Development 115, 103-115 (1992)
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Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo

BRAD H. NELSON* and DAVID A. WEISBLAT†

Graduate Group in Neurobiology and Department of Molecular and Cell Biology, 385 LSA University of California, Berkeley, California 94720, USA

*Present address Fred Hutchinson Cancer Research Center, AC-100, 1124 Columbia Street, Seattle, Washington 98104, USA
†To whom correspondence should be addressed

Summary

In leech embryos, segmental ectoderm and mesoderm are produced by a pair of sister cells located near the animal and vegetal poles, respectively. We have investigated the mechanism that localizes ectodermal and mesodermal fates along the animal-vegetal axis. The results of cleavage arrest and cell ablation experiments suggest that the full range of normal cell interactions are not required for this process. However, when the animal and vegetal hemispheres are separated by re-orientation of the first cleavage plane from meridional to equatorial, the ectodermal fate co-segregates with the animal hemisphere and the mesodermal fate with the vegetal hemisphere. Two pools of yolk-deficient cytoplasm, called teloplasm, are located at the animal and vegetal poles of the zygote, but separation of the animal and vegetal teloplasms is not sufficient for the segregation of ectodermal and mesodermal fates. Rather, complete segregation of fates requires an equatorial cleavage orientation that separates not only the two teloplasms, but also the animal and vegetal cortical regions. This, in conjunction with previous findings, indicates that ectodermal determinants are localized to the cell cortex in the animal hemisphere of the zygote. We propose that these determinants segregate to the ectodermal precursor and interact with factors in teloplasm to transform the fate of this cell from a mesodermal ground state to ectoderm.

Key words: *Helobdella robusta*, ectodermal determinants, cell ablation, cleavage arrest.

Introduction

The embryonic development of glossiphoniid leeches is highly stereotyped in terms of both cell division patterns and cell fates (Whitman, 1878, 1887; Zackson, 1984; Kramer and Weisblat, 1985; Weisblat and Shankland, 1985; Bissen and Weisblat, 1989). An early event in this process is the separation of the segmental ectodermal and mesodermal lineages, which occurs at fourth cleavage via an obliquely equatorial division of macromere D'. One daughter of this division, cell DNOPQ, is located near the animal pole and gives rise to typical ectodermal derivatives such as epidermis and neurons. The other daughter, cell DM, is located near the vegetal pole and produces typical mesodermal derivatives such as muscles and nephridia, as well as some neurons (Weisblat et al. 1984; Kramer and Weisblat, 1985; Weisblat and Shankland, 1985). We have examined the role of both cell interactions and localized factors in the determination of the fates of cells DNOPQ and DM in embryos of *Helobdella robusta*. Here we present evidence that ectodermal determinants are localized to the animal cortex of the zygote. We propose that these determinants segregate to DNOPQ during early cleavages and interact with a second set of determinants contained in yolk-deficient cytoplasm (teloplasm) to specify the ectodermal fate. In contrast, mesoderm appears to be a default fate adopted by cells that inherit teloplasm but not animal cortex.

Both cells DNOPQ and DM inherit a substantial portion of teloplasm from their parent macromere, D'. Teloplasm derives from the zygote, where it forms midway through the first cell cycle in two pools located at the animal and vegetal poles. The animal and vegetal teloplasms are visibly distinct from yolky cytoplasm and are enriched in mitochondria, endoplasmic reticulum and polyadenylated RNA (Fernandez and Stent, 1980; Fernandez et al. 1987; Weisblat and Astrow, 1989). The first cleavage division, which is slightly unequal and oriented parallel to the animal-vegetal axis, partitions both teloplasms to the larger cell, CD (Fig. 1). At second cleavage, cell CD also divides unequally, and cell AB divides equally shortly thereafter. Thus, in the four-cell embryo, cells A, B and C are approximately equal in size and contain primarily yolky cytoplasm, whereas cell D is larger and contains both the animal and vegetal teloplasms. At this time, the vegetal

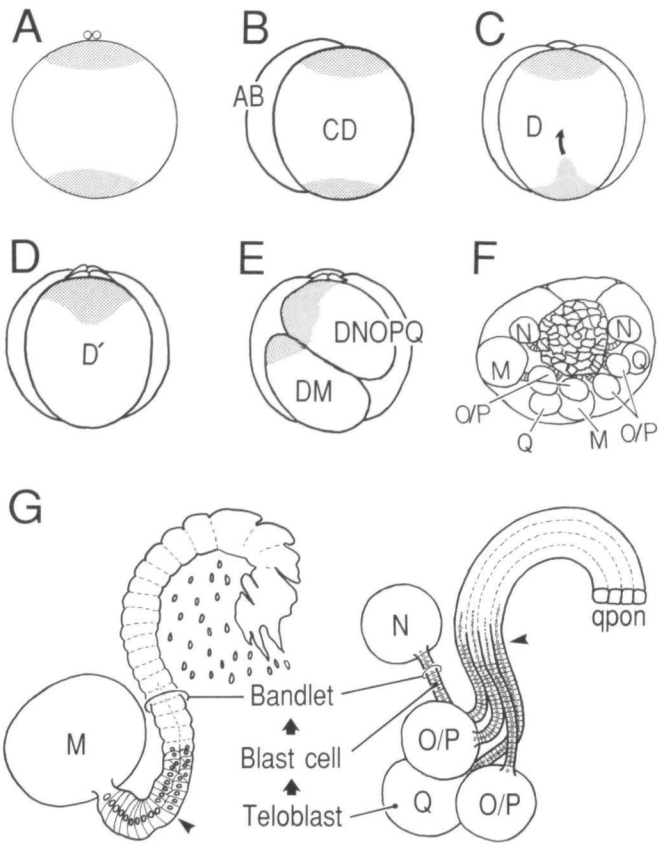


Fig. 1. Schematic summary of glossiphonnd leech development. (A) Zygote. After two polar bodies (small circles) are extruded at the animal pole (top), teloplasm (shaded regions) forms as two pools at the animal and vegetal poles. (B) 2-cell embryo. First cleavage is moderately unequal and the larger cell, CD, inherits both the animal and vegetal teloplasms. (C) 4-cell embryo. The cleavage of CD is also slightly unequal and the larger cell, D, inherits both teloplasms. The vegetal teloplasm then begins to migrate to the animal pole where it mixes with the animal teloplasm (arrow). (D) 8-cell embryo. Third cleavage is highly unequal and produces quartets of animal micromeres and vegetal macromeres. A mixture of animal and vegetal teloplasm is located at the animal pole of macromere D'. (E) 12-cell embryo. DNOPQ and DM each inherit a mixture of animal and vegetal teloplasm from macromere D' Two of the seven micromeres at the animal pole are shown. (F) Stage 7 embryo DNOPQ produces four bilateral pairs of ectoteloblasts (the N, O/P, O/P and Q teloblasts) and 13 micromeres, and DM produces one bilateral pair of mesoteloblasts (the M teloblasts) and two micromeres. Nascent bandlets associate with micromere-derived cells at the animal (future anterior) pole. (G) Stage 8. *Left* The left mesodermal teloblast and bandlet derived from DM. The arrowhead indicates the first blast cell division, which has a transverse orientation. Anterior (upper) portions of the bandlet form hemisomites (dashed lines). Migratory cells detach from the bandlet and give rise to contractile fibers of the embryonic integument. *Right* The four left ectodermal teloblasts and bandlets derived from DNOPQ. The arrowhead indicates the general location of the first blast cell divisions, which are oriented longitudinally. Lower case letters (q, p, o and n) designate bandlet identities The four ectodermal bandlets coalesce into a sheet-like structure which overlies the mesodermal bandlet, and the five bandlets together form the left germinal band (not shown). The right germinal band is a mirror-image of the left. The left and right germinal bands join at the future ventral midline (top right) to form the germinal plate (not shown).

teloplasm migrates toward the animal pole of cell D where it fuses with the animal teloplasm, thus forming a single pool (Holton et al. 1989).

At third cleavage, quartets of animal micromeres (a' - d') and vegetal macromeres (A' - D') arise by highly unequal spiral cleavages. Macromeres A', B' and C' each produce two more micromeres at the animal pole and then become multinucleate (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). In contrast, macromere D' undergoes an obliquely equatorial division at fourth cleavage to produce an animal daughter cell, DNOPQ, and a vegetal daughter cell, DM (Fernandez, 1980). DNOPQ and DM are approximately equal in size and each inherit a mixture of animal and vegetal teloplasm (Holton et al. 1989). Subsequently, DNOPQ produces four bilateral pairs of ectodermal stem cells (the N, O/P, O/P and Q ectoteloblasts) and 13 micromeres, and DM gives rise to one bilateral pair of mesodermal stem cells (mesoteloblasts) and two micromeres (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). Because DNOPQ and DM are precursors of teloblasts, they are referred to as proteloblasts (Fernandez, 1980). Each teloblast derived from DNOPQ or DM generates a chain, or bandlet, of segmental founder cells (blast cells) via a repeated series of highly unequal divisions. The ectodermal and mesodermal bandlets coalesce ipsilaterally to form the right and left germinal bands, and these in turn coalesce rostrocaudally along the ventral midline to form the germinal plate, from which segmental tissues arise (Fig. 1). On the basis of the timing and orientation of their divisions within the germinal bands and germinal plate, blast cells can be divided into seven classes (Zackson, 1984; Bissen and Weisblat, 1989), each of which ultimately contributes a distinct, segmentally iterated set of definitive progeny (Weisblat and Shankland, 1985).

