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Animal and Vegetal Teloplasms Mix in the Early Embryo of the Leech, *Helobdella triserialis*

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In embryos of the glossiphoniid leech, *Helobdella triserialis*, as in many annelids, cytoplasmic reorganization prior to first cleavage generates distinct animal and vegetal domains of yolk-deficient cytoplasm, called *teloplasm*. Both domains are sequestered to the D′ macromere, progenitor of the definitive segmental tissues, during the first three rounds of cell division. And it has been believed that during the fourth round of cell division, the obliquely equatorial cleavage of macromere D′ cleanly segregates animal teloplasm into an ectodermal precursor, cell DNOPQ, and vegetal teloplasm into a mesodermal precursor, cell DM. But here we report a hitherto unobserved cytoplasmic rearrangement between the second and the fourth divisions that seems to mix the animal and vegetal domains of teloplasm. The newly observed rearrangement consists of the movement of vegetal teloplasm toward the animal pole of cell D′ between the second and the fourth cell divisions. Animal and vegetal teloplasms form a single pool of teloplasm in cell D′ which is then divided between DM and DNOPQ at the fourth division. The movement of teloplasm was inferred by examination of embryos fixed and sectioned between the second and the fourth rounds of cleavage and was confirmed in living embryos microinjected with rhodamine 123, a fluorescent mitochondrial stain. © 1989 Academic Press, Inc.

INTRODUCTION

Here we report a previously undescribed rearrangement of animal and vegetal domains of yolk-deficient cytoplasm, called *teloplasm*, in embryos of *Helobdella triserialis*, a glossiphoniid leech. The significance of this work is in its implications for the way we think about cytoplasmic determinants in annelid embryos. Leech eggs are fertilized internally but arrest in meiosis until after they are laid. During the first cell cycle, after formation of the second polar body, two yolk-deficient domains of cytoplasm, called teloplasm, form as latitudinal rings at the surface of the zygote in the animal and vegetal hemispheres. These rings then migrate toward their respective poles and close, so that by the end of the first cell cycle there is a pool of teloplasm at each pole. The two pools of teloplasm persist for some time; they are inherited by cell CD at the first cleavage and by cell D at the second cleavage. At third cleavage, each of the four cells (A, B, C, D) divides highly asymmetrically to produce a micromere (a′–d′), from its animal end, and a macromere (A′–D′). At the fourth cleavage, cell D′ divides along an obliquely equatorial plane into a mesodermal precursor, proteloblast DM (the vegetal daughter of D′) and an ectodermal precursor, proteloblast DNOPQ (the animal daughter of D′). Cells DM and DNOPQ cleave further to make *teloblasts*, the embryonic stem cells whose iterated progeny are segmental founder cells (e.g., see Weisblat and Shankland, 1985) along with additional micromeres (Sandig and Dohle, 1988; Bissen and Weisblat, in preparation). We, and others, have assumed that DM inherits the vegetal teloplasm and DNOPQ inherits the animal teloplasm (for example, see Fernandez and Olea, 1982), consistent with the model that there are distinct mesodermal and ectodermal determinants localized in the vegetal and animal teloplasm, respectively. The present work indicates that a movement of vegetal teloplasm toward the animal pole occurs, beginning shortly before the third round of cleavage and resulting in mixing of cytoplasmic constituents between animal and vegetal pools of teloplasm, and then the mixed materials are split into cells DM and DNOPQ at the next division. This finding argues against a model in which distinct determinants of mesodermal and ectodermal fate are localized in the vegetal and animal teloplasms, respectively, and then segregate from each other during the fourth cleavage.

MATERIALS AND METHODS

Embryos

Embryos of the glossiphoniid leech *H. triserialis* were obtained from a laboratory breeding colony (Weisblat *et al.*, 1980) and cultured in HL media (Blair and Weisblat, 1984). The staging system and nomenclature used is that of Fernandez (1980) as amended (Stent *et al.*, 1982; Weisblat and Blair, 1984). In brief, the stages relevant for this paper are defined as follows (Fig. 1): stage 1 (one-cell embryo) begins with egg deposition and ends with the onset of first cleavage; stage 2 (two-

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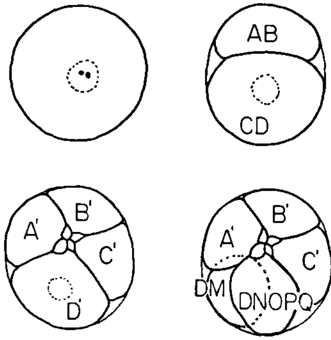

