

Centrifugation Redistributes Factors Determining Cleavage Patterns in Leech Embryos

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In the normal development of glossiphoniid leech embryos, cytoplasmic reorganization prior to the first cleavage generates visibly distinct domains of yolk-deficient cytoplasm, called *teloplasm*. During an ensuing series of stereotyped and unequal cell divisions, teloplasm is segregated primarily into cell CD of the two-cell stage and then into cell D of the four-cell and eight-cell stages. The subsequent fate of cell D is also unique in that it alone undergoes further cleavages which generate five bilateral pairs of embryonic stem cells, the mesodermal (M) and ectodermal (N, O/P, O/P, and Q) *teloblasts*. Here we report studies on the effects of centrifugation on cleavage pattern and protein composition of individual blastomeres of the leech *Helobdella triserialis*. Centrifugation partially stratifies the cytoplasm of each cell, generating a layer of clear cytoplasm in cell CD derived largely from teloplasm. After centrifuging embryos at the two-cell stage, clear cytoplasm present in cell CD and normally inherited by cell D is redistributed and can be inherited by both cells C and D at the second cleavage. The developmental fates of cells C and D in centrifuged embryos correlate with the amount of clear cytoplasm they receive. In particular, when clear cytoplasm has been distributed roughly equally between the two cells, both cell C and cell D undergo further cleavages resembling the pattern of divisions normally associated with cell D. Likewise, non-yolk-associated proteins, normally found in higher quantities in cell D than in cell C, appear evenly disbursed between the two cells under conditions which induce this fate change. These results are consistent with the idea that the fates of cells C and D are influenced by the distribution or cellular localization of cytoplasmic components. © 1987 Academic Press, Inc.

INTRODUCTION

The experiments reported here were carried out to explore the hypothesis that localization and differential segregation of cytoplasmic factors are responsible for the differences in fate that distinguish one cell from the other three in the four-cell embryo of the leech, *Helobdella triserialis*. Differential segregation of cytoplasmic determinants is one possible mechanism for restricting a cell's developmental potential during ontogeny (Wilson, 1925; Freeman, 1979). The idea that such localizations help to establish distinct cell populations is supported by experiments in nematodes, insects, ascidians, amphibians, molluscs, and annelids (reviewed in Davidson, 1976). This notion is particularly appealing for such animals as ascidians, where one or more cytoplasmic regions of the egg can be distinguished (e.g., by unique pigmentation) and where the inheritance of these regions by particular blastomeres correlates well (Whittaker, 1979) if not precisely (Nishida and Satoh, 1983, 1985) with the fate of their progeny.

Visibly distinct regions of cytoplasm also occur in the early embryos of many annelids. These have been studied primarily in the polychaete *Chaetopterus* (Lillie, 1906; Morgan, 1927; Titlebaum, 1928; Guerrier, 1970), and the oligochaete *Tubifex* (Shimizu, 1982, 1984). *Chaetopterus* embryos manifest a morphological and cytoplasmic spe-

cialization, called the polar lobe, near the vegetal pole after fertilization. Alteration of the initial cleavage pattern by compression, or stratification of the egg contents by centrifugation, such that some polar lobe material enters both blastomeres after the first cleavage, can produce mirror-symmetrical double embryos. In *Tubifex*, yolk-poor cytoplasm accumulates at the polar regions of the precleavage egg and the cell that inherits it generates the mesodermal and ectodermal precursors (teloblasts). This reorganization is believed to involve networks of microfilaments located in the subcortical and cortical cytoplasm.

In several species of glossiphoniid leeches, visibly distinct domains of yolk-deficient cytoplasm develop in the fertilized egg and persist through the first four cell cycles (Whitman, 1878; Schleip, 1914; Fernandez, 1980; Fernandez and Olea, 1982). One such domain (perinuclear plasm) forms around the nucleus in the center of the egg and two others (teloplasm) form at the animal and vegetal poles. The structural changes that follow fertilization of the leech egg, including the migration and concentration of the teloplasm, have been well characterized by Fernandez (1980). The teloplasm appears to consist of a large number of organelles, particularly mitochondria, scattered throughout a granular matrix. In contrast, the rest of the egg cytoplasm contains many yolk platelets and few organelles. Schleip used centrif-

ugation to redistribute the polar materials in embryos of *Glossiphonia*, looking for possible determinative roles for these substances. He was able to separate the cytoplasm into three zones, a lipid layer over the centripetal end, a layer of clear cytoplasm (containing the polar material), and a yolky region. His experiments yielded no embryos that developed beyond the third cleavage (Morgan, 1927). We undertook to repeat such an investigation, however, in the hope of ultimately relating it to more recent work on the role of cell lineage and cell interactions in defining fates of the progeny of individually identified teloblasts in glossiphoniid leeches.

The general features of development of glossiphoniid leeches have been extensively described (Fernandez, 1980; Stent *et al.*, 1982) and only those aspects relevant to the present research are summarized here (Fig. 1). *Helobdella* eggs are fertilized internally but are under cleavage arrest until they are laid. They are approximately 500 μm in diameter and are uniformly pink and yolky, as viewed under a dissecting microscope. During the first cell cycle (0–4.5 hr), spherical domains of yolk-deficient cytoplasm, called teloplasm, develop near the animal and vegetal poles. At the first cleavage (4–5 hr), which is slightly unequal (Settle and Weisblat, in prep-

aration), these domains are inherited by the larger daughter cell and remain near the polar surfaces (Fig. 2a, b). This cell is called blastomere CD; the smaller cell is called AB. The cleavage of cell CD (6.5 hr) is also slightly unequal and the domains of teloplasm are inherited primarily by cell D (Fig. 3a). A short time later, cell AB cleaves equally, forming cells A and B.