Astrow et al. (1987) used centrifugation to redistribute teloplasm between blastomeres of *H. triserialis* and found a correlation between the inheritance of teloplasm and the subsequent production of teloblasts, which suggests that teloplasm contains determinants for teloblast formation. Recently, we investigated the role of teloplasm in the determination of ectodermal and mesodermal fates by selectively removing either the animal or vegetal teloplasm from zygotes of *H. robusta*. We found that the two teloplasms are developmentally equipotent: each can support the formation of a full complement of ectodermal and mesodermal teloblasts and bandlets (Nelson and Weisblat, 1991). However, it appears that the *position* of teloplasm relative to the animal pole is a determinant of the fate of DNOPQ. Specifically, we found that removal of teloplasm from the animal pole of the zygote causes the nominal cell DNOPQ to assume a DM-like mesodermal identity,

producing two mesoteloblasts and no ectoteloblasts in most cases. This fate conversion is prevented if vegetal teloplasm is centrifuged to the animal pole of cell D to replace the extruded animal teloplasm. Centrifugation does not change the amount of vegetal teloplasm inherited by the nominal DNOPQ cell, but does result in more extensive contact between the vegetal teloplasm and the animal pole. In contrast to DNOPQ, the fate of cell DM does not depend on the position of teloplasm along the animal-vegetal axis; DM appears competent to produce only mesoteloblasts.

Together these findings demonstrate a dual role for teloplasm in the determination of the fates of cells DNOPQ and DM. First, both cells require teloplasm to become proteloblasts, that is, to undergo the process of teloblast formation. Second, teloplasm is required at or near the animal pole for DNOPQ to produce ectodermal rather than mesodermal teloblasts. This latter finding suggests that the animal hemisphere contains factors that normally contact and interact with teloplasm to induce the ectodermal fate in DNOPQ. One possible source of such factors are the cells adjacent to DNOPQ. Alternatively, there may be ectodermal determinants localized to the cell cortex in the animal hemisphere that segregate to DNOPQ during early cleavages. The experiments presented here support the latter hypothesis.

Materials and methods

Embryos

Embryos of *Helobdella robusta* were obtained from a laboratory colony. Standard culture conditions (Blair and Weisblat, 1984), staging criteria (Fernandez, 1980) and injection procedures (Weisblat et al. 1984) were used *H. robusta* is a newly described species of glossiphoniid leech (Shankland et al., 1992) that closely resembles *H. triserialis*, the object of most previous work on this genus

Removal of cells DNOPQ and DM

Either cell DNOPQ or DM was removed from the embryo immediately after fourth cleavage by extrusion through the vitelline membrane Both cells were pre-labeled by injecting macromere D′ with a solution of rhodamine-conjugated dextran amine (RDA; 100 mg/ml; Molecular Probes) in 0.2 M KCl. Immediately after the cleavage of macromere D′, a solution containing fluorescein-conjugated dextran amine (FDA; 75 mg/ml; Molecular Probes) and fast green (1% w/v) in 0.2 M KCl was injected into the cell that was to be removed, either DNOPQ or DM Five to ten minutes later, embryos were immobilized in a suction chamber, and a blunt micropipette (tip diameter, 10 μm) was used to tear the vitelline and cell membranes over the target cell. This caused the rapid expulsion of the cell contents to the exterior of the embryo. To facilitate expulsion, the embryo was inverted so that the opening of the suction chamber covered the extrusion site and additional suction was applied. In all cases, a small amount of loose cell remnants remained within the vitelline membrane. Embryos with intact and attached fragments of the extruded cell that were greater than about 5% of the initial cell volume were discarded; the remainder were left to develop under aseptic culture conditions.

Cleavage arrest and micromere ablation

To prevent the formation of the A-, B- and C-derived micromeres and macromeres, a mixture containing the A-chain of the lectin ricin (10 μg/ml; Sigma), FDA (30 mg/ml) and fast green (0.6% w/v) in 0.2 M KCl was injected into cell AB 40 to 55 minutes after first cleavage and then into cell C 40 to 55 minutes after second cleavage. These cells usually underwent one more round of cell division producing cells A, B, C′ and c′. The c′ and d′ micromeres were lysed 20 minutes after third cleavage by over-injecting fast green (0.4% w/v) in 0.2 M KCl. After the cleavage of macromere D′, either DNOPQ or DM was injected with RDA (100 mg/ml) in 0.2 M KCl.

Reorientation of first cleavage by compression

Upon the completion of teloplasm formation (about 45 minutes prior to first cleavage), zygotes were placed in a drop of culture medium on the inner surface of the lid of a plastic Petri dish (Falcon #3001), to which they readily adhere, and placed between two apposing silicone slabs (Dow Corning clear auto/marine sealant, Fig. 2). The slabs were then pushed closer together to form a trough approximately 300 μm high and 300 μm wide, which is slightly less than the diameter of the zygote. Zygotes were initially oriented with the animal-vegetal axis parallel to the trough. A glass coverslip was then floated above the slabs and lowered slowly by withdrawing culture medium with a paper tissue As the coverslip lowered, zygotes were compressed and forced to elongate in the trough Zygotes frequently rolled within the vitelline membrane during this procedure so that the final orientation of the animal-vegetal axis relative to the trough varied widely. In

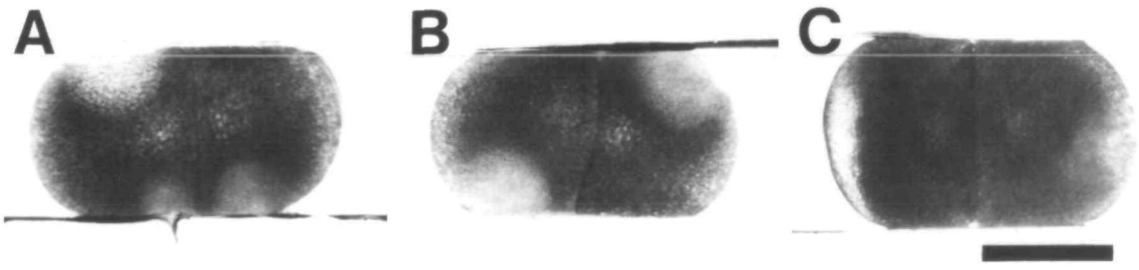


Fig. 2. Re-orientation of first cleavage by compression. Photomicrographs of living embryos still within compression troughs shortly after first cleavage. Teloplasm appears lightly shaded relative to yolky cytoplasm. Cleavage furrows are oriented perpendicular to the trough and are flanked on both sides by cell nuclei, which appear as faint spots. (A) Embryo in which the vegetal teloplasm (bottom) was bisected by a polar first cleavage plane. (B) Oblique first cleavage. The animal teloplasm is to the upper right. (C) Equatorial first cleavage. The animal teloplasm is to the left. Scale bar, 200μm.

more than 80% of cases, first cleavage was slightly unequal, and the cleavage furrow was oriented perpendicular to the trough. Thirty minutes after first cleavage was complete, the coverslip was removed from the slabs, and the embryos were freed from the trough with a jet of culture medium.

DiI labeling of the animal pole

To ascertain whether or not the compression procedure described above caused teloplasm to shift relative to the cell membrane, a small patch of membrane at the animal pole was labeled with the lipophilic fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) after second polar body extrusion. DiI was dissolved to saturation in a solution of 70% ethanol and 30% 0.2 M KCl containing 1.2% w/v fast green. This was used to fill micropipettes which were then attached to a pressure-injection apparatus. Embryos were held by gentle suction and the micropipette pierced through the vitelline and cell membranes at the animal pole. The micropipette was withdrawn slowly until the tip lay in the perivitelline space. DiI solution was released by gentle pressure for about ten seconds. The micropipette was then withdrawn completely In some cases, zygotes were labeled by impaling the animal pole with a micropipette that had been coated with a saturated solution of DiI in 100% ethanol and then air dried.

Approximately 70% of the DiI-labeled zygotes were discarded due to lysis of the cell membrane at the animal pole; the remaining 30% of zygotes formed teloplasm normally. After teloplasm had formed, the DiI spot was viewed briefly by epifluorescent illumination using a rhodamine filter set to ensure that it was in register with the animal teloplasm, which was detected by its autofluorescence when viewed with a fluorescein filter set. Zygotes were compressed as described above, left to cleave, and then released. The positions of the DiI spot and the animal teloplasm were again compared by epifluorescence microscopy. By this time, the extent of DiI diffusion in the cell membrane was such that the spot was approximately equal in size to the animal pool of teloplasm.