FIG. 1. Diagrammatic views, from the animal pole, of *Helobdella* embryos at stages relevant for this paper, including the following: stage 1, top left, with polar bodies indicated as small circles and animal teloplasm as the dotted circle; stage two, top right, with animal teloplasm again shown as a dotted circle; stage 4a, bottom left, with teloplasm as a dotted circle and micromeres a'-d' as smaller cells at the animal pole; and stage 4b, bottom right, with DNOPQ closer to the animal pole, cell DM partially hidden and represented by dotted lines, and teloplasm not shown.

cell embryo) begins at the onset of first cleavage and ends at onset of second cleavage; stage 3 (four-cell embryo) begins at the onset of second cleavage and ends at the onset of third cleavage; stage 4a (including the eight-cell embryo) begins at the onset of third cleavage and ends at the onset of fourth cleavage; stage 4b begins at the onset of fourth cleavage, when cell D' cleaves to form cells DM and DNOPQ, and ends at the onset of the cleavage of DM" to form the left and right M teloblasts. The timing of developmental events is given relative to deposition of the fertilized zygote at 23°C.

Examination of Embryos between Second and Fourth Cleavage (Stages 3-4b)

Embryos were fixed at various times between the end of the second round of cleavage and the point at which they were entering the fourth round. The selected, staged embryos were fixed, embedded in glycol methacrylate, sectioned (4 μm thickness), and stained with toluidine blue as described previously (Astrow *et al.*, 1987), then examined by standard bright-field microscopy.

Mitochondrial Labeling

Rhodamine 123 (Sigma; saturated solution in 0.2 *N* KCl), a vital stain for mitochondria (Johnson *et al.*, 1980), was pressure-injected into living embryos using standard techniques (Weisblat *et al.*, 1980). The distribution of rhodamine 123 was examined by fluorescence microscopy, using Zeiss filter set 48-77-09.

To follow cytoplasmic rearrangements occurring after teloplasm formation, embryos late in stage 1 were injected at either the animal or the vegetal pole with small volumes of rhodamine 123 (less than 10% of the cell volume). Thus, the dye was sequestered before it could diffuse from the vicinity of the injection site and only mitochondria in the teloplasm at the injected pole were labeled. In less than 20% of embryos, the fluorescence was not clearly localized; these embryos were excluded from further study. Unrestrained *Helobdella* embryos generally assume an orientation such that the animal/vegetal axis is parallel to the gravitational axis, making it difficult to observe cytoplasmic movements along the axis. To observe animal and vegetal poles simultaneously, embryos were immobilized on their sides in the channel formed by two coverslip fragments glued parallel to one another on the bottom of a plastic petri dish. The level of the medium was then lowered to the point at which surface tension held the embryos in place. Alternatively, we found that the vitelline membrane of the embryo will adhere to the surface of a fresh plastic petri dish with sufficient force to hold the embryo in any orientation. The embryos were returned to normal culture conditions until stage 4b. At stage 4b (after macromere D' had cleaved to form cells DM and DNOPQ), similarly injected embryos were placed in a solution of 50% propylene glycol (in HL medium). This solution renders cells resistant to mechanical lysis, so that cells DM and DNOPQ could be dissected away from the embryo. Propylene glycol treatment resulted in diffusion of rhodamine 123 from labeled cells after about 20 min; therefore, dissected cells were examined for fluorescent labeling within 5-15 min of treatment with proplyene glycol. Dissected cells were viewed through an image intensifier and photographed from a video monitor as previously described (Astrow *et al.*, 1987).

RESULTS

Teloplasm Movement Inferred from Examination of Sectioned Embryos

The formation of animal and vegetal pools of teloplasm was complete by about 4 hr after egg laying. At the first cleavage both pools were segregated into cell CD and at second cleavage into cell D, still remaining distinct from one another (Fig. 2a). In sections of embryos fixed at various times between second and fourth cleavage, however, changes occurred in the shape and position of the vegetal teloplasm, indicative of movement of the vegetal teloplasm to the animal pole within cell D and D'. Initially, this movement was evident as an extension of a thick strand of cytoplasm from the vegetal pool of teloplasm toward the animal pole (Fig. 2b).

