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The dorsoventral axis is specified prior to first cleavage in the direct developing sea urchin *Heliocidaris erythrogramma*

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Summary

Previous fate mapping studies as well as the culture of isolated blastomeres have revealed that the dorsoventral axis is specified as early as the 2-cell stage in the embryos of the direct developing echinoid, *Heliocidaris erythrogramma*. Normally, the first cleavage plane includes the animal–vegetal axis and bisects the embryo between future dorsal and ventral halves. Experiments were performed to establish whether the dorsoventral axis is set up prior to the first cleavage division in *H. erythrogramma*. Eggs were elongated and fertilized in silicone tubes of a small diameter in order to orient the cleavage spindle and thus the first plane of cell division. Following first cleavage, one of the two resulting blastomeres was then microinjected with a fluorescent cell lineage tracer dye. Fate maps were made after culturing these embryos to larval stages. The results indicate that the first cleavage division can be made to occur at virtually any angle relative to the animal–vegetal and dorsoventral axes. Therefore, the dorsoventral axis is specified prior to first cleavage. We argue that this axis resides in the unfertilized oocyte rather than being set up as a consequence of fertilization.

Key words: direct development, sea urchin development, dorsoventral axis, polarity determination.

Introduction

It is not certain when the dorsoventral axis is established during the development of echinoids that form a pluteus larva (Wilt, 1987). Research by Hörstadius and Wolsky (1936) and Kominami (1988) indicated that there is no relationship between the larval dorsoventral axis and the first or second planes of cleavage in *Paracentrotus lividus* or *Hemicentrotus pulcherrimus*. Recently, however, Cameron *et al.* (1989) have shown that there is a fairly good correspondence between the first two cleavage planes and the larval dorsoventral (oral–aboral) axis in *Strongylocentrotus purpuratus*.

Although Hörstadius did not find any relationship between the first two cleavage planes and the larval dorsoventral axis in *Paracentrotus lividus*, he argued that dorsoventral polarity must exist in the unfertilized eggs. This claim was supported by observations of complementary left–right or reversed dorsal–ventral symmetery properties in pairs of embryos developing from bisected eggs. These symmetry properties were revealed by staining the cut edges with vital dye before raising the bisected embryos. On the basis of their fate mapping studies, Cameron *et al.* (1989) also concluded that larval dorsoventral (oral–aboral) polarity exists prior to first cleavage but suggested that this axis is specified after fertilization. On the other hand, Kominami (1988) argued that the larval dorsoventral axis is not specified until after the fifth cleavage division in *Hemicentrotus pulcherrimus*.

Whatever the observed relationship between the early cleavage planes and the larval dorsoventral axis, this axis is not determined until some time after the 4-cell stage. This conclusion is substantiated by the results of blastomere isolation experiments which indicate that individual cells isolated from 2- and 4-cell embryos are capable of complete regulation (Dreisch, 1891, 1892, 1900, 1906; Boveri, 1907; Morgan, 1895; Von Ubisch, 1925; Hörstadius and Wolsky, 1936). In addition, other investigators have shown that the larval dorsoventral axis is easily shifted as a consequence of physical deformation or centrifugation of the eggs and embryos of indirect developing sea urchins, those that form a pluteus larva (Runnström, 1925; Lindahl, 1932*a*,*b*; Hörstadius, 1938, 1973; Pease, 1939).

Recently, we have demonstrated that the dorsoventral axis is established early during development in the sea urchin *Heliocidaris erythrogramma* (Henry and Raff, 1990). *H. erythrogramma* displays direct development *via* a non-feeding 'lecithotrophic' larva. Whereas the pluteus larval dorsoventral (oral–aboral) axis is offset approximately 45 degrees with respect to the adult dorsoventral axis in forms with indirect development, the dorsoventral axis of the *H. erythrogramma* larva is the same as that of the juvenile sea urchin which emerges during metamorphosis (Wray and Raff, 1989). The results of fate mapping studies (Wray and Raff,

1989, 1990) indicate that the first cleavage plane always bisects the *H. erythrogramma* embryo between future dorsal and ventral halves. The ventral blastomere (V) gives rise to the vestibule and most of the internal mesenchymal and coelomic cells. Furthermore, when the two first cleavage blastomeres are separated and raised in isolation, they display characteristic developmental differences that correlate with the fate mapping results (Henry and Raff, 1990). Here we present experiments designed to test whether the dorsoventral axis is specified prior to the first cleavage division in *H. erythrogramma*. Our findings indicate that the dorsoventral axis is specified prior to first cleavage. We argue that this axis resides in the unfertilized oocyte.

Materials and methods

Preparation of Gametes

Adult specimens of *H. erythrogramma* were collected from subtidal rock platforms off Sydney, NSW Australia. Gametes were obtained following the procedures of Wray and Raff (1988).

Vital dye labeling

Nile Blue sulfate dye was applied to small regions on the surface of unfertilized eggs utilizing the technique described by Henry and Martindale (1987). Dye was applied to animal, vegetal and lateral regions following reference points described in the results section. Tetramethylrhodamine isothiocyanate (no. T-2018 isomer R, Sigma, St Louis, MO), prepared as described by Wray and McClay (1988), was also used to label specific regions on the surface of fertilized eggs.

Orientation of the first cleavage plane

The first cleavage plane was oriented following the method of Rappaport (1985). Small tubes, cast in silicone, with an internal diameter of approximately 330 μm were used. Unfertilized eggs were first drawn into the submerged ends of these tubes. Sperm were then added to these elongated eggs and fertilization generally took place within five minutes. The elevation of vitelline envelopes could be monitored through the transparent walls of the silicone chambers. Immediately following fertilization, the sperm were removed by exchanging the sea water several times and the zygotes finally withdrawn from the tubes. Eggs fertilized within the tubes retained their elongated shapes when withdrawn. On the other hand, unfertilized eggs quickly rounded up after removal from the tubes. The procedure is illustrated in Fig. 1. All embryos were cultured in Millipore-filtered, pasteurized sea water (Clement, 1952) at a temperature of 22 to 25°C.