Histology and microscopy

A number of embryos with re-oriented first cleavages were examined to determine the internal distribution of teloplasm. For this purpose, embryos were fixed in 4% formaldehyde and 0.1 M Tris-HCl buffer (pH 7.4) at 4°C overnight and then rinsed several times in Tris buffer. After being dehydrated in an ethanol series, they were embedded in JB4 embedding resin (Polysciences), and sectioned at 14 μm with a glass knife microtome.

Embryos processed for epifluoresence microscopy were fixed for 1 hour at room temperature in 4% formaldehyde in 0.05 M sodium cacodylate buffer (pH 7.3). They were rinsed and manually devitellinized in 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4 3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7 4), and then incubated at room temperature for approximately 12 hours in each the following solutions: blocking solution (TBP) consisting of PBS, 2% bovine serum albumin, 1% Triton X-100, and 1 mg/ml sodium azide, a 1:100 dilution of mouse anti-leech nucleus monoclonal antibody (provided by D. Stuart, University of California, Berkeley) in TBP; several rinses of TBP; a 1:250 dilution of fluorescein-conjugated rabbit anti-mouse secondary antibody (ICN, Inc.) in TBP; and several rinses of PBS. Embryos were then post-fixed for 1 hour at room temperature in 4% formaldehyde with 5 μg/ml Hoechst 33258 in 0.1 M Tris-HCl buffer, rinsed several times in 0.1 M Tris-HCl buffer, dehydrated in an ethanol series, and cleared in methyl salicylate. Blast cell division patterns within bandlets were examined in 5 μm optical sections obtained using a confocal microscope (BioRad).

Results

Criteria for assigning ectodermal and mesodermal cell fates

Embryos subjected to cell ablation, cleavage arrest or compression were fixed after 60 hours of development (early stage 8), and cell fates were ascertained according to the following criteria which distinguish ectodermal and mesodermal bandlets in normal embryos (Fig. 1G). In mesodermal bandlets: (i) the first blast cell division occurs at a separation of 9 to 12 blast cells from the parent teloblast and has a transverse orientation (Fig. 3A); (ii) second blast cell divisions are oriented 90° relative to the first and occur at a separation of 14 to 18 blast cells from the parent teloblast; (iii) blast cell clones in the anterior (older) portion of the bandlet comprise segmentally iterated clusters of cells which are referred to as hemisomites (Zackson, 1982), and (iv) bandlets occupy a deep position in the embryo. In addition, in most experimental embryos anterior portions of mesodermal bandlets derived from sibling teloblasts were joined and gave rise to scattered cells corresponding to putative contractile fibers of the provisional integument, which are also characteristics of normal mesodermal bandlets (Fernandez and Stent, 1980; Weisblat et al. 1984).

In ectodermal bandlets: (i) the first blast cell division is longitudinal and occurs at a separation of 20 to 30 blast cells from the parent teloblast (Fig. 3D; Zackson, 1982); (ii) second blast cell divisions are also longitudinal and occur at a separation of 25 to 35 blast cell positions from the parent teloblast; (iii) anterior (older) portions of neighboring bandlets coalesce into a sheet-like structure; and (iv) bandlets occupy a superficial position in the embryo. Some experimental embryos were fixed before second ectodermal blast cell divisions had occurred; in these cases, bandlets were identified as ectodermal by the other criteria.

In approximately 10-20% of experimental embryos, cell fates could not be assigned due to ill health of the embryo, or unsatisfactory labeling of cells by lineage tracer. These embryos were discarded and were not considered further.

Alteration of cell interactions

At the time proteloblasts DNOPQ and DM arise from macromere D′, only seven other cells are present in the embryo, macromeres A′- C′, and micromeres a′- d′. *A priori*, any one or more of these cells could provide inductive signals to specify ectodermal and mesodermal fates. One test for such interactions would be to examine the fate of DM or DNOPQ in isolation. However, isolated leech blastomeres often cleave at abnormal orientations, presumably because they lack the mechanical constraints imposed by other cells (B.H. Nelson, unpublished results; Symes and Weisblat, 1992). Therefore, we investigated the role of cell

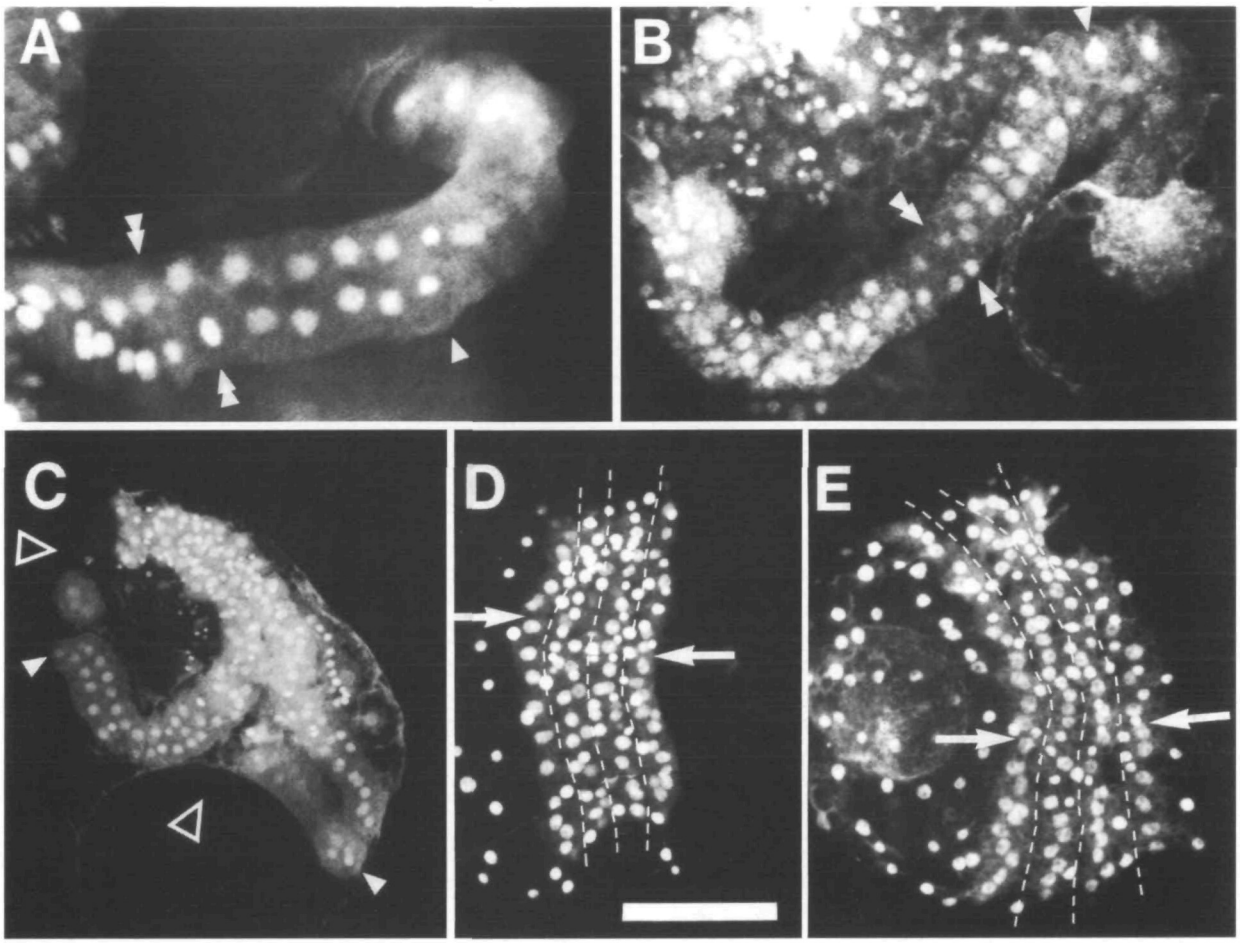


Fig. 3. Assessment of ectodermal and mesodermal fates by blast cell division patterns. Stage 8 embryos in which one blastomere was injected with RDA were processed for epifluorescence microscopy and optically sectioned at 5μm intervals on a Bio-Rad (Cambridge, Massachusetts, USA) confocal microscope. Cell nuclei appear as bright spots Two to six optical sections have been superimposed to generate each image; bandlets appear truncated because cells outside these focal planes are not visible. Anterior is to the left in A and B and up in C-E. (A) Control left mesodermal bandlet showing the characteristic orientations of the first (arrowhead) and second (double arrowheads) blast cell divisions. Part of the teloblast is visible above the bandlet (B) One of four mesodermal bandlets derived from cell VG in an oblique embryo showing the mesoderm-like first (arrowhead) and second (double arrowheads) blast cell divisions. Primary undivided blast cells are visible between the first arrowhead and the teloblast and appear bunched together due to twisting of the bandlet. (C) Two mesodermal bandlets derived from cell AN in an oblique embryo shown at lower magnification than in (A) and (B). The parent teloblasts are out of the plane of focus, but their positions are indicated by open triangles. Arrowheads point to the locations of the first blast cell divisions. Note that the anterior ends of the bandlets have a segmented appearance and are joined, two features of normal mesoderm. (D) Control embryo showing (from right to left) n, o, p and q ectodermal bandlets and the characteristic longitudinal orientation of their early blast cell divisions (arrows). Dashed lines separate the bandlets. Nuclei of micromere derivatives are also present at the left and between bandlets. (E) Four out of eight ectodermal bandlets derived from cell AN in an oblique embryo Blast cell divisions are oriented longitudinally (arrows). As in D, dashed lines separate the bandlets, and micromere-derived nuclei are also visible. Scale bar, 50μm in (A-B and D-E) and 85μm in (C).

interactions in the determination of the fates of DNOPQ and DM in two other ways.

