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Shrinkage and Expansion of Blastocoel affect the Degree of Invagination in Sea Urchin Embryos

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ABSTRACT—By immersing sea urchin embryos in seawater containing various concentrations of sucrose, we found that the level of osmotic pressure of blastocoel changed during gastrulation; the level was low around the onset of gastrulation and higher during secondary invagination. To learn how such changes in osmotic pressure related to invagination processes, embryos were shrunken or expanded by means of sucrose treatments, and the degree of invagination was monitored using Nile blue staining. The method elucidated that the cells invaginated during primary invagination occupied only top one third of archenteron at the end of secondary invagination, and that the cells constituting intestine and the posterior half of stomach were recruited into the archenteron after secondary invagination. When embryos were expanded, the degree of invagination was remarkably decreased, indicating that a higher level of expanding force hindered the fulfillment of invagination. On the other hand, shrinkage of blastocoel during secondary invagination increased the degree of invagination. Cell tracing experiments showed that the change in the degree of invagination estimated with Nile blue staining was accompanied with the change in the number of cells incorporated into the archenteron. When embryos were expanded, only the descendants of *veg2* constituted the gut rudiment, while a considerable amount of the *veg1* descendants were recruited into archenteron when embryos were shrunken. Obtained results suggest that change in the level of osmotic pressure of blastocoel would be necessary for the progress of gastrulation.

Key words: sea urchin, morphogenesis, gastrulation, osmotic pressure of blastocoel, cell fate

INTRODUCTION

Gastrulation in sea urchin embryos has been intensively studied as a model system for morphogenesis of a monolayered cell sheet (Gustafson and Wolpert, 1963, 1967), and several important factors involved in the processes of gastrulation have been elucidated. Bottle cells appearing at the center of the vegetal plate triggers the initiation of primary invagination (Nakajima and Burke, 1996; Kimberly and Hardin, 1998) and a thickened vegetal plate bends toward the blastocoel, resulting in a short stub-like gut rudiment. Extracellular matrices are necessary for the concerted movement of presumptive endodermal cells (Kinoshita and Saiga, 1979; Wessel and McClay 1987; Burke *et al*., 1991; Lane *et al*., 1993; Berg *et al*., 1996). After the primary invagination, secondary mesenchyme cells (SMCs) appear at the tip of the gut rudiment and form long filopodia that reached the inner surface of the apical plate. SMCs pull up the gut rudiment by means of long filopodia during secondary invagination (Dan and Okazaki, 1956; Gustafson and Kinnander, 1956; Hardin, 1988; Hardin and McClay, 1990). Rearrangement of cells in the gut rudiment is another important factor in forming the elongated archenteron (Ettensohn, 1985; Hardin and Cheng, 1986; Hardin, 1989).

Once, the notion was proposed that negative pressure of blastocoel triggers the initiation of invagination (Rhumbler, 1902, cited in Davidson *et al*., 1995). The proposed mechanism was not accepted, because the excised vegetal plate could undergo a morphological change similar to normal embryos (Moore and Burt 1939; Ettensohn, 1984). Due to such circumstances, the factor, “osmotic pressure of blastocoel”, has been excluded in the analysis of the mechanisms of sea urchin gastrulation. However, morphology of sea urchin embryos implies the existence of some kind of expanding force in the blastocoel. Newly hatched swimming blastulae of *Mespilia globulus* are completely spherical in shape with an extremely thinned blastocoel wall (see review, Okazaki, 1975). It is naturally supposed that such a balloon-like configuration is maintained with an expanding force, i.e., osmotic pressure of blastocoel.

In a previous study, we showed that the archenteron did not elongate to full length if blastocoel was expanded before the onset of primary invagination (Takata and Kominami,

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2001). When blastocoel was expanded after primary invagination, the archenteron elongated to full length but became more slender than that of control embryo. Thus, it is likely that the level of osmotic pressure is controlled during the processes of gastrulation. In the present study, level of the innate osmotic pressure was altered with sucrose treatment at various timing during gastrulation, and morphology of the resulted embryos was examined.

MATERIALS AND METHODS

Animals and gametes

Adults of the sea urchin *Hemicentrotus pulcherrimus* were collected and kept in aquaria supplied with circulating seawater (15°C). Gametes were handled as described before (Kominami and Masui, 1996). Milli-pore filtered seawater (MFSW) supplemented with 100 units/ml penicillin and 50 μg/ml streptomycin (Meiji Seika, Tokyo) was used throughout experiments. Embryos were cultured at 18±1°C.

Evaluation of osmotic pressure of blastocoel

By mixing MFSW and 1 M sucrose dissolved in distilled water, sucrose-containing seawater was made. Since the mixture was isotonic to normal seawater, the solution did not cause the shrinkage of embryonic cells, but caused the reduction in the size of blastocoel.

Gastrulating embryos were collected and immersed in MFSW containing various concentration of sucrose. After 20 min, embryos were treated with 0.1% formalin to stop ciliary movement and photographed. The height and width of the treated embryos were measured on photographic prints and averaged.

Shrinkage and expansion of blastocoel

To shrink the blastocoel transiently, embryos were transferred to sucrose-containing seawater (40 mM) and returned to MFSW four hr later. The treatment was done every two hr during gastrulation. On the other hand, expansion of blastocoel was carried out after the method of Dan (1952). Briefly, fertilized eggs were deprived of fertilization envelope (Showman and Foerder, 1979), and were transferred to sucrose-containing seawater (100 mM) at an early cleavage stage. An aliquot of the treated embryos were returned to MFSW every two hours during gastrulation.