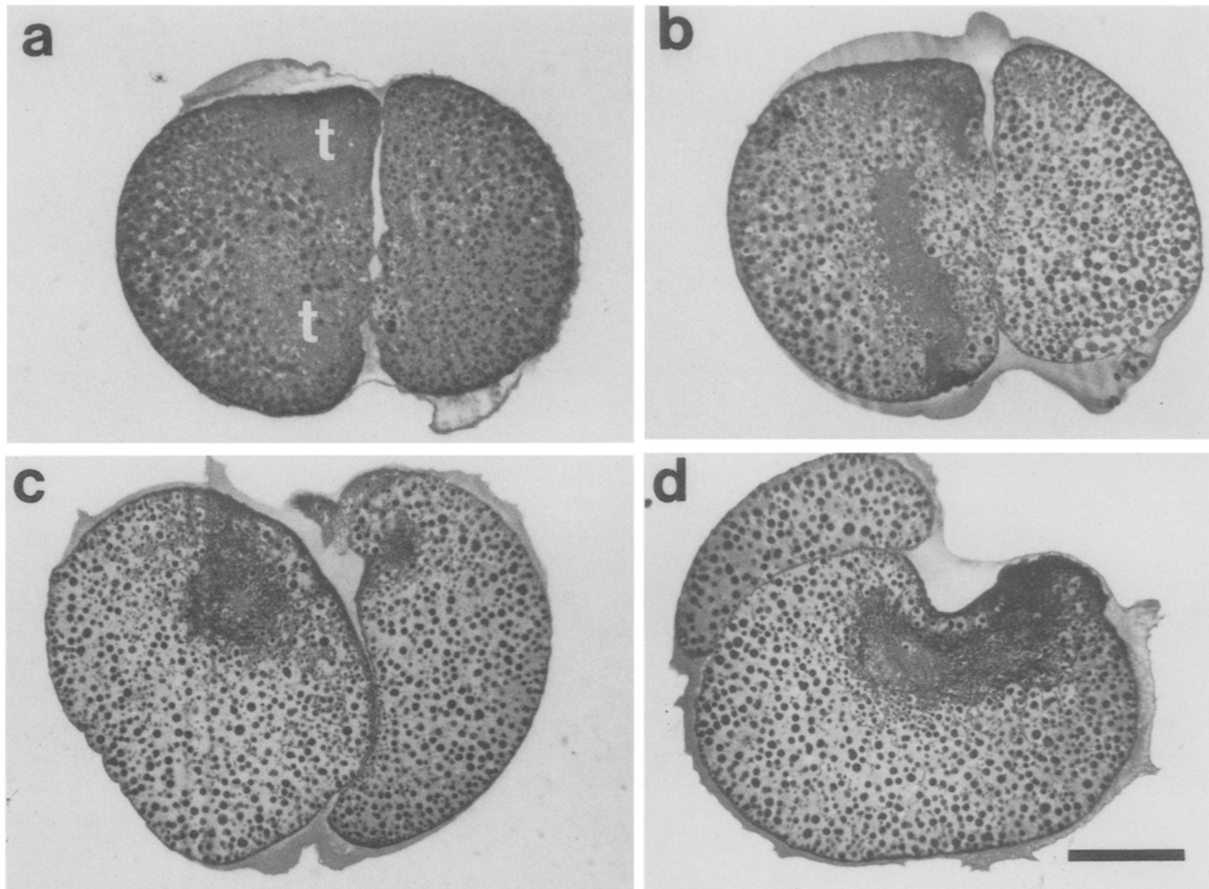

FIG. 2. Translocation of vegetal teloplasm after second cleavage. Photomicrographs of toluidine blue-stained sections through *Helobdella* embryos fixed at progressively later times between the four-cell stage and the cleavage of macromere D′ to cells DM and DNOPQ. (a) In the early four-cell stage, both animal and vegetal teloplasm (t) are well separated in cell D (left). (b) About 45 min later, shortly before formation of the first micromere from cell D (left), the vegetal teloplasm has elongated toward the animal pole, though a remnant remains close to the vegetal cortex. (c) By the time all four micromeres have arisen, less than 90 min later, only a single pool of teloplasm is evident, located at the animal pole of the D′ macromere (left). (d) As macromere D′ (bottom) cleaves, 15–30 min later, the merged teloplasms are once again divided, so that both DM and DNOPQ receive some. Animal pole is up in all panels; scale bar, 50 μm.