The four-cell embryo undergoes a sequential round of highly unequal, spiral cell divisions (stage 4a), generating four small cells (micromeres a, b, c, and d) at the animal pole and four large cells (macromeres A, B, C, and D; 6.5–10.5 hr). Micromere production is followed by the obliquely equatorial cleavage of cell D (stage 4b) into a mesodermal precursor (DM) and an ectodermal precursor (DNOPQ). Between the second and fourth rounds of division further cytoplasmic reorganization takes place, such that when macromere D cleaves to form the proteloblasts DM and DNOPQ, little of the teloplasm is evident at the vegetal pole (B. Holton, unpublished). Teloplasm is still present at the animal pole, as well as around the nucleus. Thus when D cleaves, DNOPQ inherits more of the teloplasm than does DM. The A, B, and C macromeres make additional micromeres, but cleave no further. DM and DNOPQ, on the other hand, do cleave further (stages 5–6), giving rise to five bilateral pairs of stem cells called teloblasts, plus additional micromeres (Fernandez, 1980; Ho and Weisblat, in press). DM generates one pair of mesodermal precursors (M teloblasts) and DNOPQ gives rise to four pairs of ectodermal precursors (N, O/P, O/P, and Q teloblasts).

Each teloblast generates a coherent column (bandlet) of primary blast cells (stages 6–7). Ipsilateral bandlets come together in left and right germinal bands (stage 7), which eventually coalesce into the germinal plate (stage 8), from which the segmental tissues of the leech arise. Each bandlet gives rise to distinct, segmentally iterated sets of cells according to its teloblast of origin (Weisblat *et al.*, 1984; Kramer and Weisblat, 1985; Weisblat and Shankland, 1985) and, for O/P-derived bandlets, its relative position in the germinal band (Weisblat and Blair, 1984; Shankland and Weisblat, 1984). Even the timing and orientation of the mitoses of the primary blast cells are indicative of the teloblast of origin and the bandlet in which they lie (Zackson, 1984). For example, the first mitosis in all the ectodermal bandlets is *interstitial*, meaning that the bandlet remains one cell wide, whereas the first mitosis in the mesodermal bandlets is *lateral*, i.e., the bandlet becomes two cells wide. In addition to its definitive progeny, cell DM also generates progeny, called *larval integument cells*. These cells lie between the germinal bands of the stage 7 embryo, scattered beneath a provisional epithelium which covers the germinal bands and the spaces between them. These cells are prominent when the progeny of cell DM are

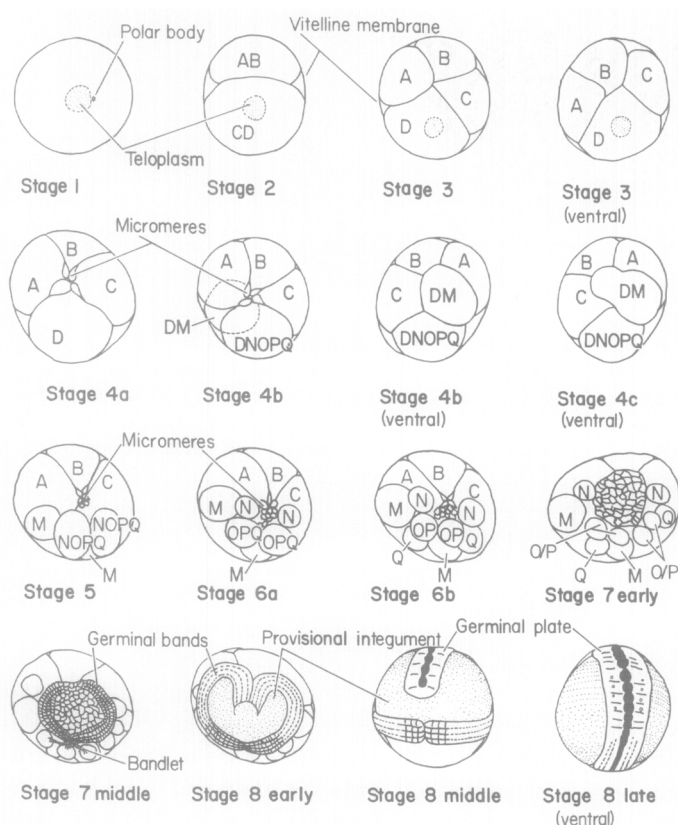


FIG. 1. Representations of *Helobdella* embryos at various stages of development. See text for description. Unless otherwise noted, each stage is viewed from the future dorsal side.