In one series of experiments, we generated embryos lacking either DNOPQ ($DNOPQ^X$ embryos) or DM (DM^X embryos) by extruding the selected cell through a hole in the vitelline membrane 10 minutes after the cleavage of macromere D′. The extruded cell was pre-labeled with both rhodamine and fluorescein lineage tracers (RDA and FDA) to confirm the success of the extrusion, whereas the surviving sister cell was pre-labeled with RDA only. In most embryos, the remaining cells rearranged within 15 minutes of the extrusion so that the surviving cell occupied a position equivalent to that of the parent macromere D′ in the 8-cell embryo (Fig. 4 A-D). Thus, cell DM in $DNOPQ^X$ embryos and cell DNOPQ in DM^X embryos occupied equivalent positions and, in principle, should have experienced identical cell interactions after the post-extrusion cell rearrangements.

In DM^X embryos (n=12), DNOPQ generated eight (n=9) or ten (n=1) ectoteloblasts which produced disorganized bandlets (Fig. 4E); in one case it produced

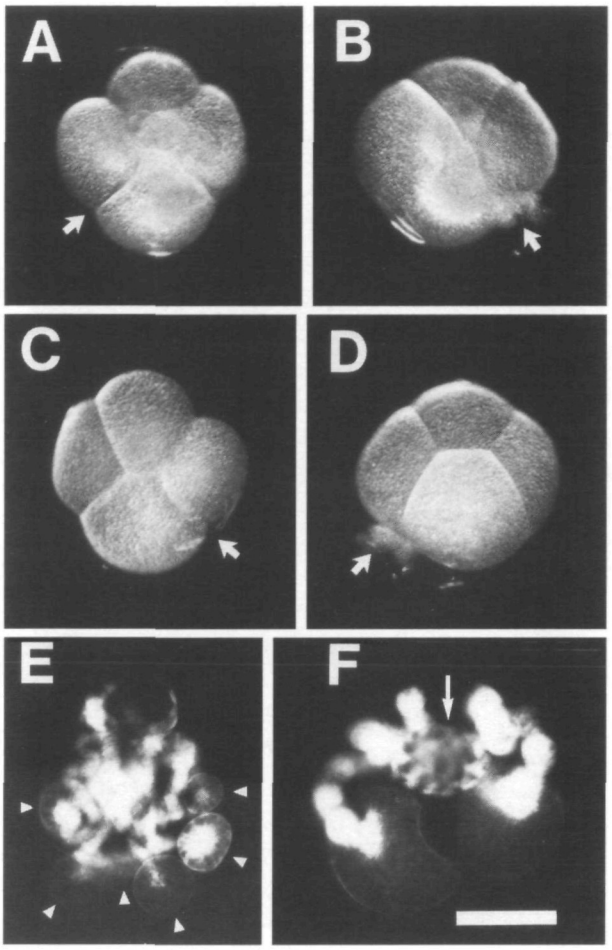


Fig. 4. Extrusion of DNOPQ and DM to test the role of cell interactions (A-D) Photomicrographs of living embryos in which DM or DNOPQ was extruded immediately after the cleavage of macromere D′. The arrows indicate the former position of the extruded cell, and the surviving sister cell, DNOPQ or DM, is at the bottom in all four photographs. (A) DM^X and (B) $DNOPQ^X$ embryos viewed from the animal pole. (C) DM^X and (D) $DNOPQ^X$ embryos viewed from the vegetal pole. Note that surviving DNOPQ and DM cells occupy equivalent positions in the embryo and appear to make the same cell contacts. (E-F) Photomicrographs of DM^X and $DNOPQ^X$ embryos at early stage 8 viewed in whole-mount under rhodamine epifluoresence Anterior is up. (E) DM^X embryo in which DNOPQ was injected with RDA and gave rise to eight ectodermal teloblasts (six are in the plane of focus and marked with arrowheads) with disorganized bandlets (F) $DNOPQ^X$ embryo in which DM was injected with RDA and gave rise to two mesodermal teloblasts and bandlets The arrow points to migratory cells that in normal mesoderm generate the contractile fibers of the embryonic integument. Scale bar, 200μm for A-D, 125μm for E-F.

six cells without bandlets, and in another it failed to divide. In $DNOPQ^X$ embryos (n=13), DM gave rise to one (n=1), two (n=7), or three (n=1) mesoteloblasts with disorganized bandlets (Fig. 4F); in other cases, DM produced several cells which lacked bandlets (n=3) or failed to divide (n=1). In no case did DNOPQ give rise to mesoteloblasts, or DM to ectoteloblasts. These results suggest that, if the fates of DNOPQ and DM are determined via cell interactions, the critical interactions must be complete within 25 minutes after the formative cytokinesis.

In a second series of experiments, the formation of the normal complement of micromeres and macromeres was prevented by injecting their precursors with the A-chain of the lectin ricin. In mammalian cells, the ricin A-chain inhibits protein synthesis by modifying 28S rRNA (Endo and Tsurugi, 1988). When injected into leech blastomeres, it arrests cleavage of the injected cell without causing lysis (D. Lans, personal communication). Cell AB and cell C were injected with a mixture of ricin A-chain and FDA, 30 to 60 minutes after they were born. They usually underwent one more cell divisions before arresting, producing cells A, B, C′ and c′. After third cleavage, micromeres c′ and d′ were lysed by over-injection of a solution of fast green in 0.2M KCl. Thus, these embryos consisted of the cleavage-arrested cells A, B and C′, and an unmanipulated macromere D′, which cleaved at an obliquely equatorial orientation as in normal embryos. Either DNOPQ (n=8) or DM (n=11) was injected with RDA. Because teloblasts in these embryos produced stunted bandlets that could not be identified reliably, the fates of DNOPQ and DM were assessed by counting the number of teloblasts produced by each. In all cases, DM produced two teloblasts, and DNOPQ generated eight to ten teloblasts. We have previously shown that when cell DNOPQ converts to a mesodermal fate as a result of removal of the animal teloplasm, it usually produces only two mesoteloblasts, and never more than six (Nelson and Weisblat, 1991). The results of these cleavage arrest experiments do not rule out the possibility of inductive interactions between cells, since molecules present in cell membranes prior to the injection of ricin A-chain could persist and mediate inductive interactions. Nevertheless, these experiments do demonstrate that the full complement of normal micromeres and macromeres is not required for DNOPQ and DM to express early aspects of their normal fates.

Re-orientation of first cleavage

The obliquely equatorial cleavage of macromere D′ that segregates segmental ectoderm and mesoderm also separates the animal and vegetal components of the cell. Therefore, to test the possibility that determinants for ectoderm and mesoderm are asymmetrically distributed along the animal-vegetal axis, we re-oriented the first cleavage plane so as to precociously separate the animal and vegetal hemispheres. We then examined the fates of the two cells produced.

For this purpose, zygotes were elongated by compression shortly after the animal and vegetal teloplasms had formed. Under these conditions, first cleavage is slightly unequal, as in normal embryos, but the furrow typically forms perpendicular to the axis of elongation (Fig. 2). Thus, by varying the orientation of embryos in

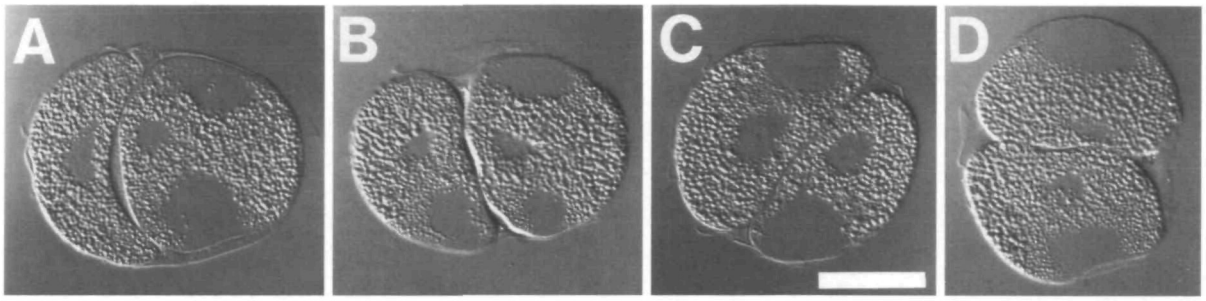


Fig. 5. Distribution of teloplasm after re-orientation of first cleavage. Meridional sections parallel to the spindle axis through 2-cell embryos viewed with Nomarski optics. The animal-vegetal axis is oriented vertically. Yolky cytoplasm has a granular appearance The animal (upper) and vegetal (lower) pools of teloplasm appear as yolk-deficient regions, as does cytoplasm surrounding the nuclei which lie adjacent to the cleavage furrows (A) Uncompressed control embryo showing the normal distribution of teloplasm within cell CD. (B) Polar embryo in which the vegetal teloplasm was bisected. (C) Oblique embryo. (D) Equatorial embryo. Scale bar, 150μm.