Micro-injection of tracer dye

One of the two first cleavage blastomeres in the elongated embryos resulting from eggs fertilized in tubes was micro-injected with lysyl tetramethylrhodamine–dextran (100 mg ml^{-1} in distilled water; no. D1817, Molecular Probes, Eugene, OR). Micro-injection was carried out following the procedures of Wray and Raff (1989, 1990). These embryos were reared to the appropriate stages in order to determine the relationship between the plane of first cleavage and the dorsoventral and animal–vegetal axes in the developing embryo. Micro-injected embryos were reared in complete darkness in order to prevent photo-bleaching of the dye. Despite the extensive manipulation of these embryos, their subsequent development and survivorship was as good as that observed in previous fate-mapping studies (Wray and Raff, 1989, 1990).

Analysis of development

Observations of external vital dye staining were made on living embryos. Micro-injected embryos were fixed at 5°C for 10 to 12 h in a 2 % solution of paraformaldehyde in sea water after 36 h of development. Fixed embryos were rinsed twice in sea water, dehydrated in ethanol, and then stored in 70 % ethanol at 5°C. Observations of fluorescence were made on fixed whole embryos in 70 % ethanol. Under these conditions, the boundary between labeled and unlabeled regions of the ectoderm could be clearly visualized. Detailed drawings were made of each specimen, and the relationship between the first cleavage plane and various developmental axes could then be determined by projecting the fluorescence labeling patterns onto the fate maps of Wray and Raff (1989, 1990). Because the ectoderm undergoes anisotropic stretching and elongation during development, this analysis must be accomplished by referring to these fate maps. The relationship between the first cleavage plane and the animal–vegetal and dorsoventral axes was expressed as two angles measured relative to a vector

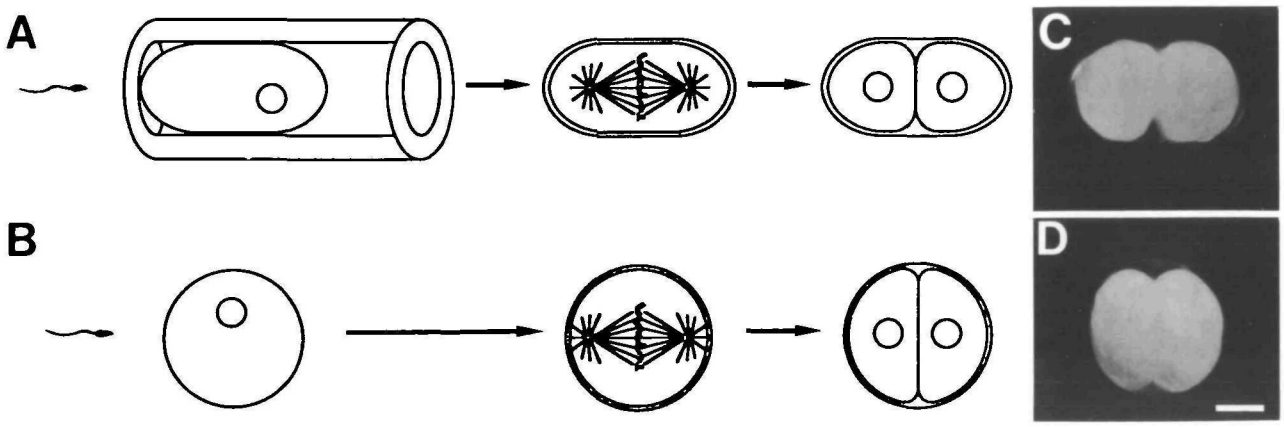

Fig. 1. Experimental procedure illustrating (A) typical equal first cleavage of elongated oocytes fertilized in 330 μm diameter silicone tubes. The rigid first cleavage spindle is forced to orient along the long axis of the zygote in most cases. (B) Equal first cleavage of undisturbed fertilized oocytes. (C and D) Corresponding light micrographs of 2-cell elongated and control embryos illustrated in A and B, respectively. Scale bar equals 150 μm.

normal to the first cleavage plane. The sign of this vector is positive in the direction of the labeled hemisphere. The first angle (α) expresses the angle relative to the animal–vegetal axis, where 0°=animal pole and 180°=vegetal pole. The second angle (β) expresses the angle relative to the dorsoventral axis, where 0° (or 360°)=the ventral side, 90°=the left side, 180°=the dorsal side, and 270°=the right side. In some cases, the topology of the labeling patterns made these angles somewhat harder to determine; the error range for all measurements is on the order of ±10°.

Results

Location of the animal–vegetal axis in unfertilized eggs

Wray and Raff (1989) observed that the fertilized eggs of *H. erythrogramma* float at the surface of the sea water with the vegetal pole facing upward. The first cleavage furrow usually begins at the downward-facing animal pole. Here we extend these observations to unfertilized eggs. We noticed, in virtually every case, that a very small protuberance is present at the float-down or presumptive animal pole of unfertilized eggs. This hillock is probably left over from the meiotic maturation divisions and serves as a useful polarity marker in these eggs. In a previous study, Wray and Raff (1989) noticed that none of the embryos survived when Nile Blue stain was applied exactly to the animal pole during first cleavage. As a precaution, therefore, we intentionally placed this stain just next to the presumed animal pole hillock when the float-down pole was marked. Exact positioning of these marks was somewhat difficult as the eggs were free-floating in the sea water. In 28 cases Nile Blue stain was applied to either the float-down pole, float-up pole or lateral side of the eggs (see Table 1). The stained eggs were then fertilized and raised to the gastrula stage. In the majority of cases (75%), these marks ended up at the animal, vegetal and lateral sides of the gastrula stage embryos, respectively, as predicted. Thus the animal–vegetal axis is recognizable in *H. erythrogramma* prior to fertilization; the animal pole is indicated by the presence of a small hillock.