Nile blue staining

Gastrulating embryos were vitally stained with 0.001% Nile blue sulfate (Chroma Gesellschaft Schmidt & Co., Germany) dissolved in MFSW for 5 min. After staining, the embryos were rinsed three times with MFSW and cultured until the desired stages. Because the dye bound to only the cells positioned outside at staining, the further recruitment of presumptive endodermal cells into the archenteron could be detected by examining the distribution of labeled cells (Kuraishi and Osanai, 1992). After staining, embryos were mounted on a glass slide and covered with a piece of cover slip. To make the observation easier, embryos were slightly squashed by absorbing excess seawater between a glass slide and cover slip with a piece of blotting paper.

Detection of alkaline phosphatase activity

Embryos were fixed with 3% formalin and were rinsed twice with MFSW. Activity of alkaline phosphatase was detected with the method of simultaneous coupling with substituted Naphthols. Naphthol AS-BI phosphate (Sigma, MO) was dissolved in DW at 50 μg/ml and its pH was adjusted to 8.3. The reaction mixture was prepared by dissolving 10 mg Fast red TR (Sigma) in 10 ml Naphthol stock solution immediately before use. Specimens were incubated in the reaction mixture at room temperature for 6 hr.

Labeling of a vegetal blastomere at the 8-cell stage

One of the vegetal blastomeres of the 8-cell stage embryo was injected with a fluorescent dye Lucifer Yellow CH (Sigma) as described previously (Kominami, 1988). For staining of the nuclei of embryonic cells, the dye-injected embryos reaching the early prism stage were immersed in MFSW containing 1 μM DAPI (Sigma) for 30 min. The embryos were then fixed with 1% formalin dissolved in MFSW and examined under an epifluorescence microscope (Olympus, BX50-FLA, Tokyo). The number of nuclei of the Lucifer Yellow-labeled cells left in the vegetal ectoderm was obtained by comparing two photographic prints taken under ultraviolet and blue light excitation. In this measurement, only the embryos that showed ventral or ventro-lateral labeling (Kominami, 1988) were used, because counting of nuclei was difficult due to the piling up of nuclei in other labeling patterns.

Immunofluorescence

For immuno-staining, gastrulating embryos were fixed successively with cold methanol and ethanol (–20°C, 20 min each). Fixed embryos were embedded in polyester wax (BDH Laboratory Supplies, Poole, England) and were sectioned at 6 μm. After removal of the wax, specimens were rinsed twice with phosphate-buffered saline (PBS) and reacted with a monoclonal antibody VE10 (Kominami and Takata, 2000) to make clear cell boundaries. After the primary reaction, specimens were rinsed twice with PBS, and then reacted with FITC-conjugated goat anti-mouse IgG (Fab fragment) antibody (Sigma). After one hr, specimens were rinsed twice with PBS. The immuno-stained sections were mounted on a glass slide with a small amount of glycerol and examined under an epifluorescence microscope.

RESULTS

Evaluation of the osmotic pressure of blastocoel

To evaluate the osmotic pressure of blastocoel, embryos were transferred to the isotonic seawater containing various concentrations of sucrose, and the change in the size of them was examined. Fig. 1A shows the time course of shrinkage of embryos (18 hr postfertilization) immersed in sucrose-containing seawater (40 mM). Embryos shrank rapidly and reached a minimum level within 6 min. After this, the size of embryos did not change significantly. On the other hand, the shrunken embryos (immersed in sucrose-containing seawater for 20 min) restored the normal size within 8 min after they were returned to MFSW (Fig. 1B). Thus, the gastrulating embryos can be regarded as a simple sphere surrounded with a semi-permeable membrane.

Using this property of embryos, we tried to evaluate the osmotic pressure of blastocoel (Fig. 2). As clearly shown in Fig. 2A, the blastocoel fluid of embryos during primary invagination (18.5 hr) was isotonic to MFSW containing 10 mM sucrose. During secondary invagination (22.5 hr), the minimal concentration of sucrose that did not cause any shrinkage of embryos increased to 14 mM (Fig. 2B).

Involution of presumptive endodermal cells during gastrulation

To know how the change in the level of osmotic pressure of blastocoel related the invagination processes, we first tried to monitor the degree of invagination with Nile blue staining. Embryos shown in Fig. 3A–C were stained with the dye at 18 hr postfertilization. All the cells that faced to the outside were

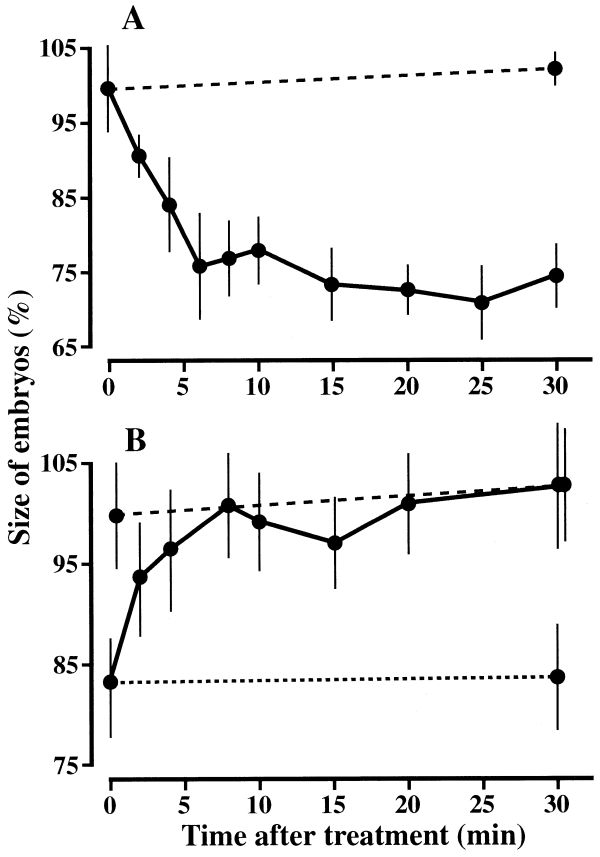

Fig. 1. Time course of change in the size of embryos upon sucrose treatment. A: Time course of shrinkage with sucrose treatment (40 mM). B: Time course of restoration from shrinkage. Broken lines in A and B indicate the change in the size of control embryos during observation. Dotted line in B indicates the change in the size of embryos treated continuously with sucrose. At each observation points, 30 embryos were examined. Embryos around the onset of gastrulation (18 hr postfertilization) were used. Vertical bars indicate S. D.