the compression chamber, one can produce two-cell embryos with varying distributions of animal and vegetal teloplasm. In this series of experiments, we defined four classes of cleavage orientation: normal, polar, oblique and equatorial (Fig. 5, Table 1). With normal cleavages, both the animal and vegetal teloplasms segregated to the larger cell CD (Fig. 5A). Polar cleavages bisected one or both pools of teloplasm; we defined the cell that was largest and inherited the most teloplasm as cell CD in such embryos (Fig. 5B). With oblique cleavages, the cleavage furrow formed adjacent to the animal and vegetal teloplasms and fully segregated the two pools to different cells (Fig. 5C). Equatorial cleavages were perpendicular to the animal-vegetal axis, or nearly so, therefore the two teloplasms were fully segregated and located as far as possible from the cleavage furrow (Fig. 5D). Approximately 95% of compressed embryos underwent normal, polar or oblique cleavages. The frequency of equatorial cleavages was lower than expected based on the orientation of zygotes in the compression chamber because, (i) zygotes elongated along the animal-vegetal axis tended to roll within the vitelline membrane, or to undertake oblique cleavage orientations (relative to the axis of elongation), and (ii) zygotes elongated along the animal- vegetal axis occasionally delayed cleavage until after they were released from compression, and then cleaved almost immediately at a normal orientation. Embryos in this latter group were discarded. For both oblique and equatorial cleavages, we define the cell that inherited the animal teloplasm as cell AN, and the other as cell VG. In most experiments, either cell AN or VG was injected with RDA.

To ascertain whether or not the compression procedure caused teloplasm to shift relative to the overlying cell cortex and membrane, we marked the animal pole of 12 zygotes prior to teloplasm formation with a spot of DiI, a fluorescent lipophilic molecule that labels the cell membrane. After teloplasm formation was complete, zygotes were compressed as usual, allowed to cleave, and then released. The DiI spot was visualized under rhodamine epifluorescence and its position compared to that of the teloplasm, which autofluoresces under fluorescein epifluorescence. We found that the animal teloplasm did not move relative to the cell membrane in most embryos, and in no case did it move more than 15 degrees of arc. Thus, the location of the animal teloplasm is a reliable indicator of animal-vegetal polarity after compression.

Although the cleavage arrest and cell ablation experiments described in the previous section did not reveal any cell interactions essential for the determination of ectodermal and mesodermal fates, we considered the possibility that novel cell interactions might arise in embryos with re-distributed teloplasm. In some experiments, one of the cells at the 2-cell stage was injected with ricin A-chain. Injected cells typically divided once and then underwent cleavage arrest. The fate of the other cell could then be examined in isolation. In general, the results obtained from these embryos were similar to those from embryos in which both cells were allowed to develop.

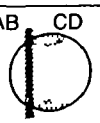
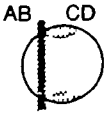
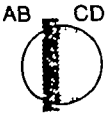
Normal first cleavage

Embryos compressed along the animal-vegetal axis had a normal distribution of animal and vegetal teloplasm at the 2-cell stage, even though the exact location of the cleavage furrow was constrained by compression. These embryos formed a normal complement of ectodermal and mesodermal teloblasts (Table 1) and most developed normally through to hatching. Thus, compression alone does not interfere with normal development. Moreover, this result implies that the zygote is radially symmetric with respect to developmental potential; that is, the animal-vegetal axis is the only axis established prior to first cleavage. If a second axis were predetermined in the zygote, one would expect that constraining the precise location of first cleavage would disrupt the development of all but a small set of embryos.

Polar first cleavage

In general, cleavages that bisected one or both pools of teloplasm did not disrupt normal development to stage 8. The cell that inherited the most animal and vegetal teloplasm, which we define as cell CD, usually

Table 1. *Fate of cells from the two-cell stage after re-orientation of first cleavage*

Group	1st Cleavage	*n*=	Number and type of teloblasts produced by AB	CD
Uncompressed control		25	(25) 0 TB	8 ETB, 2 MTB
Normal cleavage		35	(33) 0 TB	8 ETB, 2 MTB
			(2) ablated	8 ETB, 2 MTB
Polar cleavage		25	(14) 0 TB	8 ETB, 2 MTB
			(1) 0 TB	6 ETB, 2 MTB
			(1) 8 ETB, 2 MTB	0 TB
			(5) ablated	8 ETB, 2 MTB
			(2) 8 ETB, 2 MTB	ablated
			(1) 9 ETB	ablated
			(1) 0 TB	ablated
			AN	VG
Oblique cleavage		30	(4) 8-10 ETB, 2 MTB	2-4 MTB
			(6) 3-6 ETB, 1-2 MTB	2 MTB
			(1) 8 ETB, 2 MTB	4 ETB, 2 MTB
			(5) 5-8 ETB, 2 MTB	0 TB
			(2) 6-10 ETB	2-6 ETB, 2 MTB
			(1) 8 ETB	4 MTB
			(1) 8 ETB	0 TB
			(2) 7-8 ETB, 2 MTB	ablated
			(2) 6-8 ETB	ablated
			(2) ablated	8 ETB, 2 MTB
			(4) ablated	2-4 MTB
Equatorial cleavage		20	(7) 8-12 ETB	2 MTB
			(4) 8 ETB	3-4 MTB
			(1) 6-8 ETB	0 TB
			(2) 6 ETB, 2 MTB	2-4 MTB
			(1) 8 ETB	ablated
			(5) ablated	2-4 MTB

Embryos were fixed at early stage 8 and the number of ectodermal and mesodermal teloblasts was assessed. Micromeres and macromeres were also present but were not included in this analysis. Shaded boxes over zygotes indicate the range of cleavage orientations observed for each cleavage class In brackets are the number of cells displaying a given phenotype. Some cells were ablated by injection of ricin A-chain TB, teloblast, ETB, ectoteloblast and bandlet, MTB, mesoteloblast and bandlet

underwent a normal pattern of early cleavages and formed eight ectoteloblasts and two mesoteloblasts with well-organized bandlets (Table 1). Cell AB, in contrast, usually gave rise to a normal AB lineage consisting of two macromeres and some micromeres. However, in 15% of polar embryos, AB produced ectodermal and mesodermal teloblasts, and in one embryo it produced nine ectoteloblasts after inheriting approximately half the animal teloplasm.

In other annelids, equalization of first cleavage by mechanical or chemical means can result in the formation of twinned embryos (Henry and Martindale, 1987; Dorresteijn et al. 1987; Devries, 1973, 1985). Similarly, duplicated sets of ecto- and mesoteloblasts are obtained in *Helobdella* embryos when centrifugation at the 2-cell stage results in an equal distribution of teloplasm between the daughters of blastomere CD at second cleavage (Astrow et al., 1987). We did not observe twinning in our compression experiments, however, presumably because first cleavage remained unequal, so that one cell received most of the teloplasm.

Equatorial first cleavage

Complete segregation of ectodermal and mesodermal fates occurred when first cleavage was equatorial, or nearly so. In the majority of cases, the cell derived from the animal hemisphere, cell AN, gave rise to eight to twelve ectoteloblasts and no mesoteloblasts. In contrast, the cell derived from the vegetal hemisphere, cell VG, produced two to four mesoteloblasts and no ectoteloblasts (Table 1). The fates of cells AN and VG were independent of their relative sizes. The bandlets in these embryos were very disorganized but nevertheless tended to group together near the micromere cap.

