Development 120, 235-243 (1994)
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Localization of egg cytoplasm that promotes differentiation to epidermis in embryos of the ascidian *Halocynthia roretzi*

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SUMMARY

Embryogenesis in ascidians is of the mosaic type. This property suggests the presence of cytoplasmic factors in the egg that are responsible for specification of the developmental fates of early blastomeres. The epidermal cells that surround the entire tadpole larva originate exclusively from blastomeres of the animal hemisphere of early embryos. To obtain direct evidence for cytoplasmic determinants of epidermis fate, we carried out cytoplasmic transfer experiments by fusing blastomeres and anucleate cell fragments from various regions of eggs and embryos. Initially, presumptive non-epidermis blastomeres (blastomeres from the vegetal hemisphere) were fused to cytoplasmic fragments from various regions of blastomeres of 8-cell embryos of *Halocynthia roretzi*, and development of epidermal cells was monitored by following the expression of an epidermis-specific antigen, as well as by observations of morphology and the secretion of larval tunic materials. Formation of epidermis was observed when vegetal blastomeres were fused with cytoplasmic fragments from the presumptive epidermis blastomeres. The results suggested that cytoplasmic factors that promoted epidermis differentiation (epidermis determinants) were present in epidermis progenitors. Vegetal blastomeres only manifested this change in fate when fused with cytoplasmic fragments of roughly equal or larger size. Next, to examine the presence and localization of epidermis determinants in the uncleaved egg, cytoplasmic fragments from various regions of unfertilized and fertilized eggs were fused with the vegetal blastomeres. The results suggested that epidermis determinants were already present in unfertilized eggs and that they were segregated by movements of the ooplasm after fertilization. After the first phase of ooplasmic segregation, these determinants were widely distributed, with the highest activity being located in the equatorial region. There were no indications of regional differences in the activity within the equatorial region of eggs at this stage. After the second phase of ooplasmic segregation, prior to the first cleavage, the activity moved in the animal direction, namely, to the animal hemisphere, from which future epidermis-lineage blastomeres are normally formed.

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

INTRODUCTION

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. 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,c). Indeed, experiments involving redistribution of cytoplasm and transfer of cytoplasm have revealed the presence of cytoplasmic determinants that confer on muscle- and endoderm-lineage cells the ability to become muscle and endoderm cells, respectively (Whittaker, 1980, 1982; Deno and Satoh, 1984; Nishida, 1992b, 1993). In addition to tissue determinants, it was shown that cytoplasmic determinants for gastrulation are present in ooplasm (reviewed by Jeffery, 1992).

Epidermis surrounds the entire surface of the ascidian larva (Fig. 1A). This epidermis consists of approximately 800 epithelial cells in a single layer and these cells secrete transparent larval tunic materials (Monroy, 1979; Nishida, 1987). Reverberi and Minganti (1946) showed that isolated epidermis-lineage cells from 8-cell embryos autonomously develop into permanent blastulae, namely, balls of epidermis. A monoclonal antibody, Epi-2, specifically recognizes differentiated epidermal cells and larval tunic materials (Fig. 1B; Nishikata et al., 1987). Expression of the epidermis-specific antigen occurs in partial embryos that are derived from isolated epidirmis-lineage cells of 8-cell embryos (Nishikata et al., 1987). The expression of the Epi-2 antigen can also be observed in continuously dissociated embryos (Nishida, 1992a). In embryos in which cell division is arrested after the cleavage stage, the Epi-2 antigen is expressed only in epidermis-lineage blastomeres (Nishikata et al., 1987). These observations demonstrate the cellular autonomy of epidermis differentiation and suggest the existence of cytoplasmic deter-

minants in epidermis-lineage cells that direct the differentiation of epidermal cells. When fertilized eggs are bisected horizontally, the animal fragments develop into permanent blastulae (Ortolani, 1958). This observation suggests that epidermis determinants may be preferentially present in the animal hemisphere within the cytoplasm of uncleaved eggs.

In the present study, in order directly to prove the presence and localization of cytoplasmic factors that specify epidermis fate, cytoplasm was transferred to a heterotopic position and the resultant change in the fate of recipient cells was examined. Thus, non-epidermis precursor cell and cytoplasmic fragments from various regions of eggs and embryos were fused by a previously described method (Nishida, 1992b, 1993), and the differentiation of epidermis was monitored.

MATERIALS AND METHODS

Embryos

Naturally spawned eggs of *Halocynthia roretzi* were artificially fertilized and reared in Millipore-filtered (pore size, 0.45 μm) 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 plastic dishes coated with 0.9% agar. Presumptive non-epidermis (vegetal) blastomeres, namely, A4.1 and B4.1 cells of 8-cell embryos and B5.2 cells of 16-cell embryos, were isolated with a fine glass needle for subsequent fusion with cytoplasmic fragments. 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 A4.1 blastomere. After cell division had occurred in egg cells, we used only the non-divided fragments as anucleate cell fragments. Identification of nucleated and anucleate fragments by this method was verified by staining some fixed specimens with a fluorescent dye that is specific for DNA, namely, DAPI (Nishida, 1992b). In this report, we designate anucleate cell fragments as cytoplasmic fragments, although they apparently have plasma membranes. Isolated blastomeres and cytoplasmic fragments were prepared from eggs fertilized at different times such 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 fusions was about 90%. The methods for fusion have been described previously (Nishida, 1992b). In brief, a recipient blastomere and a cytoplasmic fragment were allowed to adhere firmly to one another as a result of treatment with 30% (w/v) polyethylene glycol in water. Then a single rectangular electrical pulse of 1100-1300 V/cm was applied to the adhering cells for 10-20 μsec in fusion medium (0.77 M D-mannitol in 0.25% Ca^{2+}-free artificial seawater). Higher voltage was required to fuse smaller blastomeres and cytoplasmic fragments. The cells were immediately transferred to seawater. Fused blastomeres divided with a cell cycle of normal duration and were allowed to develop until unoperated larvae hatched.