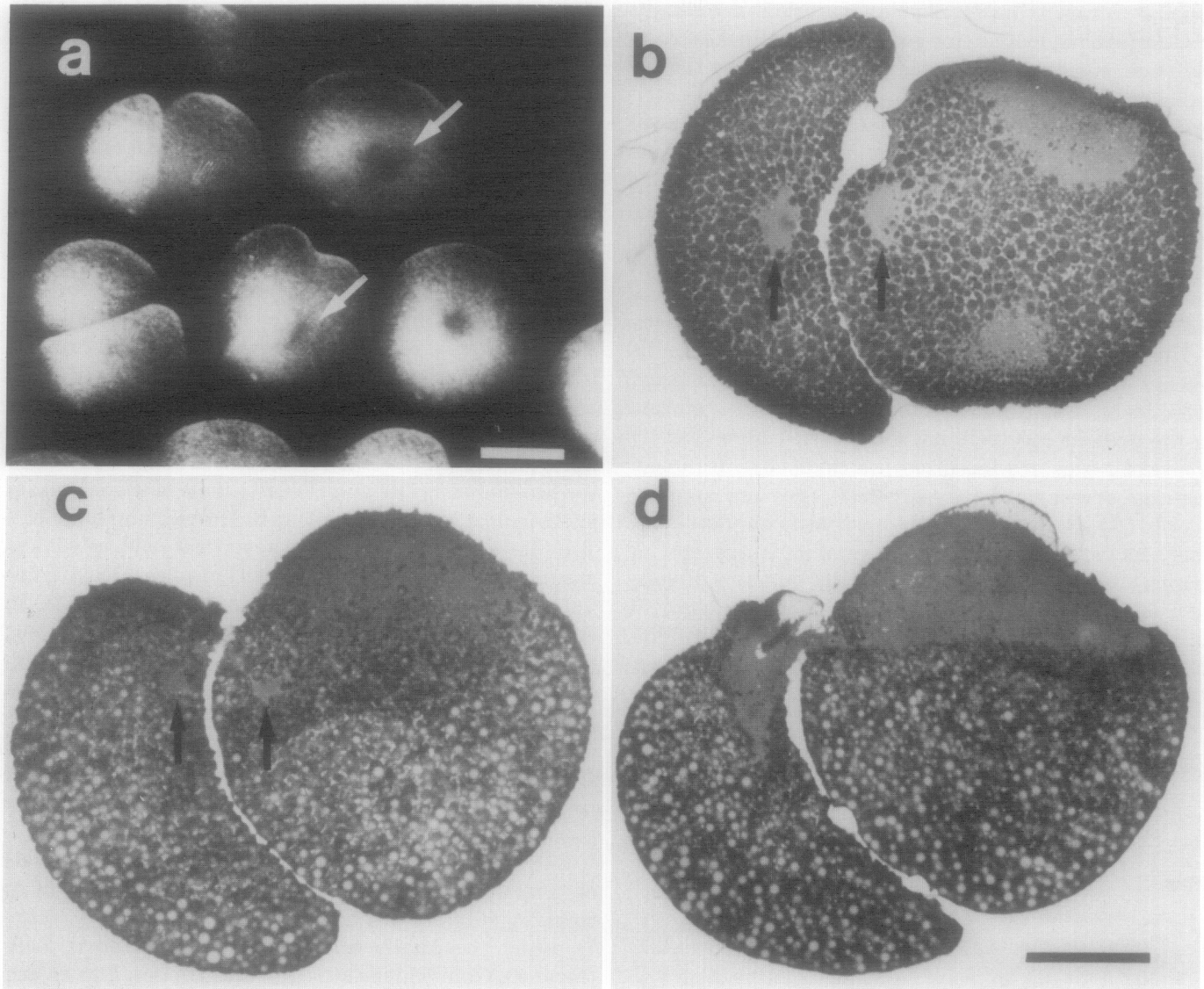


FIG. 2. Photomicrographs of one- and two-cell *Helobdella* embryos revealing the distribution of yolk-deficient cytoplasm under various conditions. (a) Live embryos at first cleavage. Photographed from above, these embryos have assumed their natural orientation with the animal pole pointing roughly out of the plane of the paper. Thus, the gravitational vector during centrifugation would be directed into the plane of the paper. Polar domains of yolk-deficient cytoplasm (teloplasm; upper arrow) are inherited by the larger cell (CD) at first cleavage (lower arrow). (b-d) Midsagittal sections of two-cell embryos stained with toluidine blue. Cell AB is to the left, cell CD to the right, and the animal pole is up in each panel. (b) Uncentrifuged embryo, showing animal and vegetal teloplasm in CD and perinuclear plasm (arrows) in both cells. (c) Embryo after low-speed centrifugation. Animal and vegetal teloplasm is now combined at the top of cell CD, but nuclei, as indicated by perinuclear plasm (arrows) are still separate. (d) Embryo after high-speed centrifugation, showing distinct layers of lipid, clear cytoplasm and yolk. Scale bar, 300 μm in (a); 100 μm in (b-d).

labeled with lineage tracers and have no counterparts in the DNOPQ-derived lineages. Thus, the presence of these cells, like the orientation of the initial mitoses and the number of teloblasts generated, serves to distinguish between the DM (mesodermal) and DNOPQ (ectodermal) cell lines.

To test for determinant substance(s) associated with the teloplasm, *Helobdella* embryos were partially stratified by centrifugation at the two-cell stage, shortly be-

fore the second cleavage. This resulted in an altered localization of the clear cytoplasm during the second cleavage. The rearrangement was apparent by inspection of living embryos under the dissecting microscope, and confirmed in sections of embryos fixed shortly after centrifugation. Developmental fates of centrifuged and control embryos were determined by direct observation and by microinjection of cell lineage tracers. To detect biochemical differences between the macromeres that