The pattern of early cleavages in equatorial embryos, described in detail in the next section, typically resulted in the formation of two pairs of proteloblasts, one derived from cell AN and the other from cell VG, which resembled cells DNOPQ and DM in terms of size,

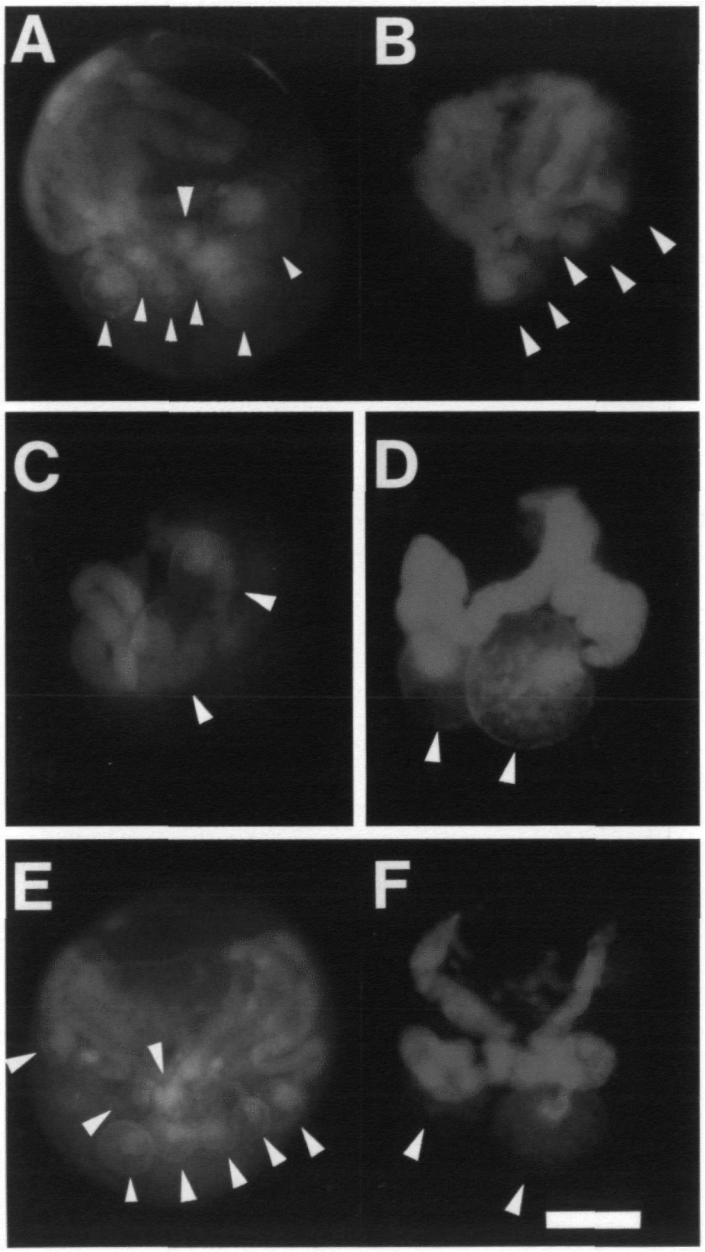


Fig. 6. The fates of proteloblasts in compressed embryos depend on their animal or vegetal origin. In embryos that underwent re-orientation of first cleavage, proteloblasts that were proximal to the micromere pole were injected with FDA and their sibling proteloblasts with RDA. Shown are photomicrographs of such embryos at early stage 8 viewed in whole-mount under fluorescein (green) or rhodamine (red) epifluorescence Arrowheads point to teloblasts. Anterior is up. Refer to the rightmost panels of Fig 7 for further clarification of which cells were injected with lineage tracer. (A) Equatorial embryo in which one proteloblast derived from cell AN was injected with FDA and gave rise to seven ectodermal teloblasts and bandlets. (B) In this same equatorial embryo, the other proteloblast derived from cell AN produced five ectodermal teloblasts and bandlets (C) In a different equatorial embryo, one proteloblast derived from cell VG generated two mesodermal teloblasts and bandlets, as did its sibling proteloblast (D). (E) Oblique embryo in which the proteloblast proximal to the micromere pole and derived from cell AN produced eight ectodermal teloblasts and bandlets, whereas its sibling proteloblast generated two mesodermal teloblasts and bandlets (F) Scale bar, 100μm.

inheritance of teloplasm, and position relative to the micromeres. We assessed the fates of these cells in a few equatorial embryos by injecting FDA into one proteloblast and RDA into its sibling. We found that both proteloblasts derived from cell AN (n=3) gave rise to four to eight ectoteloblasts (Fig. 6 A,B), whereas both proteloblasts derived from cell VG (n=2) gave rise to two or three mesoteloblasts (Fig. 6 C,D). Thus, in equatorial embryos, sibling proteloblasts can both give rise to ectoderm, or both to mesoderm, depending on their animal or vegetal origin.

Oblique first cleavage

In contrast to the complete segregation of ectodermal and mesodermal fates that resulted from equatorial first cleavages, oblique cleavages usually caused only a partial segregation of fates (Table 1). In most cases, cell AN gave rise to both ectodermal and mesodermal teloblasts (Fig. 3 C,E), whereas cell VG produced two to four mesoteloblasts and no ectoteloblasts (Fig. 3B). There was, however, some variability within this class. In 25% of cases, cell AN produced only ectoteloblasts. Cell VG produced both ectodermal and mesodermal teloblasts in 19% of embryos and no teloblasts whatsoever in 23% of embryos. In no case did cell AN make only mesoteloblasts or cell VG only ectoteloblasts. There was no apparent correlation between the fates of cells AN and VG and their relative sizes.

As in equatorial embryos, early cleavages usually resulted in the formation of two pairs of proteloblasts that resembled DNOPQ and DM, one pair derived from cell AN and the other from cell VG. However, due to the geometry of early cleavages (described in the next section), oblique embryos differed from equatorial embryos in that proteloblasts near the micromeres were derived mostly from the animal hemisphere, whereas those more distal to the micromeres arose largely from the vegetal hemisphere. In a few oblique embryos, we injected different lineage tracers into sibling proteloblasts. We found that proteloblasts derived from the animal portion of cell AN produced eight ectoteloblasts (n=7; Fig. 6E), whereas their sibling proteloblasts derived from the vegetal portion of cell AN produced two mesoteloblasts (n=6; Fig. 6F) or no teloblasts (n=1). As for the descendents of cell VG (n=3), those proteloblasts derived from the vegetal portion gave rise to two mesoteloblasts, whereas those derived from the animal portion produced seven or eight ectoteloblasts (n=2) or no teloblasts (n=1). Thus, in oblique embryos, proteloblasts derived from animal regions of either cell AN or VG tended to produce ectoteloblasts, or no teloblasts, whereas those derived from vegetal regions produced mesoteloblasts.

Early cleavage patterns following re-orientation of first cleavage

Several features of the development of embryos that underwent re-orientation of first cleavage provided insight into the mechanisms governing the orientation and timing of early cleavages. These features were also

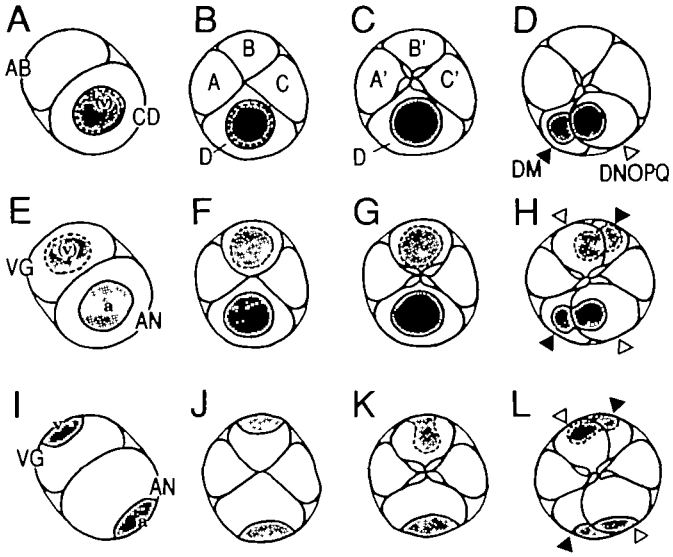


Fig. 7. Schematic summary of early development after re-orientation of first cleavage. Embryos are viewed from the prospective micromere pole, which in normal embryos corresponds to the animal pole. The positions of the animal (a) and vegetal (v) poles are indicated in the leftmost drawings and remain constant in the other drawings. The "v" in (A) and (E) is enclosed in parentheses because it is not visible from the micromere pole. Teloplasm is shaded and outlined with a solid line where it is visible from the micromere pole and with a dashed line where it is not. In A and B the animal and vegetal teloplasms overlap. (A-D) Normal embryo after the first, second, third and fourth cleavages. (E-H) Typical oblique embryo at the equivalent stages of development. In this example, teloplasm segregates to two of the four macromeres. In H, both of these macromeres give rise to one proteloblast near the micromere pole (open triangles) and another distal to the micromere pole (closed triangles) (I-L) Typical equatorial embryo. Cleavages are similar to oblique embryos, however the two pools of teloplasm are located further from the micromere pole. Note that in K the vegetal teloplasm migrates toward the animal pole, as it does during normal development (C). As in oblique embryos, macromeres that inherit substantial amounts of teloplasm give rise to one pair of proteloblasts (open and closed triangles).

observed by Devries (1973), who performed similar experiments with embryos of the oligochaete *Eisenia*.

First, the second cleavage plane in oblique and equatorial embryos was always perpendicular to the first, and was usually oriented so that the axis defined by the intersection of the two cleavage planes paralleled the animal-vegetal axis as closely as possible (Fig. 7B, F, J). [In about 25% of the equatorial embryos, the orientation of second cleavage was skewed between AN and VG, so that the distribution of the four cells was tetrahedral rather than planar.] We refer to this new axis as the "micromere-macromere axis", because it was a predictor of the orientation of third cleavage. Thus, micromeres arose by a seemingly normal round of spiral cleavages at the end of the micromere-macromere axis lying nearest the animal pole (Fig. 7C,

G, K). We define this location as the "micromere pole". Even in equatorial embryos, where the two axes were nearly orthogonal to one another, all four micromeres arose at one junction of the first two cleavage furrows; none arose from the animal pole. From these observations we conclude that the orientation of the second cleavage plane is influenced by both the first cleavage plane and the position of the animal-vegetal axis. But after the second cleavage, the orientations of subsequent cleavages are governed by the micromere-macromere axis rather than the animal-vegetal axis.