Immunohistochemical staining of an epidermis-specific antigen

Specimens were fixed for 10 minutes in methanol at −20°C. They were then stained for indirect immunofluorescence with the Epi-2 monoclonal antibody and FITC-conjugated secondary antibody by standard methods. The monoclonal antibody, Epi-2 (kindly provided by Dr T. Nishikata, Konan University, Japan), recognizes an antigen that is specific for differentiated epidermal cells and the larval tunic, which is secreted by epidermal cells (Fig. 1B; Nishikata et al., 1987).

Marking of small areas of eggs with vital stain

A fine glass needle was filled with a 1% solution of Nile Blue B in distilled water. The needle was pushed against the area to be stained and the solution was ejected by pressure (Kuraishi and Osanai, 1992).

RESULTS

In ascidian embryos. the pattern of cleavage and the developmental fates of blastomeres are invariant (Conklin, 1905b; Ortolani, 1955; Satoh, 1979; Nishida and Satoh, 1983, 1985; Nishida, 1986, 1987). The developmental fate of each blastomere of the 8-cell embryo is shown in Fig. 2. The epidermis of a larva

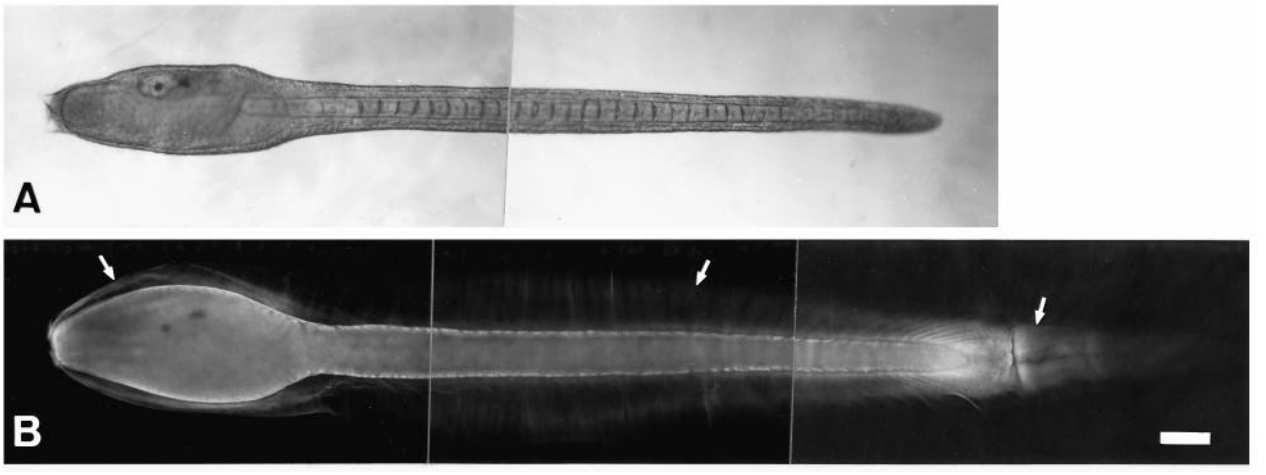

Fig. 1. (A) A larva of *Halocynthia roretzi*. The larva is surrounded by a single layer of epidermal cells. (B) A larva that was stained indirectly with the monoclonal antibody that recognizes the Epi-2 antigen. The single layer of epidermal cells and the larval tunic (arrows), which is secreted by the epidermal cells, are stained. Scale bar, 100 μm.

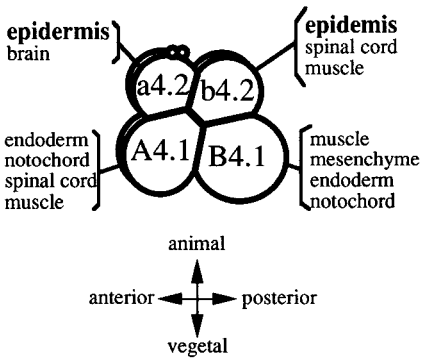

Fig. 2. Lateral view of a bilaterally symmetrical 8-cell embryo, demonstrating the orientation of the blastomeres and the major descendant tissues. Epidermis is exclusively derived from the animal blastomeres. At the next cleavage, the B4.1 blastomere divides unequally into an anterior large blastomere B5.1 and a posterior small blastomere B5.2.

originates exclusively from the four animal blastomeres of the bilaterally symmetrical 8-cell embryo, namely, the anterior a4.2 cell pair and the posterior b4.2 cell pair. The a4.2 cells give rise to the epidermis of the head and trunk regions and the b4.2 cells give rise to the epidermis of the tail region of the tadpole larva. By contrast, blastomeres of the vegetal hemisphere (A4.1 and B4.1 cell pairs) have no progeny in the epidermis. The isolated animal blastomeres of *Halocynthia* synthesize the Epi-2 antigen, which is recognized by the epidermis-specific monoclonal antibody. Vegetal blastomeres do not synthesize the Epi-2 antigen in isolation (Nishikata et al., 1987).