In embryos fixed slightly later (Fig. 2c), almost the entire pool of vegetal teloplasm was at the animal end of the cell, rather than at the vegetal end. And in embryos fixed with cell D′ in cytokinesis, the animal and most of the vegetal pool of teloplasm appeared to be fused at the animal end of the cell (Fig. 2d).

Thus, even though teloplasm was distributed into both cells DM and DNOPQ at this division (Fig. 2d), forming two separate pools again, the animal and vegetal pools had apparently fused during stages 3–4. Assuming that fusion would be accompanied by mixing of the components of the animal and vegetal teloplasms, this observation suggested that cells DM and DNOPQ each inherited teloplasm from both poles. But it remained possible that the animal and vegetal pools of cytoplasm assumed side by side positions without mixing and were resegregated as distinct entities by precise positioning of the fourth cleavage furrow.

Teloplasm Mixing Demonstrated by the Movements of Labeled Mitochondria

To test the hypothesis that the movement of vegetal teloplasm resulted in mixing the two pools of cytoplasm, we followed one component of teloplasm, mitochondria, in living embryos from the end of the first cell cycle through the cleavage of cell D′. Rhodamine 123 was injected into either the animal or the vegetal teloplasm at the end of the first cell cycle. Three hours later, by which time the embryos were at stage 3 (four cells), the dye had not diffused away from the pole into which it was injected (Figs. 3 and 4a). We take this to indicate that rhodamine 123 is rapidly and efficiently sequestered by mitochondria at the injection site. At early stage 3, injected embryos were separated into two groups, based on which pole was labeled, and then observed for movements of the labeled mitochondria.

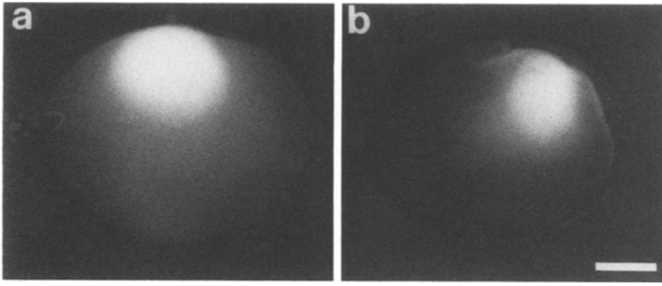

FIG. 3. Stability of polar rhodamine 123 labeling. Fluorescence photomicrographs of a live embryo into which rhodamine 123 was injected at the animal pole, just prior to first cleavage. (a) Immediately after the injection, the fluorescence is confined to the animal pole, presumably within the mitochondria of the animal teloplasm. (b) After about 5 hr, the embryo has progressed to the eight-cell stage, but the label is still confined to the animal pole of macromere D′; the vegetal pole of D′ is in view at the bottom of the embryo, but unlabeled. Scale bar, 100 μm.

The fact that the mitochondrial label was still confined to the injected pole in early stage 3 embryos (Fig. 4a) demonstrated that leakage or movement of rhodamine 123 from mitochondria is negligible over intervals as long as 3 hr. And in embryos observed 50 min later, shortly before the micromere d′ was produced, fluorescently labeled animal teloplasm was still confined to the animal pole. But in embryos whose vegetal pole had been injected with rhodamine 123, labeled teloplasm had moved toward the animal pole by this time (Fig. 4b). By the time cell D′ began to cleave to DM and DNOPQ, fluorescently labeled teloplasm that was originally located at the vegetal pole was located at the animal pole (Fig. 4c). We are able to rule out diffusion as the basis of this movement for two reasons. First, as noted above, embryos in which the animal teloplasm was labeled exhibited no change in position of the fluorescent material (Fig. 3b). And second, during the movement, the zone of *maximum* fluorescence moved toward the animal pole (Figs. 2a–2d), whereas any process based solely on diffusion would entail the point of maximum fluorescence remaining stationary. In the embryo shown in Fig. 4, note that part of the fluorescence failed to migrate, remaining instead at the vegetal cortex. This was seen in most embryos examined and correlated with a thickening of the vegetal cortex seen in sectioned embryos at this stage (Fig. 2b).