A second set of observations from compressed embryos suggested that teloplasm contains factors which affect cell cycle duration. In normal development, cell CD cleaves about 15 minutes before cell AB (Bissen and Weisblat, 1989); however, in oblique and equatorial embryos cells AN and VG often cleaved synchronously. In about half of the oblique and equatorial embryos, this cleavage was slightly unequal in both cells AN and VG, so that the two pools of teloplasm were segregated to two of the four cells. The two cells with teloplasm lay adjacent to one another in approximately half of the cases, and opposite each other in the other half. In other oblique and equatorial embryos, AN or VG divided equally, so that three or four cells inherited teloplasm. We examined the order of micromere production in 17 oblique embryos and found that it correlated with the inheritance of teloplasm. Normally, the first quartet of micromeres arises in a stereotyped sequence: cell d′ first, followed by cell c′, and then cells a′ and b′ together (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). In oblique embryos, cells that inherited the most animal teloplasm at second cleavage always made the first micromere. They were followed by cells that inherited vegetal teloplasm, and lastly by cells that did not inherit any teloplasm. In cases where teloplasm was divided equally between two cells, these cells produced micromeres at the same time. Thus, it appears that cells inheriting teloplasm undergo shorter second and third cell cycles.

A third observation from embryos with re-oriented first cleavage planes concerns the migration of vegetal teloplasm, which in normal embryos results in the mixing of animal and vegetal teloplasms during the fourth cell cycle (Figs 1C and 7C; Holton et al. 1989). In oblique and equatorial embryos, we observed that vegetal teloplasm moved from the cortex to the cell interior between the second and fourth rounds of cleavage and appeared to migrate toward the animal pole despite the fact that the animal and vegetal teloplasms were located in different cells (Fig. 7 G and K). This suggests that the migration of vegetal teloplasm does not require a direct cytoplasmic link with the animal pole.

Finally, we observed that at fourth cleavage, macromeres which inherited more than about half of the animal or vegetal teloplasm underwent an oblique and equal division much like a normal D′ macromere, yielding one proteloblast near the micromere pole that by size and position resembled DNOPQ, and another proteloblast distal to the micromere pole that resembled DM (Figs 7 D, H, L and 8). Typically, two of the four macromeres underwent such a division, so that most oblique and equatorial embryos had two pairs of proteloblasts, one derived from cell AN and the other from cell VG. In some embryos, three of the four macromeres inherited substantial amounts of teloplasm, and consequently three pairs of proteloblasts were produced. This confirms the conclusion of Astrow et al. (1987) that teloplasm contains factors which cause macromeres to undergo cleavages characteristic of the D′ lineage.

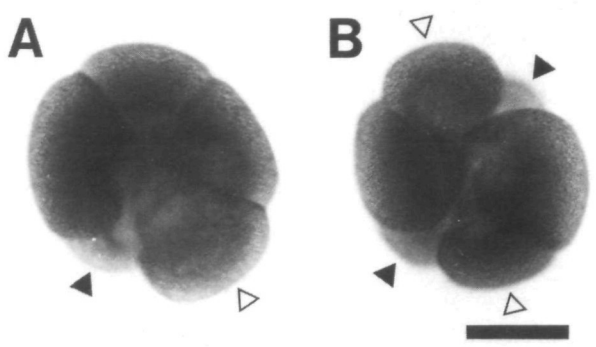


Fig. 8. Photomicrographs of living embryos after the fourth cleavage viewed from the micromere pole. (A) Normal uncompressed embryo. DNOPQ and DM are indicated by open and closed triangles, respectively. (B) Oblique embryo. Two pairs of proteloblasts are indicated by open and closed triangles. These embryos are equivalent to those diagrammed in Fig. 7 D and H. Scale bar, 150μm.

Discussion

Specification of ectoderm and mesoderm in leech

We have investigated the mechanism that specifies the fates of the ectodermal and mesodermal proteloblasts in *Helobdella* embryos. In normal development, the cleavage of blastomere D′ produces an ectodermal precursor, proteloblast DNOPQ, near the animal pole and a mesodermal precursor, proteloblast DM, near the vegetal pole. Cleavage arrest and cell ablation experiments suggest that cell interactions may not be involved in the determination of DNOPQ and DM cell fates. Instead, it appears that these fates are specified by factors localized along the animal-vegetal axis of the zygote. When the animal and vegetal hemispheres are separated by re-orientation of first cleavage, the ectodermal fate co-segregates with the animal hemisphere and the mesodermal fate with the vegetal hemisphere.

We performed two experiments to test the hypothesis that the fates of DNOPQ and DM are determined by signals from neighboring cells. First, we placed DNOPQ and DM in equivalent positions in the embryo so that they could, in principle, participate in the same cell interactions. Second, we prevented the formation of the normal complement of micromeres and macro-

meres by arresting the cleavage of their precursor cells. In both cases, DNOPQ and DM adopted their normal ectodermal and mesodermal identities. Because this is essentially a negative result, one cannot formally exclude the possibility that cell interactions are involved in the determination of DNOPQ and DM cell fates but were not revealed by these experiments. Nevertheless, these results lessen the likelihood that such interactions are involved and suggest that DNOPQ and DM adopt their respective fates in a cell-autonomous manner.

Our finding that ectodermal and mesodermal fates co-segregate with the animal and vegetal hemispheres after re-orientation of first cleavage suggests the involvement of localized determinants of cell fate. Although the two teloplasms are an obvious indication of animal-vegetal polarity in the zygote, several lines of evidence make it clear that ectodermal and mesodermal determinants are not exclusively localized to either pool of teloplasm. First, Holton et al. (1989) demonstrated that DNOPQ and DM each inherit a mixture of animal and vegetal teloplasm from cell D′. Second, we have shown that embryos with only animal teloplasm or only vegetal teloplasm nevertheless retain both ectodermal and mesodermal potential (Nelson and Weisblat, 1991). And third, in the present study we have shown that blastomeres that inherit only one pool of teloplasm as a result of an oblique first cleavage can still give rise to both ectodermal and mesodermal teloblasts.

Alternative sites for the localization of ectodermal or mesodermal determinants are in the animal and vegetal cortex and yolk, a notion that is consistent with the results of our experiments to reorient the first cleavage. When this cleavage is made equatorial, so as to separate not only the animal and vegetal teloplasm but also animal and vegetal yolk and cortex, the segregation of ectodermal and mesodermal fates is complete. In most cases the cell derived from the animal hemisphere, cell AN, gives rise to only ectoteloblasts, whereas the cell derived from the vegetal hemisphere, cell VG, produces only mesoteloblasts. However, when first cleavage is oblique, so as to separate the animal and vegetal teloplasms but not the corresponding yolk and cortical regions, there is only a partial segregation of fates. In most cases, cell AN produces both ectodermal and mesodermal teloblasts. Cell VG usually produces only mesoteloblasts, but in about 20% of embryos, generates ectoteloblasts as well.

On the basis of these results, one might postulate that ectodermal determinants are localized to the animal hemisphere, or mesodermal determinants to the vegetal hemisphere, or both. Previous studies involving extrusion of teloplasm (Nelson and Weisblat, 1991) allow us to distinguish between these alternatives. We found that the nominal cell DNOPQ is competent to produce either ectoderm or mesoderm and that it makes a decision between the two fates on the basis of the position of teloplasm relative to the animal pole. Thus, it is unlikely that mesodermal determinants are exclusively localized to the vegetal hemisphere, otherwise DNOPQ could not exhibit dual competence. In contrast to DNOPQ, cell DM seems competent to produce only mesoderm regardless of the position of teloplasm. Moreover, since the fate of cell DNOPQ seems to depend on the proximity between teloplasm and animal pole, it seems likely that the postulated determinants are in the cortex rather than in the yolk.

We propose, therefore, that ectodermal determinants are localized to the animal cortex of the zygote and extend almost as far as the equator, as shown in Fig. 9. These are inherited by all cells during the first three rounds of cleavage, but are only active in the D lineage where teloplasm is present. The cortical determinants segregate to DNOPQ and not DM at fourth cleavage. To account for the positional effect of teloplasm on the fate of DNOPQ (Nelson and Weisblat, 1991), we propose that the cortical determinants require contact with factors in teloplasm to induce the ectodermal fate. In the absence of such an interaction, as after removal of teloplasm from the animal pole, the mesodermal fate is adopted by default. As for DM, we propose that it adopts a mesodermal identity because it inherits teloplasm but no cortical determinants.