Fusion of non-epidermis precursor blastomeres with cytoplasmic fragments of blastomeres from 8-cell embryos

To prove the presence and localization of a cytoplasmic region with the ability to promote epidermis differentiation, initial experiments were carried out in which non-epidermis precursor blastomeres (A4.1 and B4.1; Fig. 2) were fused with cytoplasmic fragments from various regions of blastomeres derived from 8-cell embryos. Preparation of cytoplasmic fragments was carried out as described by Nishida (1992b). Blastomeres were bisected into anterior and posterior halves. When both fragments from a single blastomere were cultured for about 45 minutes, one of them divided. The non-divided fragments were used for fusions as anucleate cytoplasmic fragments. The cytoplasmic fragments and newly isolated recipient 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 immunostaining specific for the Epi-2 antigen (Table 1A).

When isolated a4.2 (epidermis progenitor) blastomeres were fused with cytoplasmic fragments from other a4.2 blastomeres, expression of the Epi-2 antigen was always detected (Fig. 3A). Morphologically, these embryos were so-called permanent blastulae, i.e., balls of single-layered epithelium. In every case, the surface of the embryo was smooth and epithelial cells secreted larval tunic although the larval tunic slipped off during the immunostaining procedure in most cases (see Fig. 4A,G for examples of residual larval tunic). These results indicate that epidermis differentiation is not affected by the fusion procedure. Isolated vegetal blastomeres (A4.1 and B4.1 cells) were fused with cytoplasmic fragments of animal blastomeres (a4.2 and b4.2 cells). No expression of the Epi-2 antigen was ever observed in any of the four possible combinations of donors and recipients of cytoplasm (Table 1A; Fig. 3B,C). Fused blastomeres developed into uneven cell masses without larval tunic, indicating that epidermis differentiation, in terms of morphology, also did not occur. The embryos resembled partial embryos derived merely from isolated recipient blastomeres. Thus, transferred cytoplasm seemed to have no effect on the development of recipient blastomeres in this experiment.

During fusions, we noticed that the volume of cytoplasmic fragments prepared from the animal blastomeres was much

Table 1. Expression of an epidermis-specific antigen in fused blastomeres

Cytoplasmic fragment	Recipient blastomere	Fused specimen	Successfully divided	Expression of the antigen
(A) Fusion of cytoplasmic fragments and blastomeres from 8-cell embryos				
a4.2 (0.33)*	a4.2 (0.73)	26	24	24 (100%)
a4.2 (0.33)	A4.1 (1)	20	20	0 (0%)
a4.2 (0.33)	B4.1 (1.08)	34	20	0 (0%)
b4.2 (0.32)	A4.1 (1)	45	20	0 (0%)
b4.2 (0.32)	B4.1 (1.08)	32	32	0 (0%)
(B) Fusion of cytoplasmic fragments and blastomeres from embryos at various stages				
a4.2 (0.33)	B5.2 (0.32)	30	25	23 (92%)
b4.2 (0.32)	B5.2 (0.32)	21	21	20 (95%)
A4.1 (N.D.)	B5.2 (0.32)	13	13	0 (0%)
2nd animal small (0.36)†	A4.1 (1)	62	48	4 (8%)
2nd animal large (0.98)†	A4.1 (1)	50	42	29 (69%)

The epidermis-specific antigen was detected by immunofluoresence staining with a monoclonal antibody. No distincion was made between cytoplasmic fragments of anterior and posterior origin from blastomeres at the 8-cell stage.

*Mean volumes of cytoplasmic fragments and blastomeres, relative to the volume of an A4.1 blastomere, are indicated in parentheses. N.D., Not determined.

†Cytoplasmic fragments were prepared from the animal region of uncleaved eggs after the second phase of ooplasmic segregation.

smaller than that of the recipient vegetal blastomeres. The volume of each blastomere of an 8-cell embryo was estimated by measuring the diameter of isolated blastomeres, which were almost spherical. The ratio of the volumes of the blastomeres, a4.2:b4.2:A4.1:B4.1, was 0.73:0.80:1.00:1.08 when the volume of an A4.1 cell was taken as 1.00. The mean volumes of cytoplasmic fragments prepared from the a4.2 and b4.2 cells were approximately one third of that of an A4.1 cell (Table 1A). To examine the effect of the ratio of the volume of the cytoplasmic fragment to that of the recipient blastomere on development of epidermis, we carried out two kinds of experiment. In the first experiment, the volume of recipient blastomeres was reduced and, in the second one, the volume of cytoplasmic fragments was increased (Table 1B).

B4.1 cells divide unequally, each forming an anterior large blastomere (B5.1) and a posterior small blastomere (B5.2) at the next cleavage (Conklin, 1905b; Satoh, 1979). The mean volume of a B5.2 cell was 32% of that of an A4.1 cell and was approximately equal to the volume of each cytoplasmic fragment prepared from animal blastomeres of 8-cell embryos. The B5.2 cells mainly give rise to muscle and mesenchyme and not to epidermis (Nishida and Satoh, 1985). and they did not develop into epidermis in isolation. Therefore, we chose B5.2 blastomeres as the recipients of cytoplasm. When cytoplasmic fragments of a4.2 and b4.2 cells were fused with B5.2 cells, overt expression of the Epi-2 antigen was observed in 92% and 95% of cases,, respectively (Fig. 3D,E, 5A). By contrast, embryos that were derived from fusion of a B5.2 cell and a cytoplasmic fragment of a non-epidermis precursor (an A4.1 cell) never expressed the Epi-2 antigen (Fig. 3F).