After the cleavage of cell D′, both daughter cells, DM and DNOPQ, appeared fluorescent, independent of which pole had been labeled. This indicated that both cells inherited mitochondria from both poles (Fig. 4d). To be sure that this appearance was not an artifact of light scattering by yolk platelets, embryos were dis-

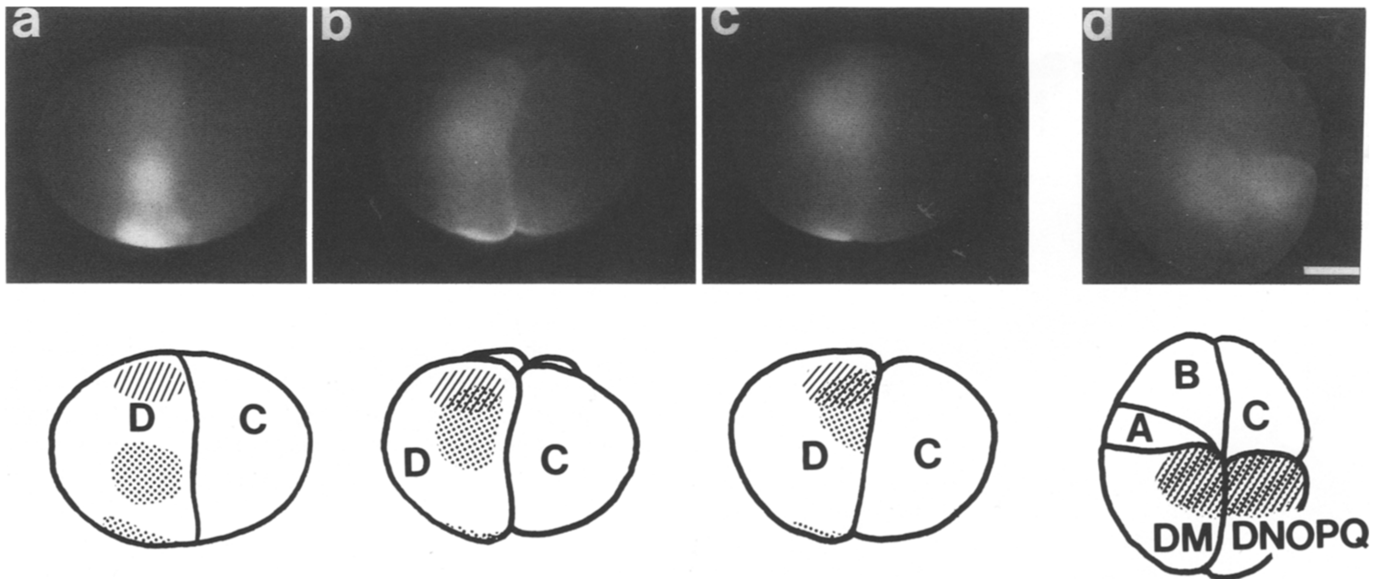

FIG. 4. Translocation of teloplasm as demonstrated by rhodamine 123 fluorescence. Fluorescence photomicrographs of a live embryo into which rhodamine 123 was injected at the vegetal pole just prior to first cleavage. Beneath each photograph is a line drawing showing cell outlines, with mitochondrial fluorescence in originally vegetal teloplasm indicated by stippling and the approximate location of animal teloplasm (not visible in photographs) by diagonal lines. (a) In the newly cleaved four-cell embryo, fluorescence is still confined to the vegetal half of cell D. (b) Shortly before formation of the first micromeres from cell D, a substantial portion of the fluorescence has shifted toward the animal pole, though some remains close to the vegetal membrane. (c) By the eight-cell stage, the bulk of the fluorescence is at the animal pole (micromeres not visible). (d) After the cleavage of macromere D′, both cells DM and DNOPQ contain fluorescence. In (a–c), the embryo is viewed from the side; in (d), it is viewed from the animal pole. Macromeres are indicated by capital letters on the line drawings, except for macromeres A and B, partially in view at the top of (b). Scale bar, 100 μm.

sected into their component blastomeres, after stabilizing them with propylene glycol treatment, and rhodamine 123 fluorescence was observed in the separated blastomeres (Fig. 5). We found that both cells, DM and DNOPQ, exhibited fluorescent label, independent of which pole had been injected initially, while the macromeres (A″–C″) remained unlabeled.