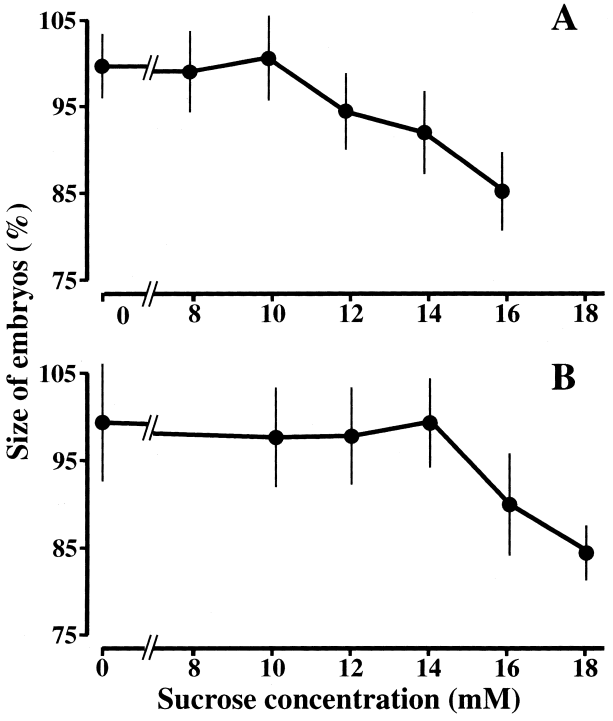

Fig. 2. Evaluation of the osmotic pressure of blastocoel fluid. Gastrula-stage embryos were immersed in MFSW containing different concentration of sucrose, and the size of them was examined 20 min later. A: early gastrula stage (18.5 hr postfertilization). B: late gastrula stage (22.5 hr). Abscissa: concentration of sucrose. Ordinate: size of embryos. At each observation points, 15 embryos were examined. Vertical bars indicate S. D.

stained (Fig. 3A). PMCs were not labeled. At the 22nd hr of development, when secondary invagination almost finished, the label was observed throughout the archenteron (Fig. 3B). SMCs observed at the space between the archenteron tip and the apical plate were also labeled (Fig. 3B, arrow). At the early prism stage (30 hr), whole digestive tract was populated with Nile blue-stained granules (Fig. 3C). The label was also observed at bulging coelomic pouches (Fig. 3C, arrow). On the other hand, embryos in Fig. 3D–F were stained with Nile blue at the 22nd hr of development. Label was not observed at spicule forming PMCs, detaching SMCs (Fig. 3D) and the invaginated archenterons (Fig. 3G). At the 26th hr of development, Nile blue labeling was observed at the bottom half of the archenteron (Fig. 3E), and the bottom two thirds of the archenteron was labeled at 30 hr postfertilization (Fig. 3F). Around this stage, the top region of the archenteron formed coelomic pouches. Thus, relative position of the boundary of Nile blue staining shifts upward to some extent. Fig. 3H show the enlarged images near the boundary between Nile blue-stained and non-stained regions at 30 hr postfertilization. Nile blue-stained granules were observed at presumptive stomach-intestine region. Even at the 48th hr of development, the boundary could be noticed (Fig. 3I).

Fig. 4 illustrates the rate of invagination estimated with Nile blue staining. Embryos were stained every two hours during gastrulation and examined four hr later. The rate is higher during primary and secondary invagination (before 22 hr). Even after the archenteron tip reached the apical plate, a considerable amount of endodermal cells were incorporated into the archenteron, although the rate became smaller.

Fig. 5 shows the pluteus-stage embryos stained with Nile blue at different timing during gastrulation. At the pluteus stage, the archenteron differentiates into esophagus, stomach and intestine, and each part gains its characteristic configuration. Thus, length ratio of Nile blue-stained region to the whole length of digestive tract does not reflect the degree of invagination. Here, scores (0–4) are given to embryos according to the position of the front of Nile blue-stained region. In the embryo stained at the onset of primary invagination (Fig. 5A), the label was observed throughout the digestive tract (score 4). If the embryos were stained slightly before the end of secondary invagination (22 hr), the label was observed both in stomach and intestine (Fig. 5B, score 3). When the embryos were stained with Nile blue at the 24th hr of development, the label occupied the post-half of stomach and intestine (Fig. 5C, score 2). In the embryo stained at the early prism stage (30 hr), the label was observed at the posterior half of intes-