Table 1. *Orientation of animal–vegetal polarity in unfertilized eggs*

Initial location of stain in oocytes	Number scored	Final location of stain in gastrula stage embryos: Animal		Lateral		Vegetal
Float-down Pole	1	0	1	0	0	0
Lateral	6	0	0	3	3	0
Float-up Pole	21	0	0	4	6	11

Diagrams at left indicate the point where Nile Blue dye was applied to the oocytes with respect to their floating orientation and the small animal pole hillock. Diagrams at top right indicate the location of the dye in the resulting gastrula stage embryos after approximately 20 h of development. Small circle indicates the position of the vegetally located blastopore.

Cleavage and orientation of elongated oocytes

Previous studies have shown that it is possible to mechanically orient the first cleavage spindle and thus the plane of cell division in a variety of zygotes by elongating or compressing them prior to cleavage (Rappaport, 1986). We utilized this approach to determine whether or not the first cleavage division sets up the dorsoventral axis in *H. erythrogramma*. From a total of 484 oocytes loaded into the elongation chambers, 352 became fertilized while still in these tubes. Fertilization proceeded very much as described by Allen (1954) for other constricted sea urchin eggs, and vitelline envelopes became completely elevated once the *H. erythrogramma* eggs were removed.

The fertilized eggs retained their elongated shapes when removed from the tubes. In 6 cases, the vitelline envelope was carefully removed with jeweler's forceps and, in all these cases, the denuded eggs retained their elongated forms, thus indicating that major changes in cytoskeletal organization take place following fertilization. This elongated shape was apparent within the empty vitelline envelopes as well. The retention of elongated shapes by the eggs of indirect developing echinoids, fertilized in tubes, has been previously noticed by several investigators, including Hörstadius and Runnström (1953) and Allen (1954).

Of the 352 *H. erythrogramma* eggs fertilized in tubes, 241 zygotes underwent the first cleavage division. In these elongated embryos, the first cleavage division took place in one of several orientations with respect to the long axis (Fig. 2). In the majority of the cases (76%), the first cleavage plane was perpendicular to the long axis and was either equal (Fig. 1A and C) or unequal. In a smaller number of cases (16%), first cleavage was slightly diagonal with respect to the long axis. In a minority of cases (8%), the first cleavage division took place more or less through the long axis. The development of these 241 cases is recorded in Table 2. The second cleavage division of these elongated embryos is variable. In many cases the second cleavage division occurred in a direction perpendicular to the first; however, in other cases the second cleavage division occurred in the same direction as the first in one or both cells. All early cleavages of the elongated embryos took place along the same time course as untreated embryos.

It is clear that the elongated shapes entrained at fertilization are sufficient to orient the first cleavage spindle in most cases. An additional set of experiments was performed to determine whether the eggs were orienting randomly when placed within the chambers. The animal or vegetal pole was marked with Nile Blue dye in a total of 36 oocytes and these randomly drawn into constriction chambers. Of these eggs, 27 were successfully fertilized and underwent the first cleavage division. For each of these latter cases, the orientation of the egg within the tubes, the first cleavage plane and

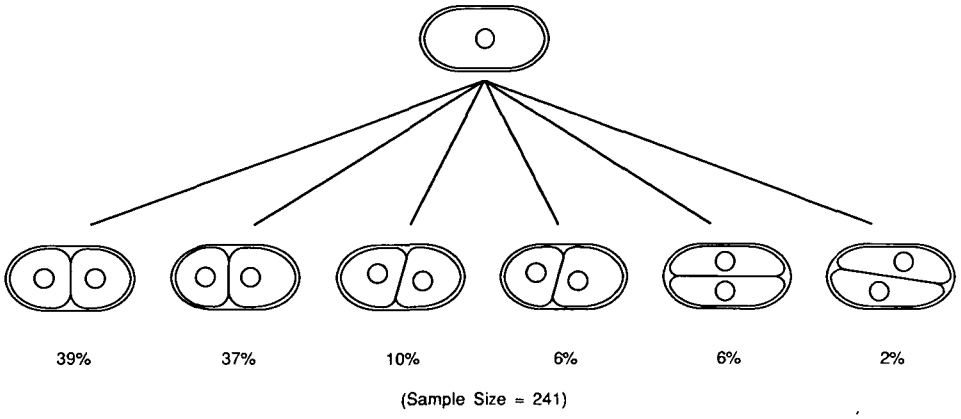

Fig. 2. Cleavage types observed in elongated zygotes resulting from eggs fertilized in 330 μm diameter silicone tubes in which one of the first cleavage blastomeres was injected with lysyl tetramethylrhodamine-dextran. Cleavage types are designated (left to right): equal, unequal, diagonal equal, diagonal unequal, longitudinal, and diagonal longitudinal. No examples of unequal longitudinal first cleavage divisions were observed. Numbers indicate the percentage of cases observed out of the 241 cases studied.

the location of the dye mark in the ectoderm of the resulting gastrula stage embryo is depicted in Fig. 3. In an additional set of experiments, one end of the elongated zygotes was stained with tetramethylrhodamine isothiocyanate immediately following fertilization while they were still within tubes. In all eleven cases, first cleavage took place perpendicular to the long axis, and the fluorescent stain remained localized at one end without shifting position (data not shown). These results show that the eggs can assume any orientation in the tubes and that this orientation does not change once they are fertilized and removed from the tubes. However, a closer examination of the data regarding the orientation of Nile Blue stained eggs reveals that they are not orienting within the tubes completely at random. In 67 % of the cases (18 out of 27), the animal and vegetal poles are in contact with the walls of the tubes. This is somewhat greater then the number of cases that should have been expected based on simple calculations of the amount of surface area a 430 μm diameter oocyte would have in contact with the wall of a 330 μm diameter tube (45 %, assuming that the volume of the egg remains constant). This bias is probably accounted for by the normal floating orientation of the eggs before they are pulled into the tubes, as stated above. Despite this, the data reveal that first cleavage can take place at any position with respect to the animal–vegetal axis, regardless of the orientation of the first cleavage plane relative to the elongation axis. Finally, the locations of the Nile Blue dye marks in the resulting gastrula stage embryos indicate that the animal–vegetal axis is not perturbed as a result of shifting the first cleavage plane.