Next, the volume of cytoplasmic fragments to be fused was increased. It is not possible to prepare large cytoplasmic fragments from animal blastomeres of 8-cell embryos. It would appear likely that epidermis determinants might be present in the cytoplasm of the animal hemisphere of uncleaved eggs because all of the epidermis-lineage cells emerge in the animal hemisphere of embryos. Therefore, we prepared cytoplasmic fragments of two relative sizes from the animal pole region of uncleaved eggs just before the first cleavage. We prepared small fragments with a mean volume that was 36% of that of an A4.1 cell, which is similar to the volume of cytoplasmic fragments of animal hemisphere. We also prepared large fragments with a mean volume that was 98% of that of an A4.1 cell, that is to say, with a similar volume to that of the recipient A4.1 blastomeres. When the small fragments were fused with A4.1 cells, only 8% of embryos expressed detectable Epi-2 antigen (Fig. 3G). By contrast, when the large fragments were used for the fusion, 69% of the embryos expressed the antigen (Fig. 3H). Epidermis differentiation was also monitored in terms of morphology. The region in which the Epi-2 antigen

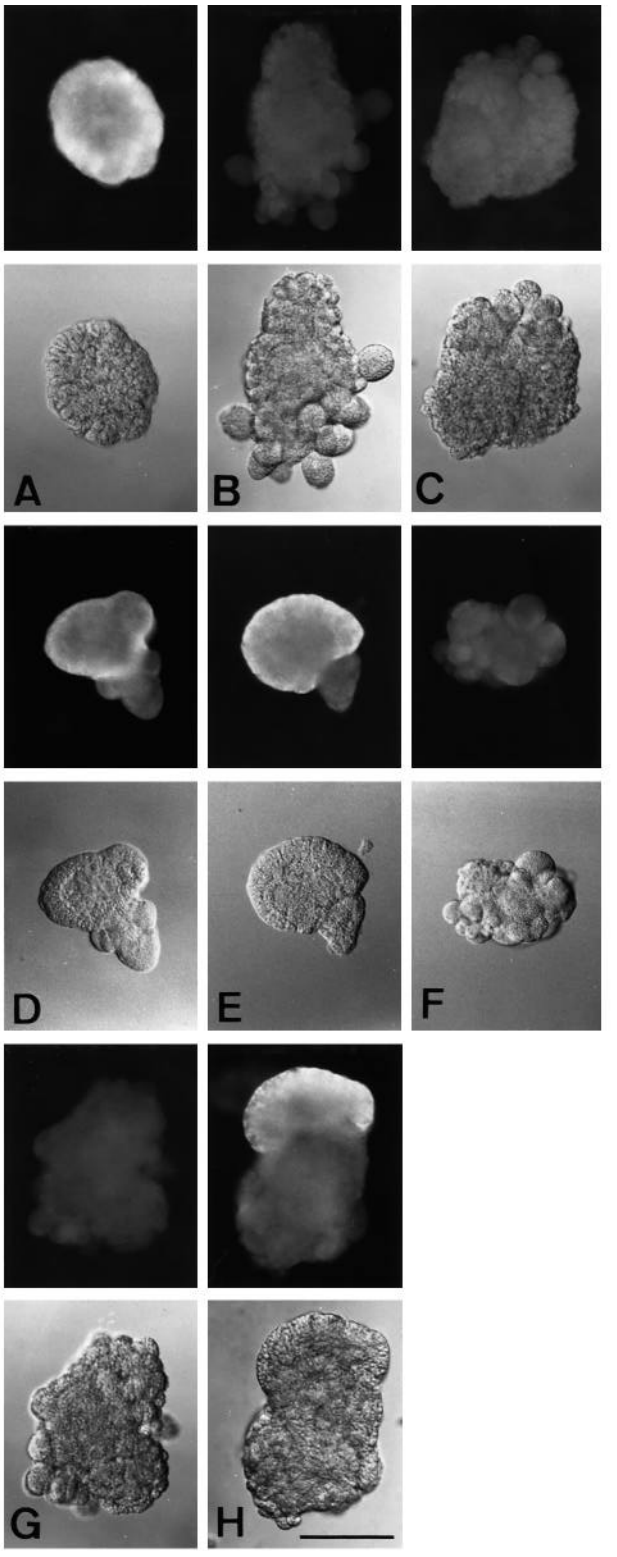

Fig. 3. Differentiation of epidermis in embryos derived from fused blastomeres. In each pair, the upper photograph shows the fluorescent image of the specimen and the lower one shows the Nomarsky image of the same specimen. (A) An isolated a4.2 (epidermis precursor) blastomere was fused with a cytoplasmic fragment of another a4.2 blastomere. Isolated A4.1 (vegetal) blastomeres were fused with cytoplasmic fragments of (B) an a4.2 blastomere and (C) a b4.2 blastomere. Isolated B5.2 (vegetal) blastomeres were fused with cytoplasmic fragments of (D) an a4.2 blastomere, (E) a b4.2 blastomere, and (F) an A4.1 blastomere. Isolated A4.1 (vegetal) blastomeres were fused with (G) a small cytoplasmic fragment, and (H) a large cytoplasmic fragment derived from the animal region of a fertilized eggs after the second phase of ooplasmic segregation. Differentiation of epidermis is evident in A, D,E and H both from the expression of the epidermis-specific antigen and the morphology. Scale bar, 100 μm.

was expressed coincided precisely with the region that had epithelial morphology and a smooth outer surface (Fig. 3). Larval tunic was also formed in the region. Epidermal cells partially covered the embryos that were derived from fusion, and non-epidermal cells were present within the embryos. These results indicate that the ability to promote epidermis differentiation is associated with the cytoplasm of presumptive epidermis blastomeres and not with that of vegetal blastomeres at the 8-cell stage. They also suggest that this ability is present in the animal region of uncleaved eggs even before cleavage occurs. The results shown in Table 1B suggest that some minimal ratio of the volume of introduced cytoplasm relative to the volume of the recipient blastomere is required for formation of epidermis.