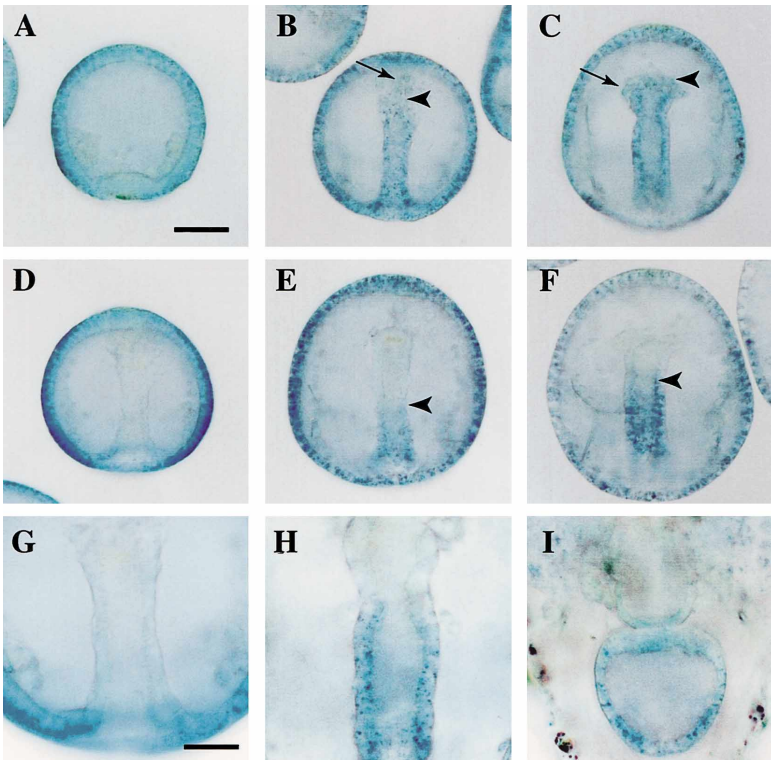

Fig. 3. Process of invagination examined with Nile blue staining. A–C: stained with Nile blue at 18 hr (at the onset of primary invagination). A: immediately after staining. B: 22 hr. C: 30 hr. D–F: stained at 22 hr (at the end of secondary invagination). D: immediately after staining. E: 26 hr. F: 30 hr. Arrowheads indicate the front of Nile blue-stained region. Difference in the size of embryos at the same stage of development (e.g. C and F) is due to the difference in the degree of compression for observation. G–I: Enlarged images near the boundary between Nile blue-stained and non-stained regions. G: immediately after staining (22 hr). The invaginated archenteron is not labeled. H: Nile blue-stained (22 hr) embryo observed at 30 hr. Nile blue-stained granules are observed at the presumptive stomach and intestine region. I: Nile blue-stained (22 hr) embryo at the 48th hr of development. Nile blue-stained granules are observed in stomach but not in esophagus. Scale bar in A is common to A–F and indicates 50 μm. Scale bar in G is common to G-I and indicates 25 μm.

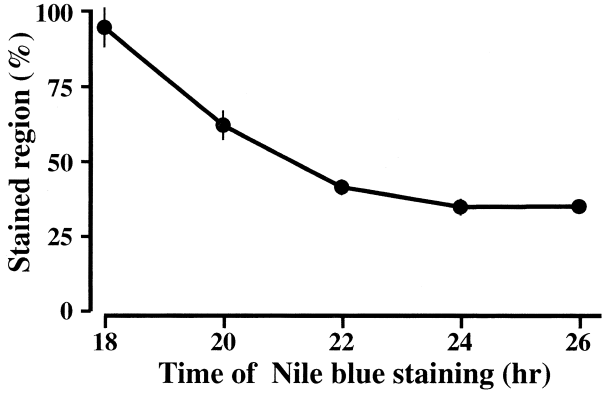

Fig. 4. Length ratio of Nile blue-stained region to the whole archenteron. Embryos were stained with Nile blue every two hr during gastrulation and examined four hours later. Abscissa: time of Nile blue staining (hr). Ordinate: ratio of the Nile blue-stained region to the length of archenteron. Each point represents the averaged value of 10 embryos. Vertical bars indicate S. D.

tine (Fig. 5D, score 1). If the label was not observed in the digestive tract at all, score 0 was given to the larvae.

Fig. 6 shows the scores obtained in the pluteus-stage embryos stained with Nile blue at different timing during gastrulation. If embryos were stained at the 18th hr of develop-

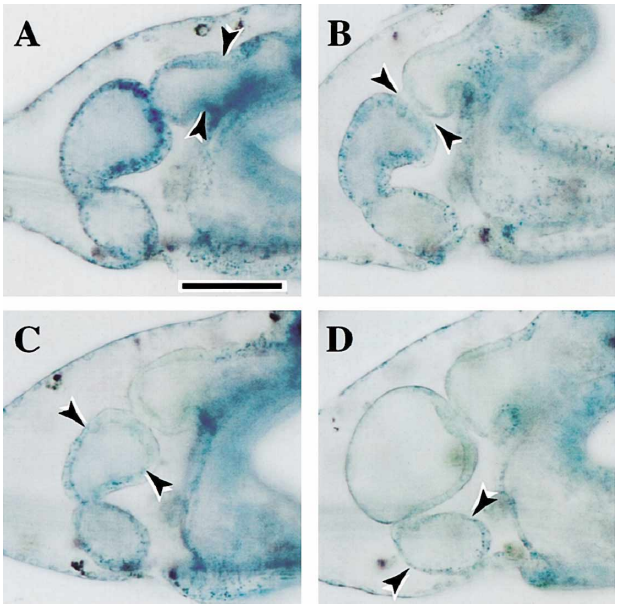

Fig. 5. Pluteus larvae stained with Nile blue during gastrulation. Embryos shown in A, B, C and D were stained with Nile blue at 18, 22, 24 and 30 hr postfertilization, respectively. Arrowheads indicate the front of Nile blue-stained region. Scale bar indicates 50 μm.