Relationship between the first cleavage plane and the dorsoventral and animal–vegetal axes

The experiments described above demonstrate that the first cleavage plane can be readily shifted in the

Table 2. *Cleavage and development of elongated and micro-injected* H. erythrogramma *embryos*

Cleavage type	Initial number created	Normal development: Number observed	Normal development: Number with interpretable patterns	Radial development: Number observed	Radial development: Number with interpretable patterns	% Survival to larva
Equal	95	21	16	43	12	67
Unequal	90	22	17	39	19	70
Dia. Equal	23	3	3	13	3	68
Dia. Unequal	15	1	0	8	2	60
Longitudinal	14	2	2	4	3	43
Dia. Longitudinal	4	1	0	2	1	75
Totals	241	50	38	109	40	

Diagrams illustrate observed differences in the first cleavage division. The abbreviation 'Dia.' stands for 'Diagonal'. Refer to text for further information.

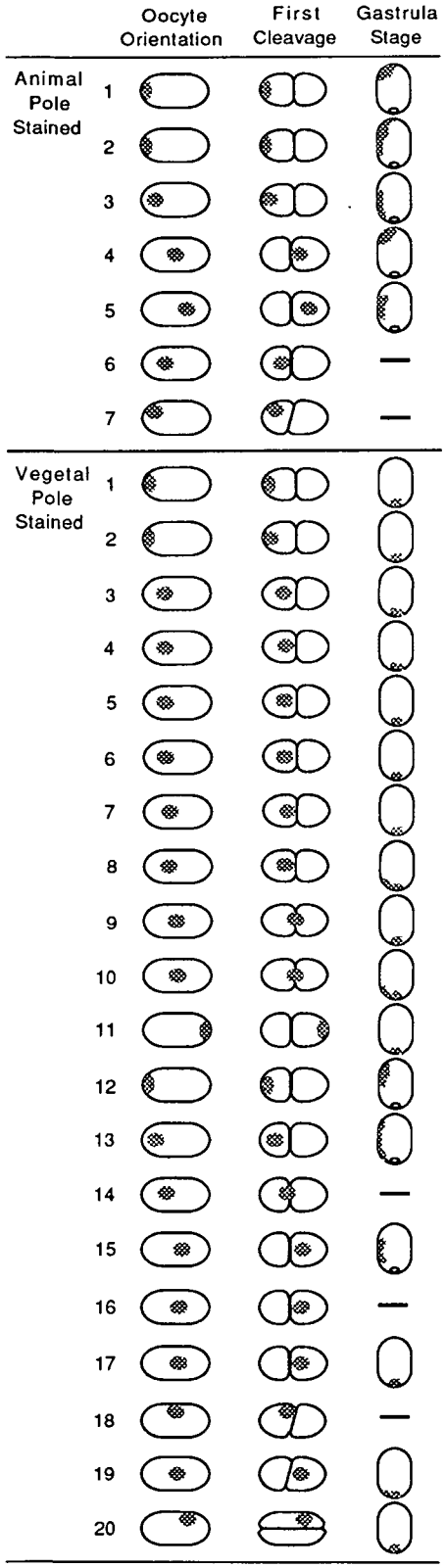

Fig. 3. Diagrams illustrating the development of elongated *H. erythrogramma* embryos in which Nile Blue dye was applied to either the animal or the vegetal pole. The location of the dye marks in the oocytes while they were being fertilized in the tubes is shown, as are the resulting 2-cell embryos. Animal-pole-stained embryos numbered four through six, and vegetal-pole-stained embryos numbered three through ten and fourteen through twenty all had their animal and vegetal poles in contact with the walls of the silicone tubes while they were being elongated. The type of first cleavage is also shown, as is the location of the dye in the gastrula stage embryos after 20 h of development. The gastrula stage embryos are oriented with their vegetal pole located downward. The blastopore is indicated by a small circle. Dashes indicate that these embryos did not complete development to the gastrula stage.

embryos of *H. erythrogramma*. Therefore, we performed a series of experiments to determine whether or not the dorsoventral axis is set up as a consequence of the first cleavage division. One of the two first cleavage blastomeres was injected with fluorescent dextran in a total of 241 elongated embryos. The first cleavage pattern and subsequent development of these embryos is recorded in Table 2. In those cases that underwent an unequal first cleavage division, the larger of the two resulting blastomeres was injected. Out of the 241 cases injected, a total of 50 developed normally but only 38 had interpretable labeling patterns. Another 109 cases developed in a radialized fashion and 40 of these latter cases had distinct labeling patterns. Radialized embryos differentiated all distinguishable cell types but lack dorsoventral polarity. Cases were deemed uninterpretable if they contained too little or no fluorescent dye or were completely labeled. In a few cases, no clear boundary between labeled and unlabeled regions could be seen and these were not scored in the final analysis. Labeling patterns in the interpretable normal embryos are recorded as two angles (α and β) relative to the animal–vegetal and dorsoventral axes, respectively, as measured from a vector normal to the plane of first cleavage (see Materials and methods). The basis for this analysis is diagramed in Fig. 4A–C. The first cleavage plane is determined from the boundary between labeled and unlabeled ectodermal regions of the embryo. The actual cases diagramed in Fig. 4A–C are shown in Fig. 5A–C. A wide variety of labeling patterns were observed and these are recorded in Fig. 6. A normal dorsal pattern is shown in Figs 4A and 5A. In only six cases did the labeling pattern correspond exactly to that of the normal fate map (α=90° and β=0° or 180°). In the example shown in Fig. 4B and 5B, the plane of first cleavage actually took place through the dorsoventral axis as would normally have been the case during the second cleavage division (α=90° and β=90°). In Figs 4C and 5C a case is shown in which the first cleavage plane took place at an oblique angle with respect to the animal–vegetal and dorsoventral axes. In two cases first cleavage took place in the equatorial plane (α=180°). Since no angle relative to the dorsoventral axis (β) could be determined in these two specimens they were not recorded in Fig. 6. These results indicate that the first cleavage plane can be made to occur with virtually any angular relationship to the animal–vegetal and dorsoventral axes.