Fusion of non-epidermis precursor blastomeres with various cytoplasmic fragments from the egg

Unfertilized and fertilized eggs before cleavage were examined for the presence and localization of cytoplasm that had the ability to promote differentiation to epidermis (Table 2A,B,D; Figs 4, 5). The 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 *Halocynia*, the first phase (0-10 minutes after insemination at 9°C) involved segregation of the transparent myoplasm to the vegetal pole. During the second phase (85-110 minutes), the transparent myoplasm

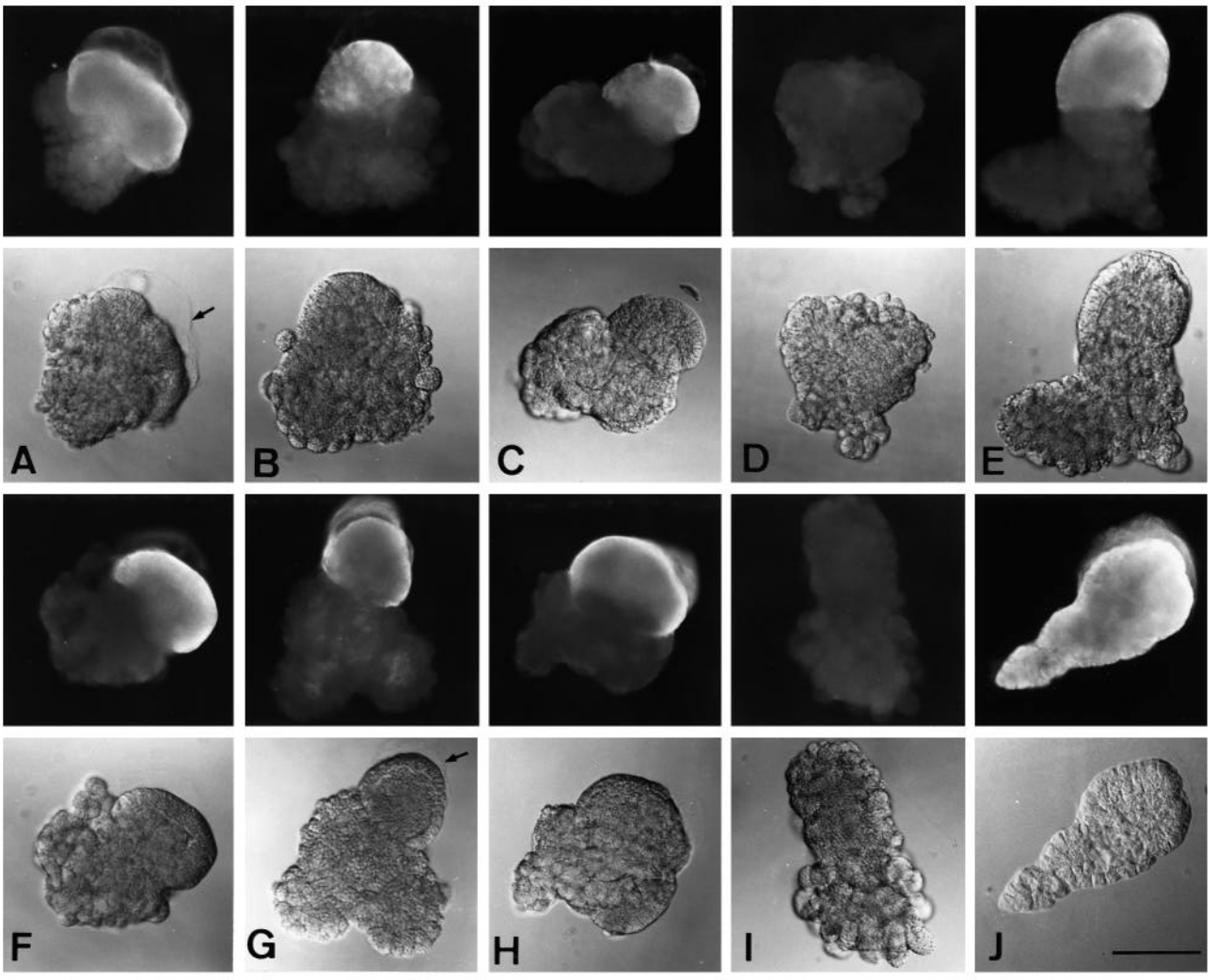

Fig. 4. Differentiation of epidermis in embryos derived from fused blastomere. In each pair, the upper photograph shows the fluorescent image of the specimen and the lower one shows the Nomarsky image of the same specimen. Isolated A4.1 (vegetal) blastomeres (except in J) were fused with cytoplasmic fragments from various regions of eggs. A4.1 blastomeres were fused with cytoplasmic fragments derived from (A) unfertilized eggs; (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; and (E) the animal region, (F) the anterior region, (G) the lateral region, (H) the posterior region, and (I) the vegetal region of a fertilized egg at the end of the second phase of ooplasmic segregation. (J) An isolated B5.2 (vegetal) blastomere was fused with a cytoplasmic fragment derived from the equatorial region of a fertilized egg at the end of the first phase of ooplasmic segregation. Arrows in A and G indicate larval tunic material that remained after the fixation and staining procedures. Differentiation of epidermis is evident in A,B,C,E,F,G,H and J. but not in D and I in terms of expression of the epidermis-specific antigen and morphology. Scale bar, 100 μm.

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.