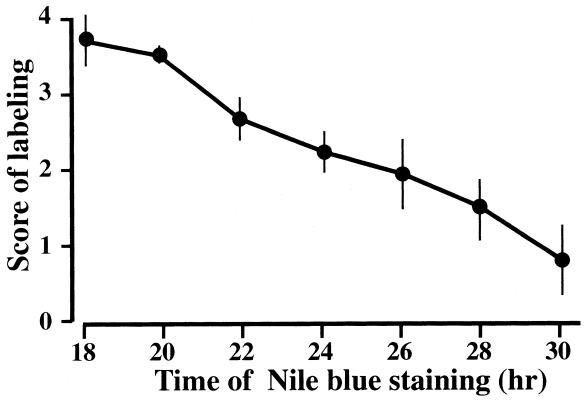

Fig. 6. Score of Nile blue staining examined at the pluteus stage. Embryos were stained with Nile blue every two hours during gastrulation and examined at the pluteus stage. Abscissa: time of Nile blue staining (hr). Ordinate: score of Nile blue staining. Each point represents the averaged value of 100 embryos. Vertical bars indicate S. D.

ment (initiation of primary invagination), the score was nearly 4.0. When embryos were stained at the 24th hr, the score was about 2.0. It is clear that the endodermal cells that constitute intestine and the posterior half of stomach are recruited into the archenteron after the completion of secondary invagination. Hereafter, this late recruitment of endodermal cells into the archenteron will be termed as tertiary invagination. Fig. 5D and Fig. 6 indicate that tertiary invagination still continues even after embryos reach the early prism stage.

Shrinkage and expansion of blastocoel during gastrulation

To learn how the innate expanding force of blastocoel related to the invagination processes, embryos were stained with Nile blue every two hours during gastrulation, and they were shrunken or expanded immediately after staining. All embryos shown in Fig. 7 were stained with Nile blue at the end of primary invagination (20 hr), and examined at the 24th (A–C) and 30th hr of development (D–F). In control embryos, the label was observed at the bottom two thirds (Fig. 7A) and almost all region of the archenteron (Fig. 7D) at 24 and 30 hr postfertilization, respectively. In the shrunken embryos, the label in the archenteron was almost similar to control embryos (Fig. 7B, E). In contrast, further involution was scarcely noticed at 24th hr, if the embryos were expanded (Fig. 7C). At the early prism stage, the label was observed only at the bottom one third of the archenteron (Fig. 7F).

In these embryos, ratio of Nile blue-stained region to the whole length of the archenteron was examined four hr later after staining (Fig. 8). Generally, shrinkage of embryos did not change the degree of invagination significantly. On the other hand, the degree became remarkably smaller in the expanded embryos. Interestingly, the effects of shrinkage and expansion of blastocoel were quite different in the embryos treated from 24 hr postfertilization; the ratio considerably increased when the embryos were shrunken, while the ratio did not change when they were expanded from the 24th hr onward. Further involution of endodermal cells into the archenteron was not observed at all when embryos were expanded from 26th hr of development onward.

Changes in the degrees of invagination in the shrunken and expanded embryos were also examined at the pluteus stage (Fig. 9). In the shrunken embryos, the scores were larger than those of control, especially when they were shrunken after the completion of secondary invagination. On the other hand, the degree of invagination was decreased when embryos were expanded. In both shrunken and expanded embryos, the score of invagination observed at the pluteus stage roughly coincided with the amount of endodermal cells incorporated into the archenteron during gastrulation (Compare Fig. 9 with Fig. 8). In another words, conspicuous regu-

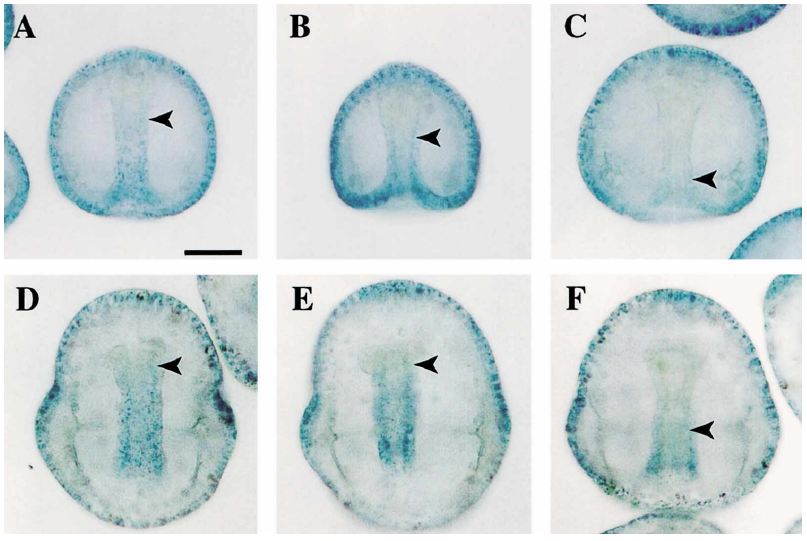

Fig. 7. Shrinkage and expansion of blastocoel. All embryos were stained with Nile blue at the end of primary invagination (20 hr). A–C: observed at 24 hr. D–F: observed at 30 hr. A, D: control embryos. B, E: shrunken embryos. C, F: expanded embryos. Arrowheads indicate the front of Nile blue-stained region. Scale bar indicates 50 μm.

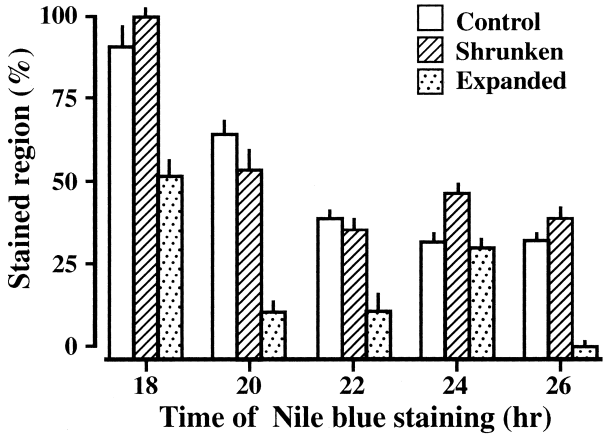

Fig. 8. Effect of shrinkage and expansion on the length ratio of Nile blue-stained region to the whole archenteron. Abscissa: time of Nile blue staining (hr). Ordinate: ratio of the Nile blue-stained region to the length of archenteron. Embryos were shrunken or expanded with sucrose treatment at the indicated time and examined at 30 hr postfertilization. Each point represents the averaged value of 10 embryos. Vertical bars indicate S. D.