Our model can also account for the development of oblique and equatorial embryos, as shown in Fig. 9. The segregation of ectodermal and mesodermal fates is complete after an equatorial first cleavage because cell AN inherits only animal cortex that is in contact with teloplasm, as do its descendent proteloblasts. Thus, these cells are determined to produce ectoderm. In these embryos, cell VG fails to inherit any animal cortex, and as a result, its descendents adopt a mesodermal fate. With an oblique first cleavage, both cells AN and VG inherit substantial amounts of animal cortex as well as a complete pool of teloplasm. They each give rise to proteloblasts that by size and position resemble DNOPQ and DM. Proteloblasts derived from the animal portion of cell AN inherit animal cortex that is in contact with teloplasm and hence adopt an ectodermal fate. Proteloblasts derived from the animal portion of cell VG also inherit animal cortex, but their inheritance of teloplasm, and the extent of the contact between their teloplasm and the cortical determinants is variable. Thus, depending on the precise distribution of teloplasm in such cells, they can generate either mesoteloblasts, ectoteloblasts, or no teloblasts at all. Proteloblasts derived from the vegetal portions of either cell AN or cell VG in oblique embryos inherit teloplasm with little or no animal cortex and therefore adopt a mesodermal identity.

According to our model for the determination of ectodermal and mesodermal fates in leech, the orientations of early cleavages are critical to ensure that animal cortex is partitioned to DNOPQ and teloplasm to both DNOPQ and DM. We have shown here that the first two cleavage planes determine the orientations of subsequent cleavages, regardless of the location of the animal-vegetal axis, and that the zygote is radially symmetric about the animal-vegetal axis with respect to developmental potential. Thus, a principle requirement for the correct partitioning of cortex and cytoplasm to DNOPQ and DM is that the first cleavage plane fall parallel to the animal-vegetal axis, the precise location

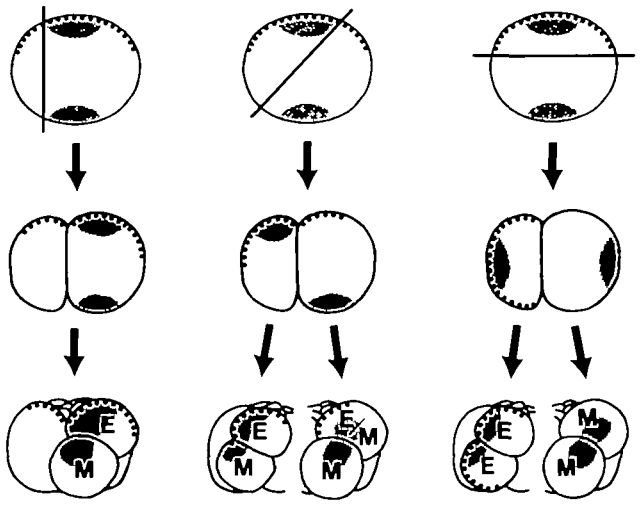


Fig. 9. Model for the determination of ectoderm and mesoderm in leech. Teloplasm is represented by shaded regions. The animal pole is up in the top row, and the prospective micromere pole is up in the middle and bottom rows *Left* Normal development. Ectodermal determinants (small black circles) are localized to the cortex in the animal hemisphere of the zygote and segregate to DNOPQ. These interact with teloplasm to induce the ectodermal fate (E). DM inherits teloplasm along with vegetal cortex, which is devoid of ectodermal determinants, and consequently adopts a mesodermal fate (M). Cells that inherit the ectodermal determinant without teloplasm do not form teloblasts. *Center* An oblique first cleavage separates the two teloplasms and bisects both the animal and vegetal cortices. Thus, both cells at the 2-cell stage have the components necessary for ectodermal and mesodermal fate determination. The cell that inherits the animal teloplasm (AN) always gives rise to a DNOPQ-like ectodermal proteloblast near the animal pole where contact between teloplasm and animal cortex is assured. It also produces a DM-like mesodermal proteloblast near the vegetal pole. The cell that inherits the vegetal teloplasm (VG) also produces a proteloblast near the animal pole; however, the fate of this cell is variable. Depending on the amount of teloplasm it inherits, and on whether or not teloplasm contacts the cortical determinants, it can produce ectoteloblasts, mesoteloblasts or no teloblasts (the production of mesoteloblasts by this cell was not observed by direct injection of lineage tracer but is inferred from the fact that cell VG often gave rise to four mesoteloblasts) VG also gives rise to a DM-like mesodermal proteloblast near the vegetal pole. *Right* An equatorial first cleavage fully separates not only the animal and vegetal teloplasms but also animal and vegetal cortex. Thus, the two cells produced contain components for either ectodermal or mesodermal fate determination, but not both. Proteloblasts derived from cell AN both inherit teloplasm in contact with animal cortex and, consequently, adopt an ectodermal identity Proteloblasts derived from cell VG inherit teloplasm without animal cortex and therefore adopt a mesodermal identity by default.

not being important. Our experience with re-orientation of first cleavage suggested the presence of a robust mechanism in the zygote that ensures that this requirement is met. With our methods, equatorial cleavages were much less common than normal, polar or oblique cleavages. This was because zygotes that were elongated on the animal-vegetal axis often cleaved at slightly oblique orientations relative to the axis of elongation, or delayed cleavage until being released from compression and then cleaved at a normal orientation. Similar results are obtained when *Eisenia* zygotes are compressed to re-orient first cleavage (Devries, 1973). Unlike normal, polar or oblique cleavage orientations, equatorial cleavages require that the spindle asters abut the animal and vegetal pools of teloplasm. It may be that teloplasm is a less permissive medium for the assembly or attachment of spindle asters compared to yolky cytoplasm. Whatever the underlying reason, the tendency for the mitotic spindle to form at any orientation other than parallel to the animal-vegetal axis ensures the correct placement of the first cleavage plane. This in turn governs the orientations of subsequent cleavages, with the result that teloplasm and animal cortex are correctly partitioned to DNOPQ and DM.

Comparison with other spiralians

Previous work on a variety of molluscan and annelid embryos has led to a general model for spiralian development in which morphogenetic determinants are localized to the vegetal cortex of the D quadrant (reviewed by Davidson, 1986). The model presented here for the determination of mesoderm and ectoderm in leech is contrary to this general scheme in that we postulate determinants localized to the animal rather than the vegetal cortex. Ours are not the first results contradictory to the general spiralian model, however. In experiments similar to those reported here, Devries (1973) used compression to re-orient the first cleavage in embryos of the oligochaete *Eisenia* and reported that, after a perfectly equatorial first cleavage, both daughter blastomeres are competent to produce ectodermal and mesodermal structures. Unfortunately, one cannot be sure of the origins of these structures because cell lineages were only inferred, rather than determined directly with lineage tracers. In another set of experiments, Devries ablated one of the daughter cells after first cleavage of compressed embryos, and again concluded that the two blastomeres were equipotent. But it appears (panels 4A and 4B in Fig. 1 of Devries, 1973) that the embryos included in this second set of experiments had undergone oblique cleavages, in which case we would expect, on the basis of our results with *Helbodella*, that both mesodermal and ectodermal structures might arise from either blastomere. Thus, it is possible that the results for *Eisenia* and *Helobdella* are the same, and that the determination of mesoderm and ectoderm in oligochaete and leech proceed by much the same mechanism.

Irrespective of how one interprets Devries' data, the experimental results from *Helobdella* and *Eisenia* contradict the notion that morphogenetic determinants are localized to the vegetal cortex of the D quadrant. It appears therefore that within the spiralian group there is more than one mechanism for localizing ectodermal

and mesodermal fates to the animal and vegetal hemispheres. Another example in which different mechanisms subserve homologous processes in annelid development has been observed in the cytoplasmic rearrangements leading to teloplasm formation in *Helobdella* and *Tubifex*. In *Tubifex*, teloplasm formation is sensitive to cytochalasin but not nocodazole, indicating that the process is microfilament-dependent in the oligochaete (Shimizu, 1982, 1984). But in *Helobdella*, by contrast, teloplasm formation is sensitive to nocodazole and tubulazole, but not to cytochalasin, suggesting that the process is microtubule-dependent in leech (Astrow et al. 1989).

Thus, we find cases in which evolution has operated to conserve embryological "ends" (e.g. assignment of ectodermal and mesodermal fates to the animal and vegetal progeny of the D quadrant, or the generation of animal and vegetal pools of teloplasm) despite the divergence of the mechanistic "means" by which these ends are achieved in different species. Presumably, this evolutionary divergence proceeds through species in which two developmental mechanisms operate in parallel with some degree of redundancy. It is worthwhile for students of comparative development to recognize that such outwardly conservative, mechanistically dynamic, evolutionary processes may operate in addition to the more commonly anticipated ones, in which outward divergence arises by small changes in relatively well conserved developmental mechanisms.

We are grateful to K Symes for devising the cell extrusion technique, J. Yost and D. Lans for advice about ricin injection, F. Wilt for suggesting the DiI labeling experiment, C. Smith for suggesting how to do it, and D. Stuart for supplying the antibody to leech nucleus. We thank D. Lans, C. Wedeen and C. Smith for critically reading this manuscript This work was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council, Ottawa, Ontario, Canada to B.H.N. and by NSF grant DCB 87-11262 to D A.W.

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(Accepted 5 February 1992)