In fusion experiments, fragments of eggs, which were approximately equal in volume to that of an A4.1 blastomere, were prepared prior to fertilization, after the first phase of ooplasmic segregation (20-50 minutes after fertilization) and after the second phase of ooplasmic 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 prepared without reference to orientation and all fragments were used for fusion with A4.1 cells. The fused cells continued to divide. Eventually, they expressed the Epi-2 antigen in 78% of cases (Table 2A; Fig. 4A). 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 fused with A4.1 cells, and the Epi-2 antigen was expressed in 17%, 47% and 10% of cases, respectively (Table 2B; Fig. 4B-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, anterior, lateral, posterior and vegetal fragments are shown in Table 2D and Fig. 4E-I. The animal fragments had the greatest ability (69%) to promote differentiation to epidermis. Fusions with anterior, lateral and posterior fragments resulted in expression of the Epi-2 antigen in 11%, 18% and 38% of specimens, respectively. It seems that the ability to promote epidermis differentiation decreases from the posterior to the anterior region within the equatorial part of the egg at this stage. By contrast, when vegetal fragments were used for fusions, no expression of the Epi-2 antigen was ever detected. Morphological observations supported these results. The region in which the Ept-2 antigen was expressed coincided with the region with epithelial morphology (Fig. 4). Larval tunic was also secreted in this region (Fig. 4A,G, arrows). Epidermal cells partially covered the aggregates of non-epidermal cells. The results suggest that cytoplasm that has the ability to promote epidermis differentiation is already present in unfertilized eggs and is segregated to the future epidermis region during ooplasmic segregation.

Examination of the localization of epidermis determinants within the equatorial ration after the first phase of ooplasmic segregation

Eggs of *Halocynthia* at the end of the first phase of ooplasmic segregation appear to be radially symmetrical along the animal-vegetal axis. The distribution of visible cytoplasmic components is also radially symmetrical when examined in paraffin sections (data not shown). However, only about half of the embryos showed epidermis differentiation when A4.1 cells were fused with the equatorial fragments from eggs at the end of the first phase of ooplasmic segregation. The variation in the results of such a fusion may be caused by localization of epidermis determinants in some areas within the equatorial region at the stage. Alternatively, the variation in the results may be due to unknown but trivial factors. Experiments were carried out in an attempt to distinguish between these two possibilities.

The design of the experiments is shown in Fig. 6. A small equatorial region of eggs was vital stained with Nile Blue at the end of the first phase of ooplasmic segregation. Then a cytoplasmic fragment was immediately cut off from the equatorial region opposite the marked area and another fragment was cut off from the stained equatorial region of the same egg. Thus, two fragments derived from opposite sides of an egg were prepared for fusion with A4.1 cells. The fused blastomeres were cultured in pairs to examine expression of the Epi-2 antigen. We would expect that if epidermis determinants were localized in a certain segment of the equatorial region, only one fused cell in each pair would differentiate into epidermis. The expression of the Epi-2 antigen was examined in seventeen pairs. In all, the expression of the Epi-2 antigen was detected in 18 out of 34 embryos (58%). In five pairs, both embryos expressed the antigen. In eight pairs, only one member of the pair expressed the antigen. In four pairs, neither embryo expressed detectable antigen. The ratio of the three types of result was about 1:2:1. It is suggested, therefore, that the appearance of the positive specimens was random. No tendency for only one member of a pair to differentiate into epidermis was observed. The result does not support the hypothesis of localization of epidermis determinants to one part of the equatorial region.

Next, equatorial cytoplasmic fragments with volumes almost equal to that of A4.1 cells were fused with B5.2 cells

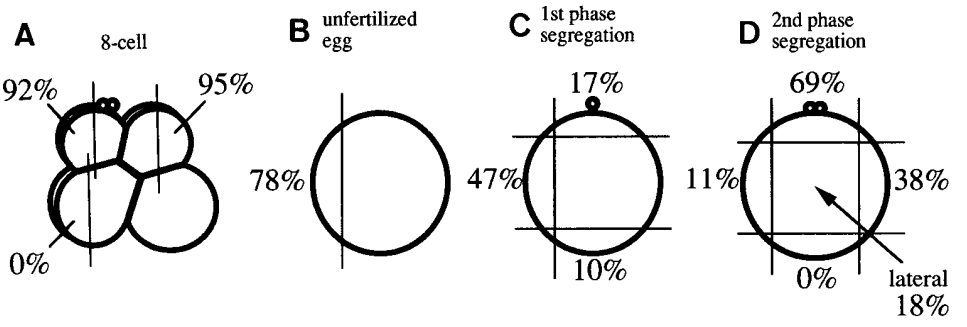

Fig. 5. Summary of the results of the present fusion experiments. Planes of sections are shown and results of the fusions described herein are presented in term of the percentages of specimens that expressed the Epi-2 antigen. The small circles at the animal pole of the eggs and embryo represent polar bodies. (A) Lateral view of an 8-cell embryo. No distinction is made between cytoplasmic fragments of anterior and posterior origin from each blastomere. (B) Unfertilized egg. (C) Egg after the first phase of ooplasmic segregation; the egg is radially symmetrical along the animal-vegetal axis. (D) Lateral view of an egg after the second phase of ooplasmic segregation, when it has become bilaterally symmetrical. Results of fusions with both the left and right fragments are shown together as fusions of lateral fragments. The recipient blastomeres in A were B5.2 blastomeres, and those in B,C and D were A4.1 blastomeres. In A and D the anterior pole is at the left of the embryo.