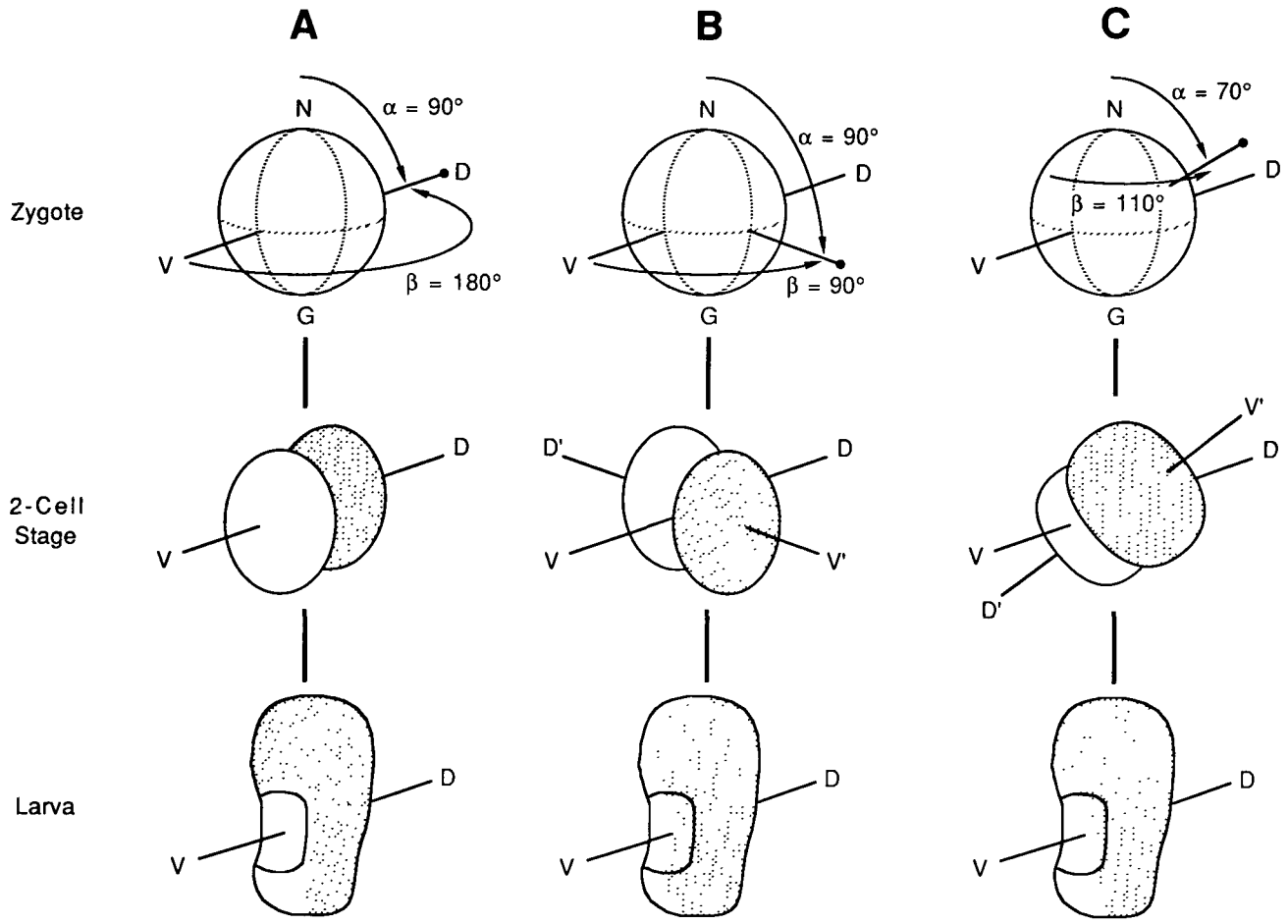

Fig. 4. Diagrams depicting observed ectodermal fate maps in larvae resulting from three representative elongated embryos in which one of the first cleavage blastomeres was injected with lysyl tetramethylrhodamine–dextran. The actual larvae from which this illustration was prepared are shown in Fig. 5. All cases illustrated displayed an equal first cleavage division. Larval views are left-frontal, and the vestibule is outlined on the ventral surface. The relationship between the first cleavage plane and the animal–vegetal (N–G) and dorsoventral (D–V) axes is depicted as two angles measured relative to a vector normal to the first cleavage plane. This vector is indicated by a line with a black dot at its end. The sign of this vector is positive in the direction of the labeled hemisphere. The convention for determining the angles (α), relative to the animal–vegetal axis, and (β), relative to the dorsoventral axis, are shown for each case as projected on the zygote (refer to the Materials and methods section for further details). (A) Example in which the labeling pattern was the same as that seen in undisturbed normal embryos in which the dorsal (D) blastomere had been injected (Wray and Raff, 1989, 1990). In this case, the first cleavage plane included the animal–vegetal axis and occurred orthogonal to the dorsoventral axis. (B) Example in which the first cleavage plane included both the animal–vegetal and dorsoventral axes (equivalent to the normally occurring second cleavage plane). (C) Example in which the first cleavage plane took place somewhat obliquely. The labels D′ and V′ in B and C indicate a possible dorsoventral axis which might have arisen if the first cleavage division had been tied to the specification of this axis.

40 radialized embryos with interpretable labeling patterns were also examined. Because these cases form with no discernible dorsoventral polarity, only the angle (α) relative to the animal–vegetal axis could be determined. As in the normal embryos, a wide range of labeling patterns was observed in these radialized embryos (Fig. 7), again indicating that the first cleavage plane can be made to occur at any angle relative to the animal–vegetal axis. Out of the 40 radialized embryos examined, 12 cases displayed labeling patterns typical of radialized embryos that arise spontaneously in undisturbed cultures (α=90°; Wray and Raff, unpublished data).

