. Scale bar, 100 μm.

Isolated a4.2 blastomeres develop autonomously into epidermis (Nishikata et al., 1987).

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

To identify the cytoplasmic region capable of promoting the expression of AP in 8-cell embryos, initial experiments were carried out in which presumptive-epidermis blastomeres (a4.2; Fig. 2) were fused with cytoplasmic fragments from various regions of blastomeres derived from 8-cell embryos. Preparation of cytoplasmic fragments of endoderm-lineage cells (cells of the vegetal hemisphere; Fig. 2), was carried out according to Nishida (1992b). First, vegetal quartets were isolated from 8-cell embryos. Using the A4.1 blastomere pair as an indicator of the anterior pole, B4.1 blastomeres were bisected into anterior and posterior halves. Finally, all the B4.1 fragments were separated from each other and from the A4.1 blastomeres. Similarly, A4.1 blastomeres were bisected into anterior and posterior halves using the B4.1 blastomeres as an indicator of the posterior pole. When the anterior and posterior fragments from a single blastomere were cultured for about 45 minutes, one of them divided. The non-divided fragments were used for fusions as cytoplasmic fragments. The cytoplasmic fragments and newly isolated a4.2 blastomeres were fused by PGEF-mediated fusion. Fused cells continued to divide and developed into multicellular embryos. They were allowed to develop until unoperated embryos hatched. Then they were prepared for histochemical staining for AP (Table 1a).

When isolated a4.2 blastomeres were fused with the anterior and posterior cytoplasmic fragments of the A4.1 blas-

Table 1. Expression of alkaline phosphatase (AP) in fused blastomeres

Cytoplasmic fragments	Operated specimens	Successfully fused	Successfully divided	AP expression
(a) 8-cell stage				
A4.1 anterior	38	35	21	15 (71%)
A4.1 posterior	46	42	30	28 (93%)
B4.1 anterior	64	60	25	21 (84%)
B4.1 posterior	62	58	28	22 (78%)
a4.2	32	31	29	0 (0%)
(b) Before fertilization				
unfertilized egg	41	30	26	18 (69%)
(c) First phase of ooplasmic segregation				
animal	52	48	45	1 (2%)
equatorial	47	44	40	2 (5%)
vegetal	44	42	42	38 (91%)
(d) Second phase of ooplasmic segregation				
animal	59	52	49	0 (0%)
vegetal	47	40	39	39(100%)
anterior	41	35	34	32 (94%)
lateral	46	46	46	37 (80%)
posterior	41	41	38	20 (53%)

AP activity was detected histochemically. No distinction was made between cytoplasmic fragments of anterior and posterior origin from a4.2 blastomeres. Unfertilized eggs were cut without reference to their orientation. In d, results of fusion of both of the left and right fragments are shown together as lateral fragments.

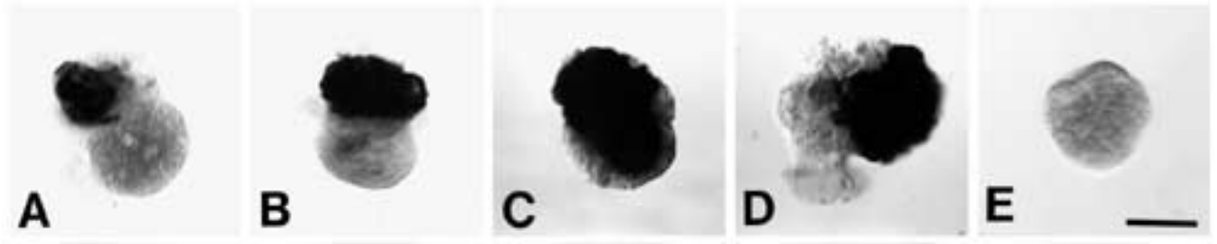

Fig. 4. Expression of AP in embryos derived from fused blastomeres. Isolated a4.2 blastomeres were fused with cytoplasmic fragments derived from (A) the anterior region of an A4.1 blastomere, (B) the posterior region of an A4.1 blastomere, (C) the anterior region of a B4.1 blastomere, (D) the posterior region of a B4.1 blastomere, and (E) the anterior or posterior region of an a4.2 blastomere from 8-cell embryos. Endoderm differentiation is evident in every case except (E). Scale bar, 100 μm.