Table 2. Expression of an epidermis-specific antigen in fused blastomeres

Cytoplasmic fragment	Recipient blastomere	Fused specimen	Successfully divided	Expression of the antigen
(A) Before fertilization				
unfertilized egg	A4.1	37	27	21 (78%)
(B) After the first phase of ooplasmic segregation				
animal	A4.1	136	95	16 (17%)
equatorial	A4.1	47	43	20 (47%)
vegetal	A4.1	57	31	3 (10%)
(C) After the first phase of ooplasmic segregation				
equatorial	B5.2	72	71	63 (89%)
(D) After the second phase of ooplasmic segregation				
animal	A4.1	50	42	29 (69%)
anterior	A4.1	46	45	5 (11%)
lateral	A4.1	40	40	7 (18%)
posterior	A4.1	56	53	20 (38%)
vegetal	A4.1	47	46	0 (0%)

The epidermis-specific antigen was detected by immunofluorescence staining with a monoclonal antibody. Unfertilized eggs were cut without reference to their orientation. In D, results of fusions with both the left and the right fragments are shown together as results of fusions of lateral fragments.

instead of with A4.1 cells. Thus, there was a decrease in the volume of the recipient cells. The volume of B5.2 cells was approximately one-third of that of the A4.1 cells, as described above. If epidermis determinants are localized in a certain region, the proportion of positive specimens should not be much greater than that formed in the previous experiment in which A4.l cells were the recipients (about 50%). The results are shown in Table 2C. Almost 90% of embryos expressed the Epi-2 antigen. Large amounts of epidermis were formed. Epidermis covered each entire embryo and excess epidermis often protruded from the embryos (Fig. 4J).The proportion of positive specimens depended to a great extenton the ratio of the volume of the cytoplasmic fragments to that of recipient cells. Again, the idea of localization of epidermis determinants in one region at the equator is not supported. Thus, in two kinds of experiment, we found no evidence for localization of epidermis determinants within the equatorial region before the second phase of ooplasmic segregation.

DISCUSSION

The results of experiments in which differentiation to epidermis was evaluated by monitoring the expression of an epidermis-specific antigen are summarized in Fig. 5. Cytoplasm with the ability to promote the expression of the Epi-2 antigen in vegetal cells is present only in epidermis progenitors (a4.2 and b4.2 cells) at the 8-cell stage (Fig. 5A). A regional difference is apparent in the ability of the cytoplasm to promote the expression of the Epi-2 antigen when fragments from various regions of eggs are fused to vegetal cells (Fig. 5B-D). The introduced cytoplasm has the ability to promote not only the expression of the Epi-2 antigen but also the development of epithelial morphology and the secretion of larval tunic. Thus, expression of several different markers of epidermis differentiation is promoted. Results of experiments with isolated blastomeres (Nishikata et al., 1987) and with dissociated blastomeres (Nishida, 1992a) indicate that fate determination and the differentiation of epidermal cells are autonomous and, thus, these processes do not require intercellular interactions. Therefore, cytoplasmic factors, which are segregated into epidermis-lineage cells (epidermis determinants) appear to be sufficient for the determination of the fate and the differentiation of epidermal cells of the larva.

When fragments of unfertilized eggs were fused with vegetal cells, a significant proportion of the specimens expressed the Epi-2 antigen. After the first phase of ooplasmic segregation, equatorial fragments had the highest ability to promote such expression. After the second phase of ooplasmic segregation, fragments other than vegetal fragments had this ability. It is, therefore, suggested that epidermis determinants are already present in unfertilized eggs and that their distribution changes during ooplasmic segregation. We have no information, at present, about the distribution of epidermis determinants in unfertilized eggs because unfertilized eggs were cut without

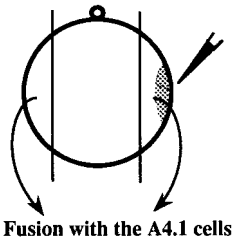

Fig. 6. Experimental design. The equatorial region of eggs was vital stained at the end of the first phase of ooplasmic segregation with Nile Blue by ejecting the dye solution through a fine glass needle. A cytoplasmic fragment was cut off from the equatorial region opposite the marked area, then another fragment was cut off from the equatorial region which had been stained. Each fragment was fused with an A4.1 (vegetal) cell, and the fused blastomeres were cultured in pairs to examine the expression of the Epi-2 antigen.