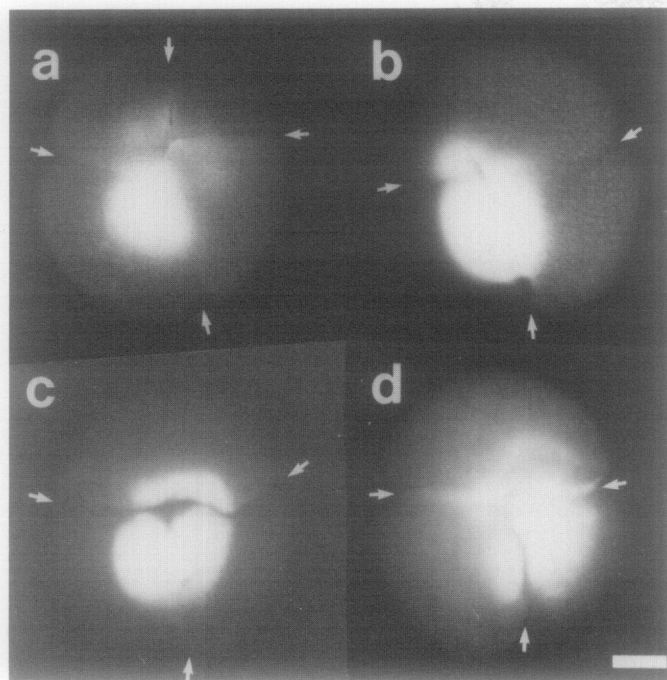


FIG. 3. Partitioning of yolk-deficient cytoplasm to nascent C and D cells. Fixed embryos were stained with acridine orange, causing yolk-deficient regions of cytoplasm to fluoresce, and photographed from the animal pole. Arrows indicate cleavage furrows. Cell D is to the lower left and cell C to the lower right in each panel. (a) Uncentrifuged four-cell embryo. Fluorescence is confined largely to cell D, corresponding to the teloplasm at the animal end of that cell. (b-d) Centrifuged embryos fixed midway through the second cleavage. Cell AB shows greater fluorescence than in uncentrifuged embryos because its clear cytoplasm has also been shifted centripetally. (b) A class C < D embryo. (c) A class C = D embryo. (d) A class C > D embryo. Scale bar, 100 μm .

might correlate with differences in cell fate, the protein compositions of cells C and D in normal and centrifuged embryos were compared using polyacrylamide gel electrophoresis (PAGE), coupled with a sensitive silver stain.

MATERIALS AND METHODS

Embryos. The embryos used in these experiments were obtained from a breeding colony of *Helobdella triseriatis* maintained as described previously (Weisblat *et al.*, 1980a). The staging system used to characterize the development of glossiphoniid leeches was that of Fernandez (1980) as amended (Stent *et al.*, 1982; Weisblat and Blair, 1984). Times of development given here apply to embryos cultured at 23°C. Times are approximate; 0 hour corresponds to the time the eggs were deposited in cocoons on the ventral surface of the parent. Living embryos were observed and photographed using a Wild dissecting microscope.

Low-speed centrifugation. For low-speed centrifugation, embryos were transferred in approximately 250 μl of HL saline (Weisblat and Blair, 1984) into 1.5-ml cen-

trifuge tubes that contained approximately 0.5 ml of Ficoll solution (50% in HL saline). The embryos floated at the interface between the HL saline and the Ficoll solution and were thus cushioned from the bottom of the tube during centrifugation. Embryos were centrifuged at the two-cell stage, approximately 75% of the way through the second cell cycle (about 90 min after the first cleavage and about 20 min before the second cleavage furrow became evident), for 5 min at 350g in a tabletop centrifuge (Microchemical Specialties, Berkeley, CA) controlled by a variable voltage transformer. After centrifugation, the embryos were removed from the tube, rinsed twice in HL saline and returned to normal culture conditions. Embryos centrifuged in this manner generally developed for at least 3 days, at which time uncentrifuged siblings had reached developmental stage 8 (coalescence of the germinal bands); however, most centrifuged embryos did not survive beyond this point. Centrifugation caused yolk to leak into the perivitelline space of some embryos, presumably because their cell membranes were damaged. Such embryos were discarded.

High-speed centrifugation. Embryos were also stratified by high-speed centrifugation to separate yolk cytoplasm from clear cytoplasm for biochemical analyses. Embryos in HL saline were allowed to settle onto a cushion of 50% Ficoll (see above) and then centrifuged for one minute at 10,000g in an Eppendorf microcentrifuge. Embryos were rinsed in HL saline, transferred to 50% proylene glycol (see below, PAGE), and cut into two parts (the clear cytoplasmic layer and the yolk layer). These parts were immediately placed in sample buffer, as described below.

Injection. Methods for injecting blastomeres with lineage tracers were the same as those detailed elsewhere (Weisblat *et al.*, 1984). Some embryos were injected with horseradish peroxidase (HRP; Sigma, type IX), allowed to develop to stage 7, and then fixed in glutaraldehyde and reacted with benzidine and hydrogen peroxide (Weisblat *et al.*, 1984). Other embryos were injected with either fluorescein- or rhodamine-conjugated dextran (FDA and RDA, respectively) (Gimlich and Braun, 1985) and later fixed with formaldehyde and stained with the DNA-specific fluorescent dye Hoechst 33258 (Weisblat *et al.*, 1980b). Embryos injected with FDA or RDA were viewed and photographed under the fluorescence microscope using appropriate filters (Weisblat *et al.*, 1980b). Some fixed embryos were cleared in 70% glycerol and examined in whole mount. Others were dehydrated in an ethanol series, embedded in glycol methacrylate (JB-4 embedding media; Polysciences, Inc.), and sectioned with glass knives on a Sorvall microtome. For weakly fluorescent embryos, the microscope image was viewed with an image intensifier (Varo 3603-1), in combination

with a video camera (Dage-MTI series 68), and photographs of these embryos were taken from a video monitor (Conrac QQA 15/C). Microinjection reduces the average viability of embryos, especially those that have been centrifuged. Embryos that were clearly unhealthy, as characterized by swelling, apparent breakdown of cellular boundaries, or change in color from pink to white (Blair, 1982) were discarded. In these experiments, the survival rate after injection varied from 70 to 100% for noncentrifuged embryos and from 0 to 100% for centrifuged embryos.

Histology. The distribution of yolky and yolk-deficient cytoplasm was examined in embryos fixed at the two-, three-, and four-cell stages. Two-cell embryos were dehydrated, embedded, and cut into 4- μ m sections as above, then stained with 0.15% toluidine blue in 25 mM sodium borate buffer for 10 to 15 sec, and then rinsed in distilled water. Fixed three- and four-cell embryos were stained for 30 min in a solution of acridine orange (0.5 mg/ml in 0.2 M MES buffer, pH 3.5), rinsed twice in buffer, and cleared in glycerol.