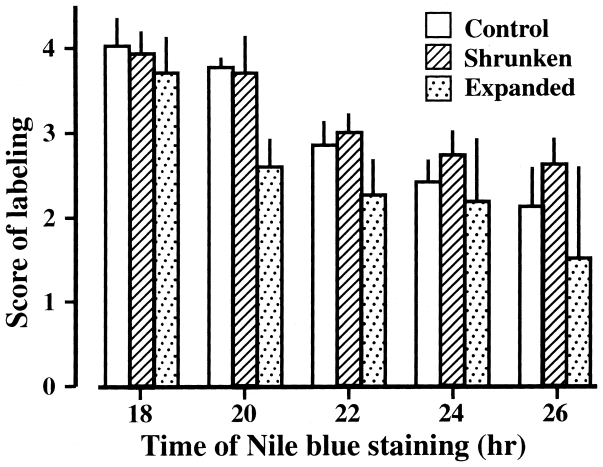

Fig. 9. Effect of shrinkage and expansion on the score of Nile blue staining. Abscissa: time of Nile blue staining (hr). Ordinate: score of labeling. Embryos were shrunken or expanded with sucrose treatment at the indicated time and examined at 48 hr postfertilization. Each point represents the averaged value of 100 embryos. Vertical bars indicate S. D.

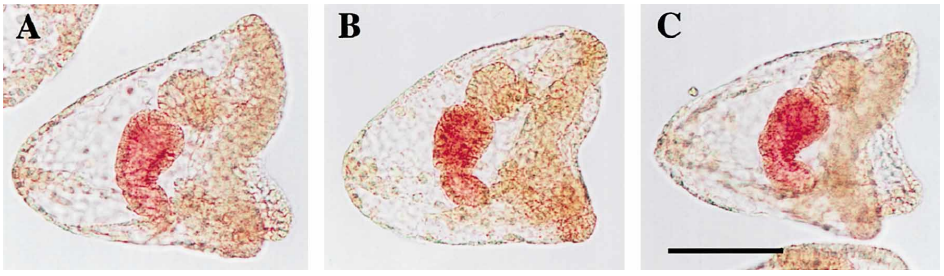

Fig. 10. Alkaline phosphatase activity detected at the early pluteus stage. A: a control embryo. B: an embryo shrunken during 23–27 hr. C: an embryo expanded at 20 hr. Alkaline phosphatase activity is detected only in stomach and intestine in all types of embryos. Scale bar indicates 100 μm.

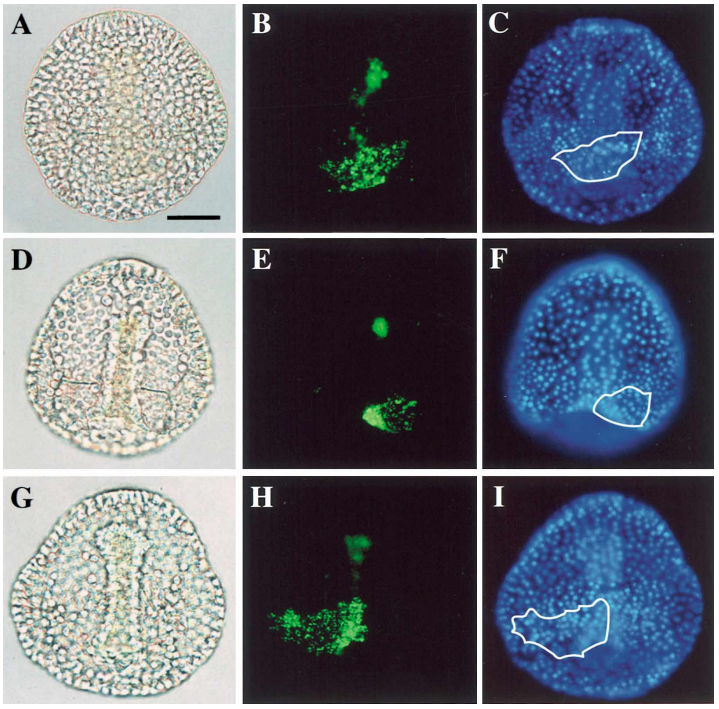

Fig. 11. Early prism-stage embryos labeled with Lucifer yellow A-C: a control embryo. D-F: a shrunken embryo (from 24 hr). G-I: an expanded embryo (from 20 hr). All embryos were observed at the 28th hr of development. A, D, G: bright field, B, E, H: fluorescence images obtained with B (blue light) excitation. Descendants of a vegetal blastomere of the 8-cell stage are fluorescent. C, F, I: fluorescence images obtained with UV excitation. DAPI-stained nuclei are fluorescent. White lines encircle the distributed region of cells labeled with Lucifer yellow. Scale bar indicates 50 μm.