tomeres, overt development of AP activity was observed in 71% and 93% of cases, respectively (Fig. 4A,B). When a4.2 cells were fused with the anterior and posterior cytoplasmic fragments of the B4.1 cells, expression of AP was observed in 84% and 78% of cases, respectively (Fig. 4C,D). In these cases, cells expressing AP were located within a single cluster. Differentiation of epidermis always occurred in other part of the embryos. Epidermal areas had smooth surfaces and secreted transparent larval tunic materials. Differentiation of epidermis in these cases substantiates the notion that the recipient of cytoplasm was an epidermis-precursor cells. In control experiments, a4.2 blastomeres were fused with cytoplasmic fragments of other a4.2 blastomeres. The expression of AP was never observed in these cases (Fig. 4E). The fused blastomeres developed into permanent blastulae, that is to say into balls of epidermis. These results suggest that the ability to promote endodermal expression of AP is associated with the cytoplasm of presumptive-endoderm blastomeres and not with that of presumptive-epidermis blastomeres at the 8-cell stage.

Fusion of presumptive-epidermis blastomeres and various cytoplasmic fragments of the egg

Uncleaved eggs were examined for the presence and localization of cytoplasm with the ability to promote the expression of AP. Ooplasm of ascidian eggs undergoes dramatic movements after fertilization, and this process is known as ooplasmic segregation (Conklin, 1905b; Hirai, 1941; Sawada and Osanai, 1981; Jeffery and Meier, 1983; Sardet et al., 1989). Movement of the ooplasm occurs in two phases between fertilization and the first cleavage. In *Halocynthia*, the first phase (0-10 minutes after insemination at 9°C) involves segregation of transparent myoplasm to the vegetal pole. During the second phase (85-110 minutes), the transparent myoplasm moves towards the future posterior pole and forms a crescent-shaped domain (see Fig. 5 in Nishida, 1992b). The first cleavage occurs at 160 minutes.

Bates and Jeffery (1987a) reported that non-nucleate fragments of fertilized eggs do not develop AP activity, an indication of a requirement for a nucleus in *Styela*. We con-