Polyacrylamide gel electrophoresis (PAGE). Stage 4a embryos (4 macromeres and 4 micromeres) were dissected in 50% propylene glycol (PG), 0.15% NaCl, 3 mM EDTA, and 2.5 mM EGTA. PG made the embryos more resistant to puncture and this facilitated the isolation of individual cells. Because PG also killed the embryos, batches of embryos were kept in HL saline and then transferred, three at a time, to PG for dissection. Protein patterns of cells collected in this way were indistinguishable from patterns of cells collected by dissecting embryos in HL medium.

Dissected cells were immediately transferred to a small volume of sample buffer. Sample buffer for cells being prepared for one-dimensional PAGE consisted of 3% SDS, 60 mM Tris, pH 6.8, 1% sucrose, and 10% 2-mercaptoethanol. For two-dimensional PAGE the dissected cells were first placed in 1% SDS, 50 mM Tris, pH 7.4, 4 mM EDTA, 2 mM EGTA, and 2% 2-mercaptoethanol. After all of the embryos had been dissected the extract was diluted so that the SDS concentration was 0.25% and Nonidet-P40 was added to 5%. The samples were made 8 M in urea just prior to two-dimensional PAGE. When samples had to be stored before PAGE they were stored at -20°C in their respective sample buffers. Protein concentrations were determined using the procedure of Peterson (1977, 1979), so that equal amounts of protein from each sample would be loaded on the gels. One- and two-dimensional PAGE were performed according to the protocols of Laemmli (1970) and O'Farrell (1975), respectively (as summarized in the Hoefer Scientific Instruments Catalog, San Francisco, CA). The slab gels for both protocols contained an acrylamide gradient from 7.5 to 15%.

Protein patterns were revealed by the sensitive silver stain protocol of Morrissey (1981), modified in that gels were fixed for 40 min in 50% methanol, rinsed three times in 5% acetic acid over the period of 1 hr, and not post-fixed.

RESULTS

Low-Speed Centrifugation

No attempt was made to orient embryos prior to centrifugation, but their preferred orientation is such that the centripetal pole of the stratified embryo is approximately coincident with the animal pole. Thus, later, the micromeres were born from the centripetal pole of centrifuged embryos. Low-speed centrifugation prior to the second cleavage produced cells with a semistratified appearance (Fig. 2c) and with a clearly shifted center of gravity, so that centrifuged embryos were self-righting like a roly-poly toy. A zone of clear (yolk-free) cytoplasm covered the entire centripetal pole of the embryo. This zone occupied about one fifth of the volume of cell CD and a much smaller fraction of cell AB. We presume that this clear cytoplasm is approximately the same as the yolk-deficient cytoplasmic domains (i.e., teloplasm) present prior to centrifugation. But, because of the possibility that centrifugation transfers cytoplasmic factors from yolk to teloplasm, altering the composition as well as the position of each, we will reserve the term teloplasm for reference to uncentrifuged embryos. The centrifugal portions of both cells appeared uniformly yolky under the dissecting microscope, although in sectioned embryos, a gradient in size of yolk platelets was apparent, with larger platelets lying more centrifugally (Figs. 2c and 2d). Both the visibly apparent stratification and the self-righting tendencies of centrifuged embryos had essentially disappeared by the third cleavage.

Centrifugation did not significantly affect the timing or orientation of the second cleavage. The cleavage proceeded meridionally; the cleavage furrow passed at a right angle to the clear layer in cell CD. Thus, both cells C and D received some portion of the clear cytoplasm. There was considerable variability, however, in the exact position of the cleavage furrow with respect to both the midpoint of the cell and of the clear cytoplasm (Fig. 3). This variability probably reflected deviations from coincidence between the animal-vegetal and centripetal-centrifugal axes during centrifugation. Thus, both the partitioning of clear cytoplasm between cells C and D and the size of the cells themselves varied among centrifuged embryos. Centrifuged embryos were classified with respect to the relative amounts of clear cytoplasm found in cells C and D during or shortly after the second cleavage, independent of the relative sizes of the cells. In the majority of cases (65%), cell D received the bulk

of the clear cytoplasm and cell C inherited relatively little; this class of embryos is referred to as $C < D$ (Fig. 3b). However, in a substantial fraction (30%) of the embryos, referred to as class $C = D$, the teloplasm was distributed approximately equally to cells C and D (Fig. 3c). Finally, in the remaining 5% of the embryos, referred to as class $C > D$, clear cytoplasm was sequestered largely into cell C and relatively little was inherited by cell D (Fig. 3d).

Centrifuged embryos cleaved in synchrony with non-centrifuged, sibling embryos. Cleavage of cell AB followed shortly after the cleavage of CD. Then, both centrifuged and noncentrifuged embryos produced a primary quartet of micromeres. These tended to be somewhat larger in centrifuged embryos but seemed normal otherwise.

At the fourth cleavage, some centrifuged embryos deviated dramatically from the stereotyped cleavage pattern. Normally, cell D cleaves obliquely equatorially, generating cells DM and DNOPQ. In 75% of centrifuged embryos cell D divided, as usual and in synchrony with controls, albeit with a slightly more meridional orientation of the cleavage furrow (Fig. 4a). But in 20% of the embryos, two cells, C and D, cleaved. These cleavages were both more meridional than normal. We will refer to this abnormal cleavage pattern as symmetric cleavage. Symmetric cleavage generated embryos that contained six large blastomeres, instead of the normal five (Fig. 4b). The abnormal vegetal and animal daughters

of cell C were mirror symmetric to the DM and DNOPQ daughters of cell D and we refer to them as cells CM and CNOPQ, respectively. A small fraction of centrifuged embryos (<5%) exhibited yet another novel cleavage pattern, in which only cell C underwent an equatorial cleavage that gave rise to two large cells (CM and CNOPQ). This cleavage pattern resulted in an embryo with the correct number of large blastomeres, but which appeared inverted with respect to a normal embryo (Fig. 4c). We will refer to this cleavage pattern as inverted.