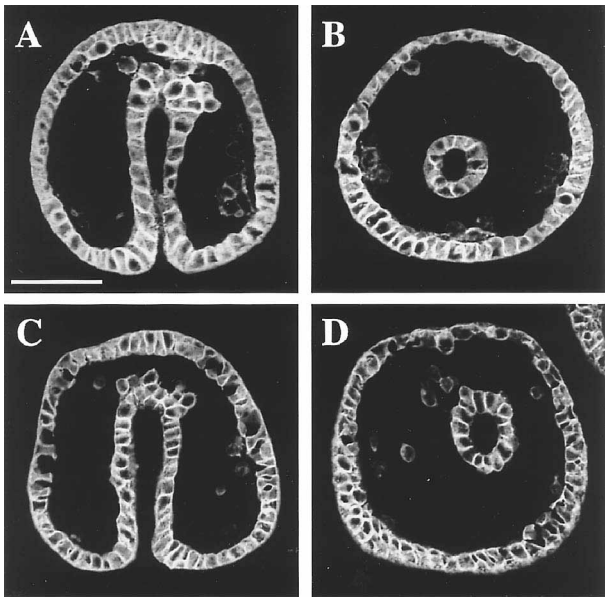

Fig. 12. Histological sections of the early prism-stage embryos. A, B: control embryos. C, D: shrunken embryos (from 24 hr). A, C: frontal section. B, D: transverse section. Specimens were immuno-stained with a monoclonal antibody VE10 that binds to the cell surface of all blastoderm. Scale bar indicates 50 μm.

lation of the amount of archenteron cells does not occur during successive stages.

In sea urchin embryos, endogenous alkaline phosphatase activity is seen only in stomach and intestine. As described above, the amount of presumptive endodermal cells was altered with shrinkage and expansion of blastocoel. Do the excess cells incorporated into the archenteron in the shrunken embryos differentiate into the endodermal cells? Do the presumptive endodermal cells express alkaline phosphatase activity, even if they are not recruited into the archenteron? As shown in Fig. 10, only the cells incorporated into the stomach and intestine developed the activity. This indicates that the blastoderm cells remain to be unspecified until rather later stage of development, and that the fate of cells around the blastopore depends solely on their position.

Cell-tracing and histological observation

Cell-tracing experiment was undertaken, to make sure that the change in the amount of invagination detected with Nile blue staining was accompanied with the change in the number of cells incorporated into the archenteron (Fig. 11). In control early prism-stage embryo (28 hr), about 60 (59.9±9.6, n=7) cells derived from a vegetal blastomere of the 8-cell stage embryo were observed in the vegetal ectoderm (Fig. 11A–C). If embryos were shrunken at the 24th hr of development, a smaller number of labeled cells (45.9±9.2, n=9) were left in the ectoderm (Fig. 11D–F). Difference in the numbers of labeled cells between control and the treated embryos is statistically significant (t_{cal}=2.76). On the other hand, the number of labeled cells left in the ectoderm increased slightly (66.8±12.0, n=10, t_{cal}=1.19), when the embryos were expanded from the 20th hr onward (Fig. 11G–I). These results show that Nile blue staining detects the change in the number of archenteron cells.

Further, the number of cells constituting the archenteron was estimated on histological sections of control and the shrunken embryos (Fig. 12). In control embryos, 11.6±1.0 (n=14) cells were observed along the axis of archenteron, while 11.0±1.5 (n=11) cells were observed in cross section of it (Fig. 12A, B). On the other hand, 12.8±1.3 (n=21) and 13.2±1.6 (n=17) cells were observed in frontal and cross section of archenteron in the shrunken embryos treated with sucrose at the 24th hr of development (Fig. 12C, D). The observed numbers of cells in control and the treated embryos are statistically significant (t_{cal}=2.79 and 3.25 for frontal and cross sections, respectively). From these values, the numbers of cells in the archenteron of control and the treated embryos were estimated to be 128 and 169, respectively.

DISCUSSION

Osmotic pressure of blastocoel

In terms of mechanics, sea urchin gastrula might be viewed as a thin elastic shell filled with liquid, and the elastic tension of the shell counterbalances the osmotic pressure of the liquid. This point of view may explain why newly hatched blastulae are almost spherical and why the blastocoel wall becomes thinner during gastrulation. This study first estimated the level of osmotic pressure of blastocoel of sea urchin gastrula and suggests that the level of it changes during gastrulation; osmotic pressure of the blastocoel fluid rises during secondary invagination by 4 mM compared with that during primary invagination (Fig. 2). This rise (equivalent to 0.08 atmospheric pressure) seems to be enough to maintain the spherical shape and to expand the blastocoel wall gradually, although mechanical properties of blastocoel wall remain to be solved (Davidson *et al.*, 1999).

In the present study, expanding force of blastocoel was canceled by immersing embryos in sucrose-containing seawater. The timing of this treatment could be precisely controlled as shown Fig. 1. On the other hand, expanding force was increased with pre-loading of sucrose in the blastocoel. The concentration of sucrose used to load sucrose in the blastocoel was 100 mM. This concentration seems to be much higher than the physiological one, because the innate osmotic pressure of blastocoel ranges 10–14 mM so far as examined (Fig. 2). It should be noted, however, that the blastocoel is small when sucrose is enclosed in the blastocoel (before hatching). During swimming and mesenchyme blastula stages, embryos become considerably expanded. The volume of blastocoel of early gastrula was about four times larger than that of newly hatched blastulae. Hence, the concentration of sucrose entrapped in the blastocoel is calculated to be less than 25 mM. Thus, the force operating in the experimentally expanded embryos seems to be within the physiological level. In fact, the morphology of the treated embryos did not differ so much from control embryos, although the treated embryos were certainly expanded (compare Fig. 7A and C). Unlike the

shrinkage experiment, the duration of the expansion could not be controlled. In most cases, however, the effect was noticed during 3–4 hr, judging from the external morphology and the size of the expanded embryos.