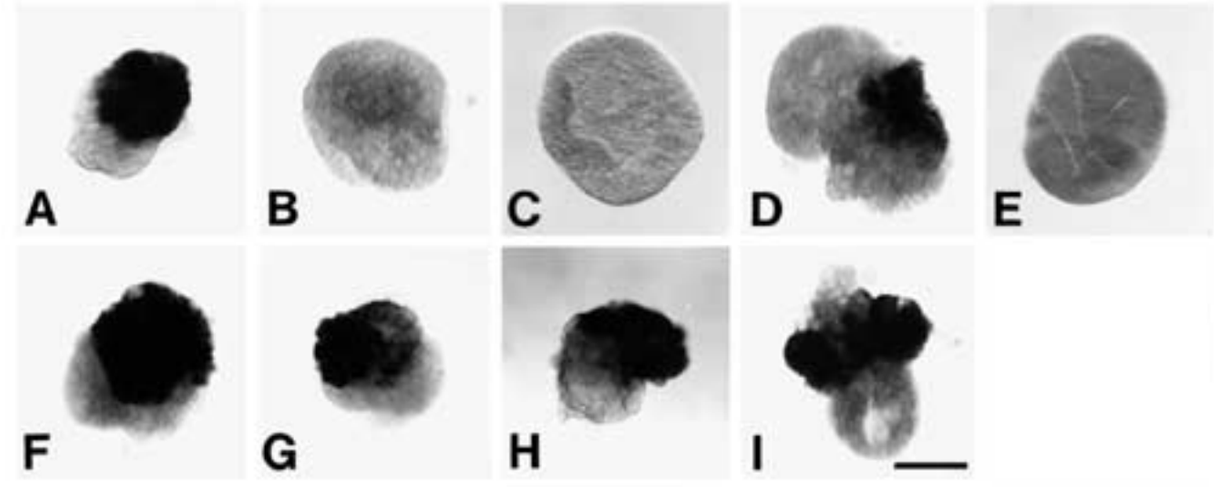

Fig. 5. Expression of AP in embryos derived from fused blastomeres. Isolated a4.2 blastomeres were fused with various cytoplasmic fragments derived from (A) unfertilized egg; (B) the animal region, (C) the equatorial region, and (D) the vegetal region of a fertilized egg at the end of the first phase of ooplasmic segregation; (E) the animal region, (F) the vegetal region, (G) the anterior region, (H) the lateral region, and (I) the posterior region of a fertilized egg at end of the second phase of ooplasmic segregation. Differentiation of endoderm is evident in A,D,F,G,H and I, but not in B,C and E. Scale bar, 100 μm.

firmed this result in *Halocynthia* embryos. Fertilized eggs were bisected along the animal-vegetal axis after the second phase of ooplasmic segregation. Nucleate fragments developed into larvae which exhibited AP activity (14 out of 14 cases). In contrast, non-nucleate fragments, cultured until controls hatched, did not developed AP activity (none out of 16 cases).

In fusion experiments, 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). In the unfertilized egg, the first meiotic spindle is situated at the animal pole. However, it is not possible to discern the spindle in unfixed, unstained specimens. Consequently, fragments were made without reference to orientation and all fragments were used for fusion with a4.2 cells. The fused cells continued to divide. Eventually, they developed AP activity in 69% of cases (Table 1b, Fig. 5A). After the first phase of segregation, eggs were radially symmetrical along the animal-vegetal axis, having a polar body at the animal pole, and myoplasm at the vegetal pole. Animal, equatorial, and vegetal cytoplasmic fragments were prepared separately for fusion with a4.2 cells. Fused cells divided and developed into multicellular embryos. Among these embryos, only in cases of fusion of vegetal fragments, did expression of AP occur at a high frequency (91%; Table 1c, Fig. 5B-D). After the second phase of segregation, eggs are bilaterally symmetrical, having polar bodies at the animal pole and myoplasm at the future posterior pole. The results of experiments with animal, vegetal, anterior, lateral, and posterior fragments are shown in Table 1d and Fig. 5E-I. When animal fragments were fused with a4.2 cells, AP activity was never observed. By contrast, when vegetal fragments were used for fusions, expression of AP occurred in all cases. Fusions with anterior, lateral and posterior fragments resulted in the expression of AP in 94%, 80%, and 53% of the specimens, respectively. It seems that the ability to promote the expression of AP gradually decreases from the anterior to the posterior region within the equatorial part of the egg. In the cases where AP-expressing cells were generated, the cells that expressed AP were always present as a single cluster. Differentiation of epidermis was also observed in other parts of the embryos. The results suggest that cytoplasm which has the ability to promote endodermal expression of AP is already present in unfertilized eggs and is segregated to the future endoderm region during ooplasmic segregation.