Although cell D did not cleave equatorially in these embryos, it may have participated in the second round of micromere production. The relative amounts of clear cytoplasm in cells C and D after centrifugation correlated well with the cleavage patterns of these cells (Fig. 5). Embryos in class $C < D$ cleaved normally 96% of the time. Embryos in class $C = D$ often (70%) exhibited the abnormal, symmetric cleavage. And finally, embryos in class $C > D$ generally (82%) showed the inverted cleavage pattern.

Although centrifugation affected the size of cells C and D as well as their cytoplasmic composition, the appearance of novel cleavage patterns was correlated with the distribution of clear cytoplasm and not with the relative size of cells C and D. For example, in one experiment 38 embryos were centrifuged and two embryos, in which cell D was larger than cell C but cell C received more clear cytoplasm, were separated and observed. Both these embryos cleaved by the inverted pattern. In the

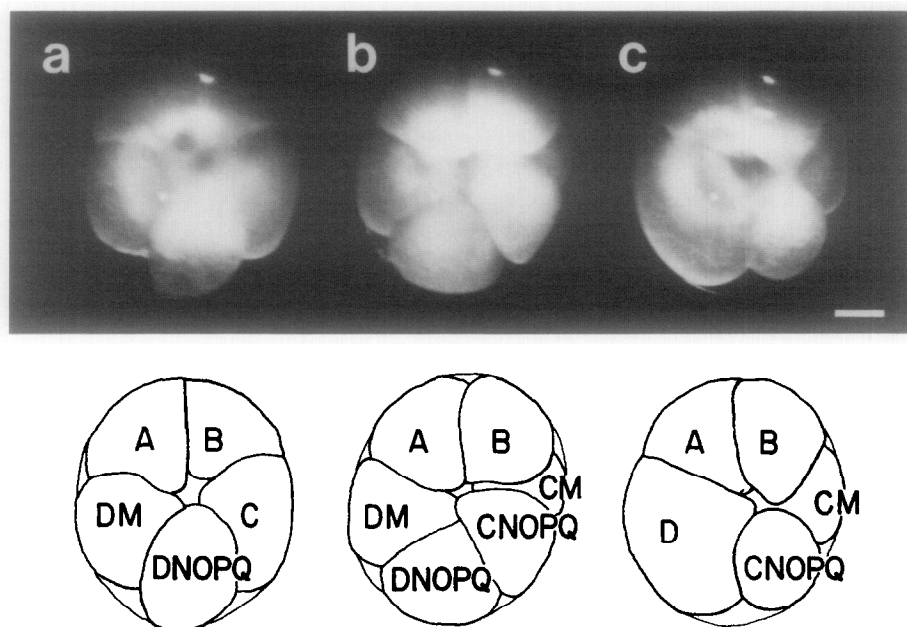


FIG. 4. Cleavage patterns induced by centrifugation. Top: photographs of centrifuged embryos at stage 4b. Bottom: tracings of the same embryos, indicating cell designations. (a) Normal cleavage pattern (virtually indistinguishable from that observed in uncentrifuged controls). (b) Symmetric cleavage. (c) Inverted cleavage. Scale bar, 100 μ m.

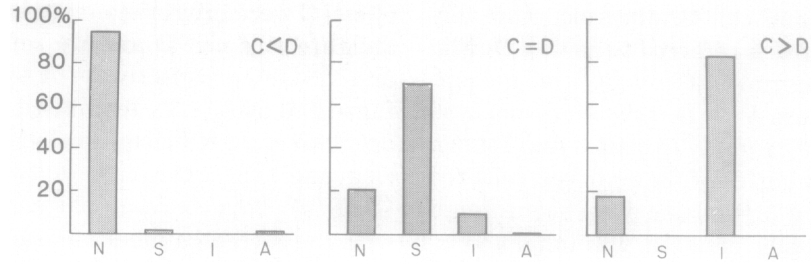


FIG. 5. Correlation of inheritance of yolk-deficient cytoplasm at second cleavage with the pattern of the fourth cleavage. Cleavage patterns are abbreviated as follows: N, normal; S, symmetric; I, inverted; A, abnormal embryos not falling into any of these classes.

same experiment, four other embryos were separated in which cell D was larger than cell C, but the clear cytoplasm was equally distributed. Three of the four cleaved symmetrically and one cleaved normally. Finally, another three embryos were separated in which cells C and D were of equal size, but cell C inherited more clear cytoplasm. Two of these three cleaved with the inverted pattern and one cleaved symmetrically. (In the remaining embryos, cytoplasmic composition and relative cell size predicted the same fates.) Thus, among nine embryos in which the fates predicted on the basis of size differed from those predicted on the basis of the distribution of clear cytoplasm, seven (78%) followed the fate predicted by cytoplasmic distribution and only one (11%) followed the fate predicted by cell size.

Subsequent Development

Centrifuged embryos which had normal initial cleavage patterns frequently generated two germinal bands that were organized in the characteristic heart-shaped pattern by late stage 7, as revealed by Hoechst staining. As in uncentrifuged controls, bandlet cells were derived from the teloblast progeny of cells DM and DNOPQ. Centrifuged embryos that cleaved normally survived better than any other centrifuged embryos except for those few that cleaved in the inverted pattern (see below). Some normally cleaving, centrifuged embryos survived through stage 8 and presumably were capable of fully normal development.