Invagination processes elucidated with Nile blue staining

Processes of invagination in sea urchin embryos have long been divided into two phases, primary and secondary invagination. In these years, however, several studies have shown that the presumptive endodermal cells are recruited into the archenteron even after the completion of secondary invagination (McClay and Logan, 1996; Logan and McClay, 1997; Martins *et al*., 1998; Ransick and Davidson, 1998). Such late invagination of presumptive endodermal cells was first found in starfish embryos using Nile blue staining (Kuraishi and Osanai, 1992).

In the present study, the same method was employed to examine the degree of invagination in sea urchin embryos, and several aspects of invagination processes were elucidated (Fig. 4, 6). (1) The cells invaginated during primary invagination occupy only top one third of the archenteron at the end of secondary invagination. (2) The presumptive endodermal cells are continuously convoluted during secondary invagination. (3) The cells constituting intestine and the posterior half of stomach are recruited into the archenteron after the completion of secondary invagination.

Cell-tracing

As described above, Nile blue staining is a useful method to know the relative amount of invagination. However, we could not know the actual number of cells recruited into or excluded from the archenteron upon shrinkage or expansion of embryos. By the early prism stage, most of blastomeres have undergone 11 cycles of cell divisions in *Hemicentrotus pulcherrimus* embryos (Kominami, 2000). As a result, one vegetal blastomere formed at the 3rd cleavage gives rise to 128 cells. Among these, 64 cells are derived from blastomeres of *veg1* layer. As shown in Fig. 11A–C, about 60 cells were left in the ectoderm after the completion of secondary invagination in control embryos. This indicates that the archenteron was mostly composed of *veg2*-derived cells. On the other hand, a considerable amount of *veg1*-derived cells were incorporated into the archenteron in the shrunken embryos, because only 46 cells were left in the ectoderm (Fig. 11D–F). The difference was about 14. From this value, the number of archenteron cells in sucrose-treated embryos is supposed to be larger than control embryos by 56 (there are 4 vegetal blastomeres in the 8-cell stage embryo). The numbers of archenteron cells in control and the sucrose-treated embryos obtained on histological sections were 128 and 169, respectively (Fig. 12). The difference in the numbers of cells (45) is somewhat smaller than the value calculated above. This is probably due to the fact that the number of descendent cells recruited into the archenteron differs among four vegetal blastomeres of the 8-cell stage embryo, as has been reported in other sea urchin species (Logan and McClay, 1997). Thus, change in the degree of invagination estimated with Nile blue staining reflects the change in the number of cells recruited into the archenteron upon expansion and shrinkage of blastocoel during gastrulation.

Osmotic pressure and invagination processes

Around the onset of primary invagination, the expanding force of blastocoel seems to become weak, because only a short archenteron was formed when the blastocoel was expanded from the beginning of primary invagination (Takata and Kominami, 2001). In the present study, this was ascertained with Nile blue staining. The degree of invagination was considerably decreased when the embryos were expanded during primary invagination (Fig. 8, 9). A low level of osmotic pressure may enable the inward bending of the thickened vegetal plate. In *M. globulus*, only a small gut rudiment is formed during primary invagination. It is of interest that *M. globulus* embryos remain to be completely spherical even after the occurrence of primary invagination, suggesting that the level of osmotic pressure is rather high.

At the end of secondary invagination (24 hr), effects of shrinkage and expansion of blastocoel were different from those during primary invagination (20 hr). The expansion did not affect the degree of invagination. The shrinkage of embryos increased the amount of endodermal cells (Fig. 8, 9). The level of osmotic pressure is supposed to be rather high at the later phase of secondary invagination. Due to this, cells around the blastopore cannot be pulled into the blastocoel, while the archenteron is stretched along the animal-vegetal axis. It is naturally supposed that a considerable amount of cells are recruited into the base of archenteron if such expanding force is canceled by shrinkage.

Why is the level of expanding force higher during secondary invagination? By this stage, the ectodermal layer becomes thinned considerably. Nevertheless, such thinned ectodermal layer should afford the scaffold with which filopodia of SMCs exert the force that pulls up the archenteron tip toward the apical plate. In fact, the constituent cells of the archenteron are extremely stretched along the animal-vegetal axis at the later stage of secondary invagination (Kominami and Takata, 2000). The ectodermal layer might mechanically strengthen by the presence of a rather high level of expanding force of blastocoel.

In *Scaphechinus mirabilis*, the archenteron continuously elongate throughout the invagination processes (Kominami and Masui, 1996). SMCs that connect the archenteron and the inner surface of the apical plate are not observed. The archenteron cells are not stretched along the animal-vegetal axis and not rearranged (Kominami and Takata, 2000). Thus, a higher level of expanding force of blastocoel seems to be unnecessary. It is interesting to note that the *S. mirabilis* embryos become flattened as early as gastrulation starts. This indicates that the expanding force of blastocoel is rather weak during gastrulation.

Our experiments clearly showed that tertiary invagination was considerably hindered in expanding embryos (Fig. 8,

9). Further, the number of the vegetal blastomere-derived cells left in the ectoderm was much smaller than in control embryos when embryos had been shrunken from the 24th hr of development (Fig. 11D–F). These indicate that decrease in the level of expanding force is one of the prerequisites for occurrence of tertiary invagination. As shown in Fig. 7A and D, embryos become considerably expanded during invagination processes. The blastoderm cells around the blastopore would be passively convoluted into the blastocoel when the blastodermal layer expands due to the presence of low level of expanding force.

The present study showed how the level of osmotic pressure of blastocoel related invagination processes. However, mechanical properties of blastocoel wall and more precise change in the level of expanding force of blastocoel should be elucidated for the further understanding of the mechanics and mechanism of sea urchin gastrulation.

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