Discussion

Previous studies have shown that the first cleavage plane in the direct developing sea urchin *H. erythrogramma* normally bisects the zygote into prospective dorsal and ventral halves (Wray and Raff, 1989, 1990). Furthermore, blastomere isolation experiments reveal that a segregation of dorsoventral developmental potential takes place at first cleavage (Henry and Raff, 1990). Here we show that the establishment of dorsoventral polarity is not directly tied to the first cleavage division. Through experimental manipulation, the first cleavage division can be made to occur at

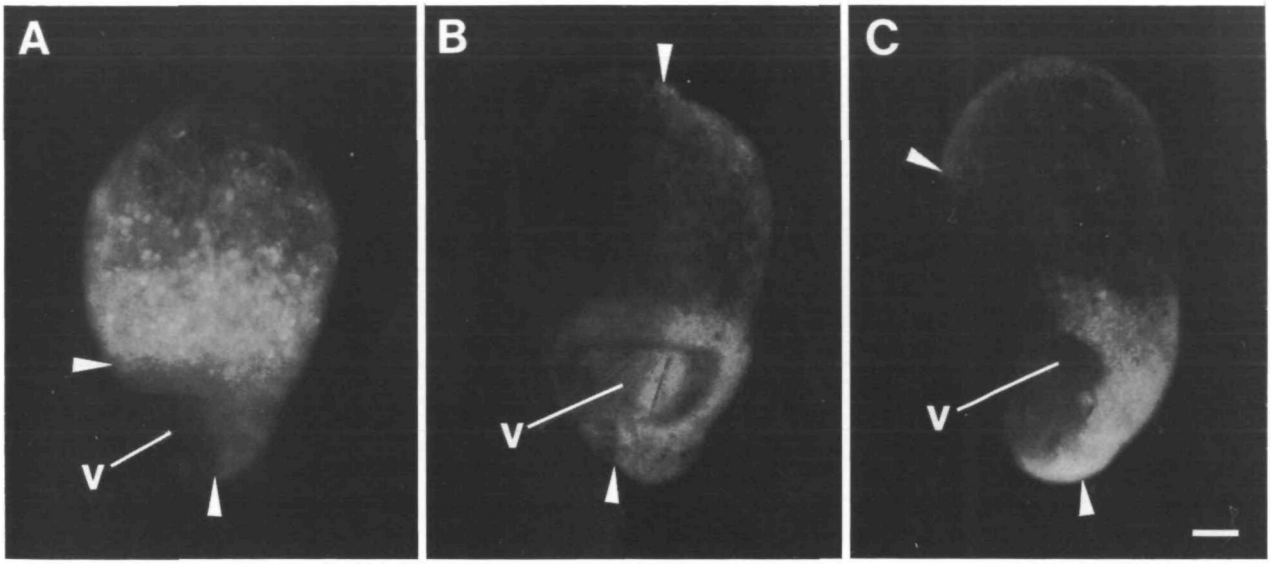

Fig. 5. Fluorescence light micrographs of three elongated embryos (after 36 h of development) in which lysyl tetramethylrhodamine–dextran was injected into one of the first cleavage blastomeres. These three cases are illustrated schematically in Fig. 4. All cases shown resulted from embryos in which the first cleavage division took place equally. White arrowheads indicate the boundary between labeled and unlabeled regions of the ectoderm. (v=vestibule). (A) Left–frontal view of a larva in which the first cleavage plane included the animal–vegetal axis and occurred perpendicular to the dorsoventral axis. The vestibule is unlabeled on the ventral side of the larvae. This is typical of a normal dorsal (D) blastomere labeling pattern (Wray and Raff, 1989, 1990). (B) Frontal view of a larva with a bilaterally symmetrical labeling pattern. In this example the first cleavage plane included both the animal–vegetal and dorsoventral axes (equivalent to the normally occurring second cleavage plane). (C) Frontal view of an example in which the first cleavage plane took place somewhat obliquely. Scale bar equals 50 μm.

virtually any angle relative to the animal–vegetal or dorsoventral axes. Therefore, dorsoventral polarity must reside within the egg or the zygote prior to first cleavage.

This does not, however, mean that the dorsoventral axis is absolutely fixed prior to first cleavage. In fact, there were a large number of cases (44 %) in which the angular relationship between the first cleavage plane and the dorsoventral axis (but not necessarily the animal–vegetal axis) was the same as that seen in undisturbed embryos, i.e. β=0° or 180°. This percentage is somewhat greater than would be expected if there was no relationship between the dorsoventral axis and the plane of first cleavage. Given the degree of accuracy with which we could determine these angles (±10°) one would expect that 11 % (40°÷360°=11 %) of the cases should lie along the lines β=0° or 180° if the first cleavage plane occurs at random with respect to the dorsoventral axis. The percentage actually observed (44 %) suggests that in a proportion of the experimental sample analyzed (44 %−11 %=33 %) the prospective dorsoventral axis was shifted by changing the plane of first cleavage in *H. erythrogramma*. Since the first cleavage plane always corresponds with the frontal plane in undisturbed embryos, one might assume that some mechanism operates to preserve the correct angular relationships between cleavage and symmetry properties during normal development. Such a mechanism may be active in some elongated embryos such as those that displayed unequal, diagonal and longitudinal forms of cleavage. The experimental treatment may not have been entirely effective in changing the cleavage plane in these forms. Therefore, the proportion of cases in which the dorsoventral axis appears to have been shifted may be even smaller. Nevertheless, in a majority of cases (56 % *versus* 33 %), there was a retention of intrinsic polarity properties, despite dramatic changes in the shape of the eggs and the experimentally imposed orientation of the first cleavage plane. Therefore, while the dorsoventral axis is specified prior to first cleavage in *H. erythrogramma*, it is not rigidly determined.

In the eggs and embryos of echinoderms that develop *via* a feeding larva, the larval dorsoventral axis appears to be more readily altered or entrained by various experimental treatments (Runnström, 1925; Lindahl, 1932*a*,*b*; Hörstadius, 1938, 1973; Pease, 1939). For example, Lindahl (1932*a*) claimed that constriction of sea urchin eggs in capillaries frequently entrained larval dorsoventral polarity. He noted that the first end to enter the tubes, opposite the site of fertilization, often became the ventral side of the developing embryo. We certainly did not observe such an effect in our experiments with *H. erythrogramma*.