DISCUSSION

Yolk-free pools of cytoplasm called teloplasm confer a particular developmental potential, that of cleaving to generate teloblasts, to recipient cells in embryos of the leech *H. triserialis* (Astrow *et al.*, 1987). During the first two cell divisions, the animal and vegetal pools of teloplasm remain separate as they are segregated first into cell CD and then into cell D. As have others, we previously assumed that animal and vegetal teloplasms remained separate through micromere formation as well, and that when cell D′ cleaved to form a mesodermal precursor, DM, and an ectodermal precursor, DNOPQ, the vegetal pole teloplasm was inherited by DM and the animal pole teloplasm by DNOPQ. Accordingly, a reasonable hypothesis was that animal teloplasm contained ectodermal determinants and that vegetal teloplasm contained mesodermal determinants. Specialized cytoplasms are known in other organisms, such as ascidians, whose embryos exhibit distinct ectodermal, endodermal, and muscle-specific cytoplasms assumed to contain various tissue-specific cytoplasmic determinants (Whittaker, 1979).

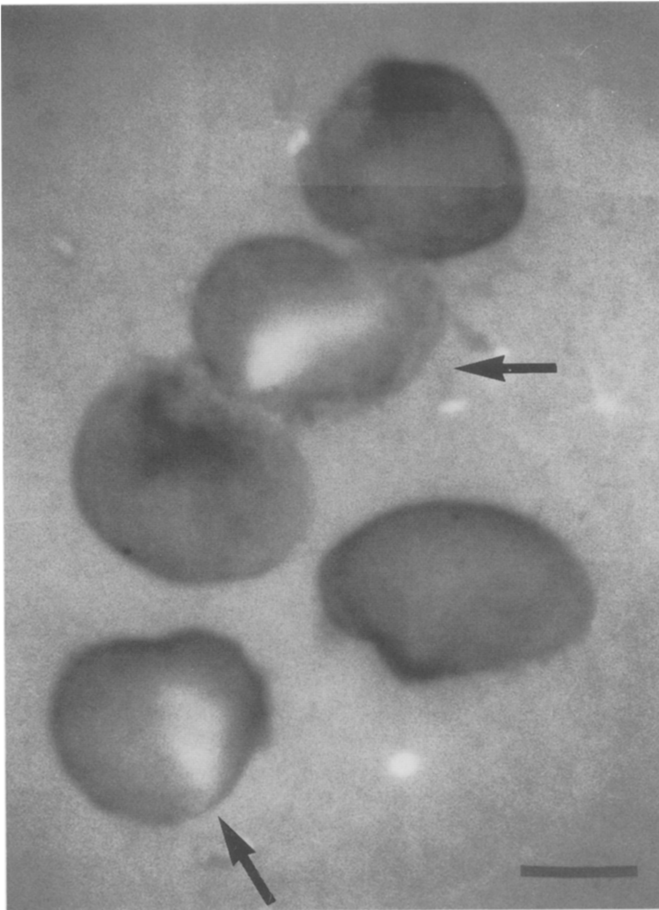

FIG. 5. Animal and vegetal teloplasms mix by stage 4b. Embryos were injected with rhodamine 123 at either pole and allowed to develop until stage 4b. Then the five large blastomeres of individual embryos were separated from one another in 50% propylene glycol and viewed by fluorescence microscopy, using an image intensifier and a video camera (see Materials and Methods). The cells of one such embryo are shown. Labeled mitochondria are evident in both cells DM and DNOPQ (arrows). Scale bar, 100 μm.

In examining sectioned *Helobdella* embryos, however, we found that the vegetal pool of teloplasm migrates toward the animal pole after second cleavage and seems to become coextensive with the animal pool. Moreover, when mitochondria in one pool of teloplasm were labeled with rhodamine 123, we observed that both cells DM and DNOPQ inherited fluorescent label, suggesting that the two pools of teloplasm mix during this period. Thus, cells DM and DNOPQ normally receive mitochondria, and therefore possibly other cytoplasmic components as well, from *both* pools. The formation of yolk-deficient cytoplasmic domains appears to be similar in various annelids, including other glossiphoniid leeches (Whitman, 1878; Schleip, 1914; Fernandez, 1980; Fernandez and Olea, 1982; Fernandez *et al.*, 1987) and in a far more distantly related species, the oligochaete *Tubifex hattai* (Shimizu, 1982, 1984, 1986). Thus, we suspect that this apparent mixing of animal and vegetal teloplasms may be a general phenomenon in annelid development. And Shimizu (in preparation) has observed similar movements of vegetal pole plasm in *Tubifex.*