In contrast, the bandlets of embryos in which both cells C and D divided symmetrically were disorganized, and did not show the heart-shaped pattern that is typical of stage 7 embryos. These embryos invariably died during stage 8. The symmetrically cleaving embryos appeared to contain supernumerary teloblasts and bandlets; however, the disorganization in these embryos prevented a direct count of bandlets stained only with Hoechst 33258. For this reason, the subsequent development of centrifuged embryos was examined further by labeling individual proteloblasts (DM, DNOPQ, CM,

and CNOPQ) with either HRP, FDA, or RDA and then examining the embryos for the presence of lineage tracer in progeny cells.

This technique revealed that both cells CM and CNOPQ generated teloblasts and bandlets. In fact, cell CM, in embryos that underwent symmetric cleavage, often produced progeny that were indistinguishable from those of a normal DM cell (Fig. 6; Table 1). That is, cell CM formed two teloblasts and bandlets and some non-bandlet cells. The blast cells in the bandlets divided laterally (Figs. 7a and 7b). The non-bandlet cells descended from cell CM (Figs. 7c and 7d) appeared morphologically similar to larval integument cells. The labeling pattern of CM-derived cells, however, frequently deviated from control DM labeling patterns. For example, only one teloblast and bandlet were formed in 38% of the embryos examined and putative larval integument cells were detected in only about 50% of the embryos in which CM was injected with RDA.

Similarly, the lineage of cell CNOPQ resembled the lineage of cell DNOPQ in normal, uncentrifuged embryos. DNOPQ normally generates the four pairs of ectodermal teloblasts, N, O/P, O/P, and Q, each of which forms a bandlet. RDA injected into cell CNOPQ generally labeled several teloblasts and bandlets in the stage 7 embryo (Fig. 8). The number of labeled teloblasts from successful CNOPQ injections ranged from 3 to 8 (Table 1). Just as with control DNOPQ injections, no labeled larval integument cells were found.

Centrifuged embryos in which the inverted cleavage pattern was observed were similar to normal embryos from stage 6 onward. Inverted embryos contained two germinal bands arranged in the typical heart-shaped pattern. Because of the oblique cleavage of cell D, normal stage 6 embryos exhibit a slight right/left asymmetry with respect to the M teloblasts. The left M teloblast is normally located at the surface of the embryo, and lies closer to the animal pole than the right M teloblast, which is partially obscured by the yolky macromeres. This asymmetry was reversed in inverted embryos. That is, the right M teloblast was prominent, while its left-