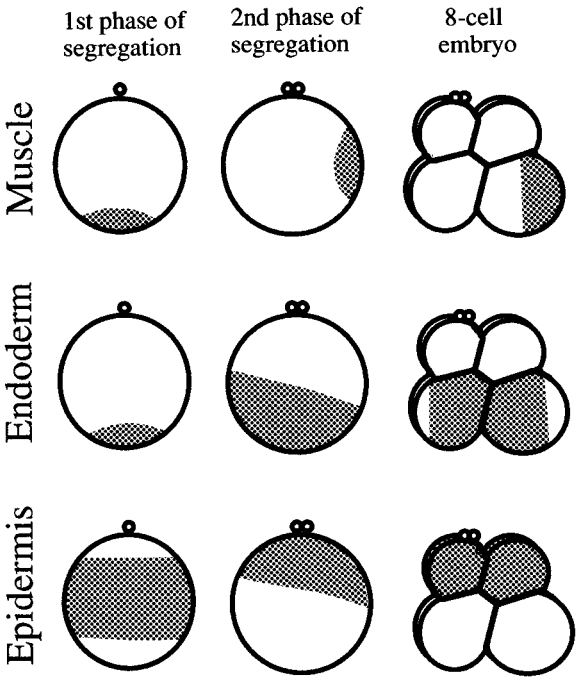

Fig. 7. Comparison of the movements of cytoplasmic determinants of three kinds of tissue, namely, muscle, endoderm and epidermis, during ooplasmic segregation. The results of the present and previous fusion experiments (Nishida, 1992b, 1993) are represented schematically. Shaded areas represent locations of cytoplasmic determinants. Note that the illustrations show surface views of eggs. We have no information about the distribution of cytoplasmic determinants in central regions of eggs. The animal pole is at the top. Eggs after the first phase of ooplasmic segregation are rotationally symmetrical, while eggs after the second phase of ooplasmic segregation and 8 cell embryos are bilaterally symmetrical. In these illustrations, anterior is to the left and posterior is to the right. The locations of determinants are drawn on the basis of the simplest supposition that they are each distributed in a continuous region. The areas of shading are tentatively based on the proportion of positive specimens in each fusion experiment. Although all three kinds of the determinant are present in the cytoplasm of unfertilized eggs, no information is available about their distribution, because unfertilized eggs were cut without reference to or knowledge of their polarity.

reference to or knowledge of their polarity. After the first phase of ooplasmic segregation, the determinants are present with their highest activity in the equatorial region of the egg. Then they move towards the animal pole. After completion of ooplasmic segregation and before the first cleavage, the determinants are already located at the site of formation of epidermis-lineage cells, namely, the animal hemisphere. It is noteworthy that the inferred movements of the determinants of epidermis during ooplasmic segregation coincide closely with the movements of cytoplasm that is referred to as the ectoplasm and has been described in the eggs with colored cytoplasm of some species of *Styela* (Conklin, 1905b,c; Jeffery et al., 1983). The ectoplasm is defined as clear cytoplasm that is darkly stained in sections by hematoxylin. It originates from the contents of the germinal vesicle and it is distributed in the animal hemispheres of unfertilized eggs. During the first phase of ooplasmic segregation, ectoplasm streams in the vegetal directions, following the myoplasm. Thus, three cytoplasmic regions are stratified along the animal-vegetal axis in the following sequence: yolky cytoplasm, ectoplasm and myoplasm. Ectoplasm is situated just below the equator. During the second phase of ooplasmic segregation, the ectoplasm moves with the sperm aster in the posterior direction and then spreads within the animal hemisphere. Unfortunately, in *Halocynthia* the ectoplasm is invisible in living eggs. Even in paraffin sections, in the absence of distinct criteria, we cannot identify the ectoplasm with certainty, although yolkless cytoplasm is associated with the mitotic apparatus, as described by Conklin (1905c).

There is no evidence for the localization of epidermis determinants within the equatorial region before the second phase of ooplasmic segregation. After the second phase of ooplasmic segregation, the ability to promote differentiation to epidermis decreases from the posterior to the anterior region within the equatorial part of the egg. This result may reflect the fate map of the embryo since the most anterior part of the animal hemisphere develops into the brain and sensory organ, and muscle and spinal cord originate from the lateral part of the animal hemisphere. By contrast, the region of presumptive epidermis extends down to the equator in the posterior part of the animal hemisphere (Nishida, 1987).

In most cases in our experiments, epidermal cells partially covered the embryos that were derived from fused cells, and nonepidermal cells were located within the embryos. Therefore, introduced determinants of epidermis may not diffuse in fused cells, and they may only be inherited by some descendent cells, as similarly suggested in the case of muscle and endoderm determinants (Nishida, 1992b, 1993). The determinants may be immobilized by the cytoskeleton (Jeffery and Meier, 1983) or may be anchored to the plasma membrane. Elucidation of the nature, as well as mode of action and manner of localization, of epidermis determinants awaits their isolation and characterization. The Epi-2 antigen first appears as the early tailbud stage during embryogenesis. Expression of the antigen is blocked by treatment with an inhibitor of transcription if treatment is initiated before the late gastrula stage (Nishikata et al., 1987). It is suggested that the gene for the antigen is expressed at the gastrula stage. Thus, determinants of the epidermis may be regulatory molecules, responsible for the activation of a cascade of specific genes that ultimately results in synthesis of proteins specific for differentiated epidermis. The determinants might either be localized molecules or they might be localized active forms of widely distributed molecules.

For comparison, the inferred movements of muscle determinants (Nishida, 1992b), endoderm determinants (Nishida, 1993) and epidermis determinants are represented schematically in Fig. 7. The three kinds of cytoplasmic determinant move in different directions during ooplasmic segregation. It will be of great interest to determine the way in which the egg segregates the three kinds of cytoplasmic factor to different regions. Prior to the onset of the first cleavage, three kinds of determinant settle at sites that correspond to region of the future fate map.

We have proposed the presence of three kinds of determinant on the basis of the results of fusion experiments. However, there is an another possibility that we cannot ignore: cells may autonomously assume a certain developmental fate when they

contain none of the postulated determinants. For example, epidermis determinants may not exist, and cells without any kind of determinant may develop into epidermis. Since cytoplasmic determinants did not diffuse in fused cells, we cannot exclude such a possibility. Logically, this possibility can be adopted for the formation of only a single kind of tissue. Therefore, determinants for at least two out of the three kinds of tissue should exist.

The author thanks Dr N. Satoh (Kyoto University), Dr W. R. Jeffery and Dr B. J. Swalla (Bodega Marine Laboratory) and all members of our laboratory for useful discussions. Thanks are also due to Dr T. Nishikata (Konan University) for providing the monoclonal antibody, Dr T. Numakunai for supplying living materials, Dr R. Kuraishi for teaching the method for the vital staining of eggs, and all other members of the Asamushi Marine Biological Station for facilitating the author's work there. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (no. 04740401).

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(Accepted 14 October 1993)