The observation that cells DM and DNOPQ both inherit mitochondria from both poles does not eliminate the possibility that distinct ectodermal and mesodermal determinants are localized within the animal and vegetal teloplasms. Such determinants might simply be segregated much more precisely than are the mitochondria during cleavage of the D′ macromere. Another possibility is that, by stages 3–4, when mixing occurs, mesodermal and ectodermal determinants are confined to regions of cytoplasm that do not participate in the mixing. Indeed, the cortex overlying the vegetal pole often remained thicker even after the vegetal teloplasm had migrated away from this region, as if not all the teloplasm had migrated, or as if some component of the teloplasm had remained at the vegetal pole. During cleavage of macromere D′, this vegetal cortex is inherited by cell DM.

These possibilities notwithstanding, the simplest interpretation of our observations of mixing by animal

and vegetal teloplasms between the second and the fourth cleavages in *Helobdella* embryos is counter to the classical notion that these cytoplasmic domains contain distinct ectodermal and mesodermal determinants. Yet even so, the two teloplasms need not be equivalent. For example, each pool of teloplasm might contain a unique factor that is necessary but not sufficient to initiate the cleavages that generate the teloblasts. According to this model, mixing the animal and vegetal teloplasms would be required to activate the cleavage program, in the same sense that one must mix part A and part B to prepare epoxy glue. This type of cytoplasmic mixing has been proposed as the mechanism by which cortical rotation establishes the dorsal axis in an amphibian, *Xenopus laevis* (Gerhart *et al.*, 1983). On the other hand, the idea that the animal and vegetal teloplasms in annelids *are* truly equipotential is supported by work on the oligochaete, *Eisenia* (Devries, 1973). As in the leech, pole plasms form and are normally inherited by one cell (CD) at the first cleavage. But when embryos are compressed, reorienting the first cleavage such that each nascent cell (AB and CD) receives *either* the animal *or* the vegetal pole plasm, both cells cleave in a pattern similar to that of a normal CD blastomere, and normal embryonic structures are produced in duplicate. This result suggests that teloplasm mixing is no more essential for normal development than is the maintenance of separate animal and vegetal teloplasm.

Our results and others, therefore, impose constraints on the classical notion that distinct ectodermal and mesodermal determinants are localized within the animal and vegetal teloplasms, respectively. Moreover, they are consistent with the counterhypothesis that these teloplasms are functionally equivalent. In that case, we must prepare to examine new hypotheses regarding the mechanism(s) by which ectodermal and mesodermal progenitor blastomeres become distinct in the leech embryo. A priori, the difference between ectodermal and mesodermal progenitors could be ascribed to quantitative differences in the amount of teloplasm they inherit. But the results of experiments in which teloplasm is redistributed by centrifugation are not consistent with this notion. In such experiments, supernumerary teloblasts are produced, indicating that the amount of teloplasm is not limiting the numbers of teloblasts produced, and yet the vegetal daughters of the C′ and D′ macromeres (cells CM and DM) never produce more than two (M) teloblasts each (Astrow *et al.*, 1987). This leads to a final class of explanation, that the distinction between ectodermal and mesodermal progenitors is imposed by influences not contained in teloplasm. For example, the presence or absence of interactions with the micromeres at the animal pole is one such possibility. Another is that "generic" teloplasm interacts with an animal/vegetal gradient of some substance(s) laid down in the cortical cytoplasm during oogenesis. Interactions with micromeres have been considered important in the development of sea urchin embryos (for a recent review, see Wilt, 1987) and especially in equal cleaving molluscs (van den Biggelaar and Guerrier, 1979) where contact with primary quartet micromeres is the signal that causes one of the four equipotent macromeres to become the mesentoblast. Micromere derivatives have also been demonstrated to influence on cell fates later in *Helobdella* development (Ho and Weisblat, 1987). And as alluded to earlier, cortical factors are thought to be one factor in establishing the dorsal axis of *Xenopus* (Gerhart *et al.*, 1983).

At present then, available evidence leads us to favor a two-step model for the determination of ectodermal and mesodermal progenitors in *Helobdella*. In the first step, the presence or absence of teloplasm determines whether a cell becomes a teloblast precursor (proteloblast) or a macromere. In the second step, factors *external* to the teloplasm determine whether a given proteloblast becomes a mesodermal or an ectodermal precursor.

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