Claims have been made that dorsoventral polarity is morphologically discernible in the eggs of three other species of direct developing echinoderms: the holothurians *Cucumaria frondosa* and *Psolus phantapus* (Runnström and Runnström, 1920), and the starfish *Asterina gibbosa* (Hörstadius, 1925). In all three cases, the eggs usually appear to be elongated in an axis perpendicular

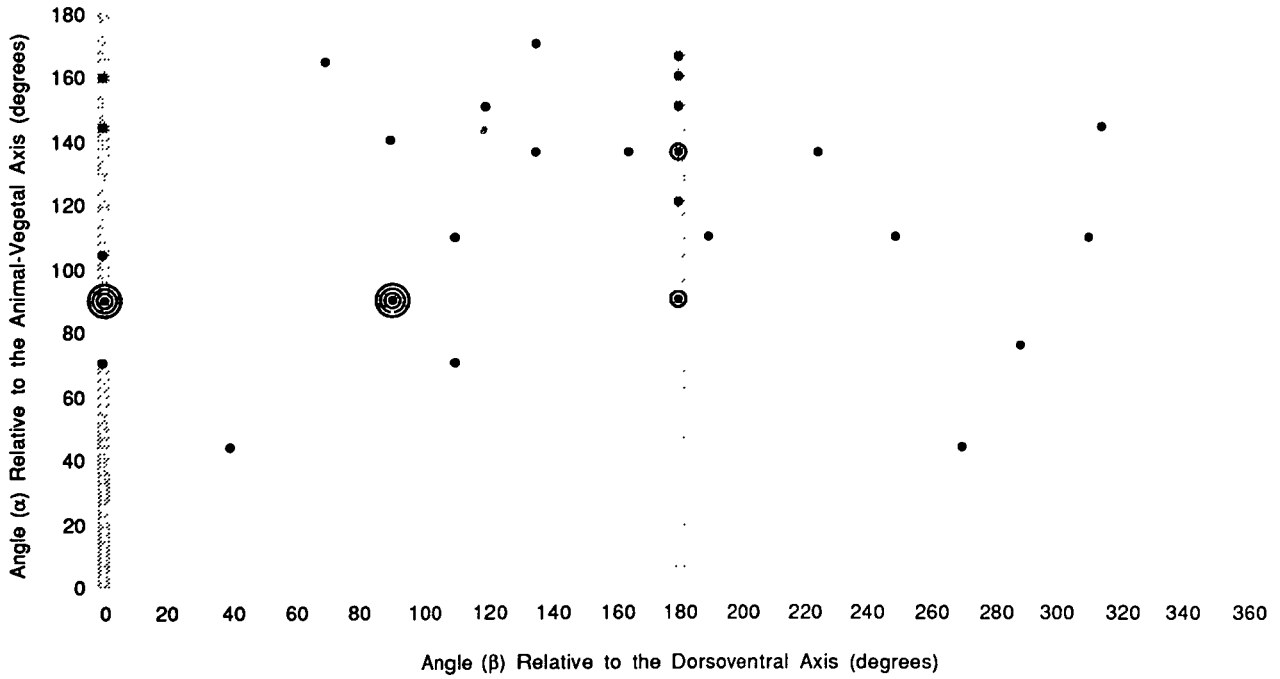

Fig. 6. Graphic representation of fluorescence labeling patterns in 36 normally developing larvae resulting from elongated embryos in which one of the two first cleavage blastomeres had been microinjected with lysyl tetramethylrhodamine–dextran. Each specimen is represented by a single point. Multiple cases with identical labeling patterns are indicated by a corresponding number of concentric circles. The labeling patterns are represented by two angles measured relative to a vector normal to the first cleavage plane. The angle (α), relative to the animal–vegetal axis, is read on the ordinate while the angle (β), relative to the dorsoventral axis, is read on the abscissa. Refer to Fig. 4 and the text for further details. Points located along the shaded horizontal line (α=90°) are cases in which the first cleavage plane included the animal–vegetal axis. Points located along the two vertical shaded lines (β=0° or 180°) are cases in which the angular relationship between the first cleavage plane and the dorsoventral axis (but not necessarily the animal–vegetal axis) was the same as that seen in undisturbed embryos (Wray and Raff, 1989, 1990). Specimens with completely normal ventral (V) or dorsal (D) blastomere labeling patterns (Wray and Raff, 1989, 1990) are located where α=90° and β=either 0° or 180°, respectively. Refer to Fig. 4 and the text for further details. In two additional cases, the first cleavage plane occurred along the equatorial plane (α=180°). Since no angle relative to the dorsoventral axis could be determined for these two cases, they are not recorded in this graphic representation.

to the animal–vegetal axis. The poles of this elongated axis are said to be the future ventral and dorsal poles, the latter of which appears to be heavier in *P. phantapus* (Runnström and Runnström, 1920). In the case of *A. gibbosa*, the first cleavage division appears to bisect the embryo into dorsal and ventral halves (Hörstadius, 1925), as is the case in *H. erythrogramma*.

The results of the Nile Blue marking experiments presented here indicate that the animal–vegetal axis is rigidly specified in the eggs and embryos of *H. erythrogramma*. This is in general agreement with the results of previous studies using indirect developing echinoids (Morgan and Spooner, 1909; Hörstadius, 1953; Harvey, 1956), which demonstrate that experimental manipulations do not affect animal–vegetal polarity.

It is notable that the angle α was found to be greater than 90° (more label in the vegetal hemisphere) in most of the injected cases examined in this study. Wray and Raff (unpublished) also observed a greater number of cases, with vegetally labeled cells in their fate mapping studies of *H. erythrogramma*. These results could suggest that the injection affects animal–vegetal polarity formation. However, as mentioned earlier, the eggs and embryos orient naturally with their vegetal sides upwards. It is easier to inject the uppermost blastomere. It seems more likely that this accounts for some of the bias in these samples. In nearly half of the cases, cleavage took place unequally, and in these cases the larger blastomere was always injected. One would expect that the larger blastomeres would contain vegetal material more frequently than the smaller blastomeres, and these would contribute to the formation of more vegetal progeny. Clearly, injection does not appear to influence dorsoventral polarity, since a similar number of injected embryos with dorsal *versus* ventral patterns were observed. In addition, Wray and Raff (unpublished) did not observe any bias in dorsoventral fates in their original fate mapping studies of *H. erythrogramma*.