DISCUSSION

The results are summarized in Fig. 6. Cytoplasm with the ability to promote the expression of AP in presumptive-epidermis blastomeres is present only in endoderm-lineage (A4.1 and B4.1) cells at the 8-cell stage (Fig. 6A). A regional difference is apparent in the cytoplasm's ability to promote the expression of AP when fragments from various regions of eggs are fused to presumptive-epidermis cells (Fig. 6B-D). Results of experiments with isolated blastomeres (Whittaker, 1990, and this report) and with dissociated blastomeres (Nishida, 1992a) indicate that fate determination and the initial events in the differentiation of endodermal cells (i.e., expression of AP) are autonomous and, thus, these processes do not require cellular interactions. Therefore, cytoplasmic factors, which are segregated into endoderm-lineage cells, (endoderm determinants) appear to be sufficient for determination of the fate and expression of the features of endoderm cells of the larva. Terminal differentiation of endoderm (e.g. the formation of a gut, branchial sac, and pharynx) occurs much later during metemorphosis and may not be mediated by determinants inherited from the egg.

At the 8-cell stage, the ability to promote the expression of AP was higher in the posterior fragments than in the anterior fragments of the A4.1 cells (Fig. 6A). Similarly, the ability was higher in the anterior fragments than in the posterior fragments of the B4.1 cells. These results may reflect the fate map of the embryo, since the posterior part of the A4.1 cell and the anterior part of the B4.1 cell, namely, the central parts of the vegetal hemisphere, are future endoderm regions. By contrast, the most anterior part of the A4.1 cell is the site of formation of notochord-lineage and spinal cord-lineage cells, and the most posterior part of the B4.1 cell is the site of formation of mesenchyme-lineage and muscle-lineage cells (Conklin, 1905b; Ortolani, 1955; Nishida, 1987).

When fragments of unfertilized eggs were fused with an a4.2 cell, a significant proportion of the specimens expressed AP. After the first phase of segregation, only vegetal fragments had the ability to induce expression of AP. After the second phase of segregation, fragments other than

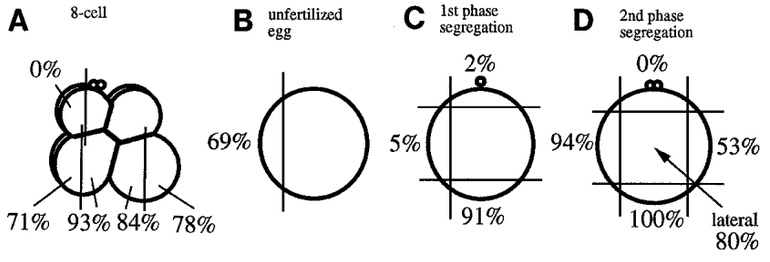

Fig. 6. Schematic representation of the results of the present fusion experiments. Planes of sections are shown and results of the fusions described herein are presented in term of percentages of specimens that expressed AP activity. Small circles at the animal pole of eggs and the embryo represent polar bodies. (A) Lateral view of an 8-cell embryo. (B) Unfertilized egg. (C) Egg after the first phase of segregation; the egg is radially symmetrical along the animal-vegetal axis. (D) Lateral view of an egg after the second phase of segregation, when 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.

animal fragments have this ability. It is suggested that endoderm determinants are already present in unfertilized eggs and that their distribution changes during ooplasmic segregation. Initially, these determinants move to the vegetal pole, then their distribution extends in the equatorial direction. After the completion of ooplasmic segregation, and before the first cleavage, the determinants are already localized to the site of formation of endoderm-lineage cells, namely, the vegetal hemisphere.

Endoderm cells are rich in yolk granules. Therefore, the portion of the ooplasm that is rich in yolk granules has been called the endoplasm (Conklin, 1905a). The inferred movements of endoderm determinants, as mentioned above, do not precisely coincide with the movement of the so-called endoplasm. The yolk-rich endoplasm moves towards the animal pole during the first phase of ooplasmic segregation (Conklin, 1905b; Jeffery et al., 1983), while endoderm determinants are localized at the vegetal pole region just after the first phase of ooplasmic segregation. During the second phase of ooplasmic segregation, endoplasm moves back into the vegetal hemisphere and it is eventually inherited by endoderm-lineage blastomere.

In our previous paper (Nishida, 1992b), it was suggested that, after the first phase of segregation, muscle determinants are localized only at the vegetal pole, being similar in this regard to endoderm determinants. Although, during the second phase of segregation, muscle determinants move towards the posterior pole, it is suggested that the distribution of endoderm determinants extends in every direction, i.e., in the posterior direction as well as in the anterior and lateral directions. During the second phase, it seems that the cytoplasm of the vegetal pole moves in a posterior direction. The movement of muscle determinants corresponds to this visible movement of cytoplasm, while that of endoderm determinants does not. It will clearly be of interest to determine the way in which the egg segregates the two kinds of the cytoplasmic factor.

Differentiation of epidermis was always observed in the embryos derived from the fused cells because the recipients of the cytoplasm were presumptive-epidermis blastomeres. Cytoplasm introduced by fusion did not suppress the epidermal fate of these cells. Endoderm cells were formed as a single cluster in the embryos. Therefore, introduced endoderm determinants may not diffuse in fused cells, and they may only be inherited by some descendant cells, as suggested similarly in the case of muscle determinants (Nishida, 1992b). They may be immobilized by the cytoplasmic skeleton (Jeffery and Meier, 1983). Another possibility is that they may be anchored in a plasma membrane introduced with the fused fragment. After fusion, yolk granules diffused evenly in fused cells, suggesting that it is unlikely that endoderm determinants attach to yolk granules.

AP activity appears during gastrulation in normal embryogenesis (Whittaker, 1977; Bates and Jeffery, 1987a). Treatment with an inhibitor of translation before gastrulation abolishes the appearance of AP activity. This result suggests that translation of the mRNA for the enzyme is required for the appearance of AP activity (Whittaker, 1977). However, inhibitors of transcription do not prevent the appearance of AP activity even if the treatment is started before fertilization (Whittaker, 1977; Bates and Jeffery, 1987a). Such drugs can suppress the appearance of markers of differentiation of other tissues. This result suggests that maternal mRNA for AP is present in the egg cytoplasm. It may be that the localized cytoplasmic factor required for development of AP activity is actually the maternal mRNA for AP itself. However, there is also evidence against this possibility. Non-nucleate fragments of fertilized eggs do not develop AP activity, an indication of a requirement for a nucleus (Bates and Jeffery, 1987a; and this paper). Cloning of the gene(s) for AP, which would allow the presence of maternal mRNA to be examined directly, as well as the identification of other markers of endoderm differentiation, are now required.

Endoderm-lineage cells lie in the central region of the vegetal hemisphere. They are the first cells to invaginate during gastrulation. Ortolani (1958) bisected fertilized eggs near the equator and showed that the animal fragments develop into permanent blastulae. Bates and Jeffery (1987b) showed that when small region of vegetal-pole cytoplasm are removed from the zygote between the first and second phase of segregation, the embryos do not gastrulate. The deletion of cytoplasm at the vegetal pole could be mimicked by UV irradiation of fertilized eggs near the vegetal pole (Jeffery, 1990). These results suggest that factors that specify the site of gastrulation, which are called axial determinants, are localized in the vegetal-pole region after the first phase of segregation. By contrast, removal of a small region of vegetal-pole cytoplasm after the second phase of segregation had no effect on embryogenesis. Bates and Jeffery (1987b) suggested that axial determinants spread throughout the entire vegetal hemisphere during the second phase of segregation. The inferred movements of the axial determinants seem to correspond closely to those of determinants of the expression of AP. However, it has been reported that when 5-15% of the total cytoplasm is removed from the vegetal pole after the first phase of segregation, the embryos fail to gastrulate but do develop AP activity (Bates and Jeffery, 1987b). The UV-irradiated embryos that fail to gastrulate express AP activity, indicating the development of AP activity is UV insensitive (Jeffery, 1990). Therefore, the cytoplasmic factors required for gastrulation and for expression of AP activity may not be the same.

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