It may be of some significance that the first cleavage plane took place along either what normally would have been the first (α=90°, β=0° or 180°), second (α=90°, β=90° or 270°) or third (α=0° or 180°, β=unknown) cleavage planes in a number of cases (12 out of 38 cases). These three planes may represent more stable

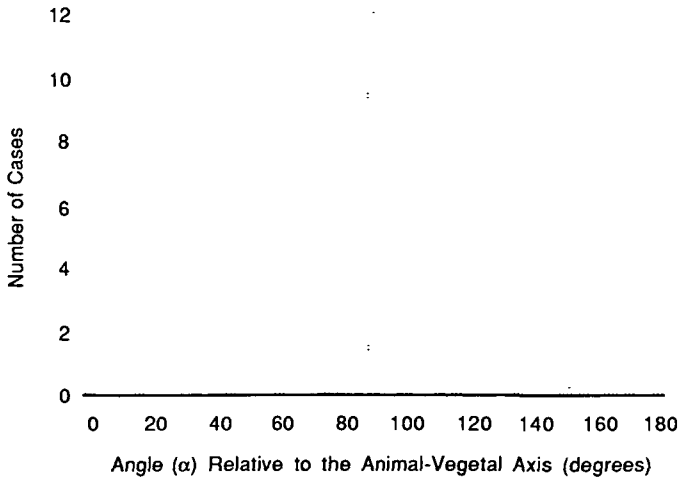

Fig. 7. Histogram representing fluorescence labeling patterns in 40 radially developing larvae resulting from elongated embryos in which one of the two first cleavage blastomeres had been microinjected with lysyl tetramethylrhodamine–dextran. Since radially developing embryos have no discernible dorsoventral polarity, the labeling patterns are represented as a single angle (α) measured between a vector normal to the first cleavage plane and the animal–vegetal axis. Typically, $\alpha=90°$ in radially developing embryos that arise spontaneously from undisturbed cultures (Wray and Raff, unpublished data). Refer to Fig. 4 and the text for further details.

positions, which bias where cleavage can take place. This may be a further indication that a scaffold of animal–vegetal and dorsoventral symmetry properties is set up in the egg or zygote.

In a previous study (Henry and Raff, 1990), we demonstrated that a segregation of developmental potential takes place as a result of the first cleavage division in *H. erythrogramma*. We proposed that an asymmetric segregation of maternal determinants, responsible for the specification of what are classically termed 'vegetal' cell fates, takes place between the two resulting blastomeres, since one of these blastomeres differentiates most vegetal structures such as the endoderm and mesoderm. We further proposed that this segregation of vegetal determinants is coupled with the establishment of the dorsoventral axis, as most vegetal cell types are derived from the ventral half of the embryo. The results of the present investigation do not support the hypothesis that ventral differentiation is linked to the pattern of distribution of vegetal determinants. If this were the case, one would expect from the fate mapping of elongated embryos that those labeled cells with more vegetal cell fates ($\alpha>90°$) would also be more ventral in character ($\beta<90°$ or $>270°$). This was not the case. Thus the specification of ventral cell fates and the dorsoventral axis appears to be dissociable from the specification of vegetal cell fates.

Although the results indicate that dorsoventral polarity exists prior to first cleavage in *H. erythrogramma*, we cannot unequivocally determine whether this property resides in the oocyte or is set up as a consequence of fertilization. We believe, however, that the site of fertilization was constrained when the *H. erythrogramma* eggs were fertilized within the 330 μm diameter tubes. For example, tetramethylrhodamine isothiocyanate does not appear to diffuse past one end of the elongated eggs while constricted in these tubes, and most eggs took very long to become fertilized if sperm was added only to the end of the tube located farthest from the egg. It is likely, therefore, that the elongated eggs were fertilized on the end where they are located closest to the opening of the tube (refer to Fig. 1). Since there is generally no relationship between the long axis of these zygotes and the dorsoventral axis, we favor the hypothesis that this axis is specified maternally.

Hörstadius (1973) concluded that the unfertilized eggs of indirect developing echinoids possessed larval dorsoventral polarity, but there is actually no definitive evidence supporting this point. Several investigators have examined the role of the sperm in setting up embryonic polarity; however, no clear relationship has been determined (reviewed by Hörstadius, 1973). Recently, Schroeder (1980) and Schatten (1981) found no relationship between the site of fertilization and the first cleavage plane in the indirect developing sea urchins *Paracentrotus lividus*, *Arbacia lixula*, and *Lytechinus variegatus*. On the other hand, Schatten (1981) did observe that the first cleavage plane occurs within 8° of the path of male pronuclear centripetal migration in the zygotes of *L. variegatus*. On the basis of these results, Cameron *et al.* (1989) suggest that centrosomal migration and position following fertilization could serve to orient the future larval dorsoventral (oral–aboral) axis since they have observed a correlation between this axis and the first two cleavage planes. We are planning to investigate the role of these factors in establishing developmental polarity in *H. erythrogramma* in the near future.

Our results are analogous to those obtained for the amphibian *Xenopus laevis*. During normal development in *X. laevis*, there appears to be some variability in the correlation between the first cleavage plane and the plane of bilateral symmetry (Klein, 1987; Danilchik and Black, 1988; Masho, 1990). Experiments clearly indicate, however, that the first cleavage division can be dissociated from the plane of bilateral symmetry (Black and Vincent, 1988). Therefore, there is no causal relationship between the first cleavage plane and the establishment of the dorsoventral axis in *X. laevis*; rather, the dorsoventral axis is established relative to the subcortical rotation of cytoplasm that takes place following fertilization (Vincent *et al.* 1986).

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