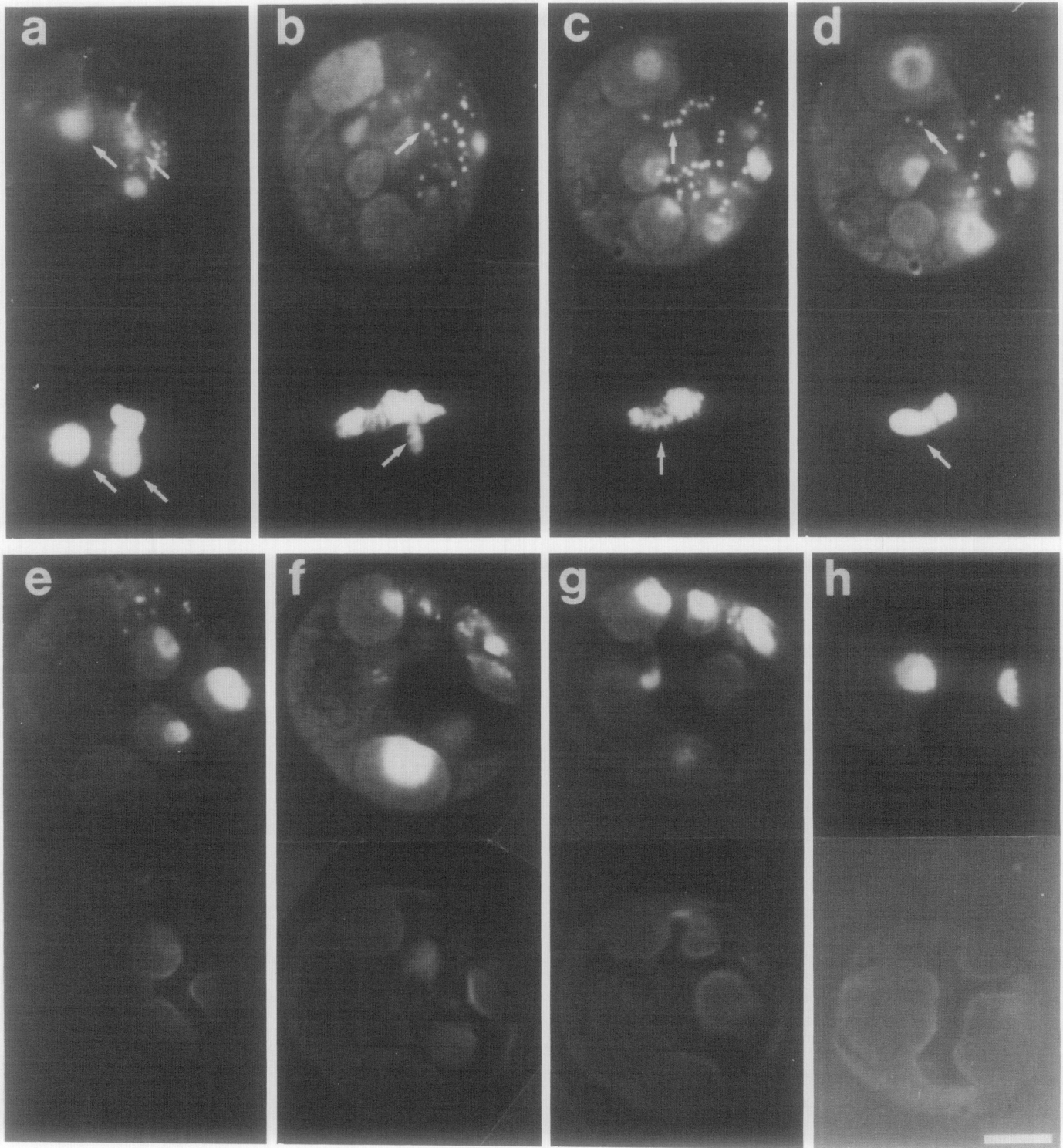


FIG. 6. Progeny of cell CM in a class C = D embryo. Fluorescence photomicrographs of eight 15 μm thick sections from a serial series through an embryo in which cell CM had been injected with RDA two days prior to fixation. Top of each panel: Hoechst 33258 fluorescence reveals that a total of 16 teloblasts arose in this embryo, not all of which appear in the sections shown here. Bottom of each panel: Rhodamine fluorescence, showing only the progeny of the injected cell CM. Arrows indicate the two labeled teloblasts, in (a), and their labeled bandlets, in (b-d). These sections were viewed with an image intensifier and TV camera and photographed from a video monitor. Panels (a-d) are adjacent sections; (e-h) are at intervals through the rest of the embryo. Scale bar, 100 μm .

TABLE 1
NUMBER OF TELOBLASTS DERIVED FROM INDIVIDUAL PROTELOBLASTS IN CENTRIFUGED EMBRYOS EXHIBITING SYMMETRIC CLEAVAGE^a

Proteloblast injected	Number of teloblasts derived from injected proteloblast								Total examined
	1	2	3	4	5	6	7	8	
CM	6	9	0	0	0	1	0	0	16
CNOPQ	0	1	1	2	1	2	2	3	12
DM	0	4	0	0	0	0	0	0	4
DNOPQ	0	0	0	0	0	0	2	1	3
Control DM	0	3	0	0	0	0	0	0	3
Control DNOPQ	0	0	0	0	0	0	0	3	3

^a In all embryos, the proteloblasts were injected with a lineage tracer at stage 4b. Labeled teloblasts were counted in serial, 30 μ m thick sections through stage 7 embryos. Fluorescent label appearing in the identical location in two consecutive sections was taken to be from the same cell.

hand homolog was obscured. Inverted embryos generally continued to develop normally through stage 8.

Protein Comparison

The protein composition of early blastomeres was first analyzed using two-dimensional PAGE. Homologous individual blastomeres were isolated by dissection and 6 to 12 cells were pooled to obtain sufficient amounts of protein for analysis. Two-dimensional gels resolved over 200 proteins belonging to a single cell type. Comparisons of the protein spot patterns from cells A, B, C, and D revealed no consistent qualitative differences. Thus, we detected no proteins unique to any of the blastomeres. Significant differences between these cells became evident, however, when the relative amounts of individual proteins were compared. These differences could easily be seen using one-dimensional PAGE, so this technique was used to obtain the results below.

Because it seemed likely that the relative differences in protein content of cells A, B, C, and D arose from differences in the amount of yolk-deficient cytoplasm in the four cells, we sought to distinguish between yolk-associated proteins and those associated with non-yolky cytoplasm. To that end, four-cell embryos were centrifuged for 1 min at 10,000*g*, which stratified their intracellular components far more extensively than 5 min at 350*g*. Such embryos contained three distinct zones: an opaque white cap at the centripetal pole; a clear yolk-free layer; and a region of yolky cytoplasm comprising the centrifugal two-thirds of the embryo. Embryos subjected to high-speed centrifugation never cleaved. Such embryos were bisected into centrifugal and centripetal pieces; the centripetal fragment contained predominantly clear cytoplasm and the centrifugal fragment contained predominantly yolky cytoplasm. In this experiment, no distinction was made between individual macromeres. Fragments from 20 to 30 embryos were pooled and aliquots containing equal amounts of protein

were compared by one-dimensional PAGE (Fig. 9). The yolky fraction yielded about 12 prominent protein bands, whereas the yolk-deficient fraction separated into over 100 bands. A comparison of these fractions with one-dimensional gels of cells C and D showed that cell C contained much more of the yolk-associated proteins, while cell D contained relatively more of the non-yolk proteins. In particular, two protein bands (50 and 80 kDa) associated with the clear, centripetal cytoplasm appeared in much higher (>10-fold) amounts in cell D than in cell C (Fig. 9).

Finally, we sought to correlate these results with the low-speed centrifugation, which suffices to alter embryonic cell fates. For this purpose we took advantage of the fact that it is possible to reliably predict which embryos will undergo the symmetric cleavage by observing the distribution of clear cytoplasm at the second cleavage of partially stratified embryos. Centrifuged (350*g*) embryos of class C = D (which would be expected to exhibit 70% symmetric cleavage) were dissected and their C and D macromeres were pooled separately and analyzed in parallel with the samples described above by one-dimensional PAGE. The protein patterns from such modified C and D cells were indistinguishable from each other; each has about the same proportions of the proteins that were normally associated with the clear cytoplasm and were normally enriched in D cells (Fig. 9).

DISCUSSION

Correlation between Determinative Factor(s) and Teloplasm

The work reported here examines cytoplasmic differences between blastomeres with different fates in embryos of a glossiphoniid leech. In normal development, the segregation of yolk-deficient cytoplasm (teloplasm) correlates with differences in fates of macromere D compared to macromeres A, B, and C (Whitman, 1878; Muller, 1932). Attempts to examine the role of the tel-

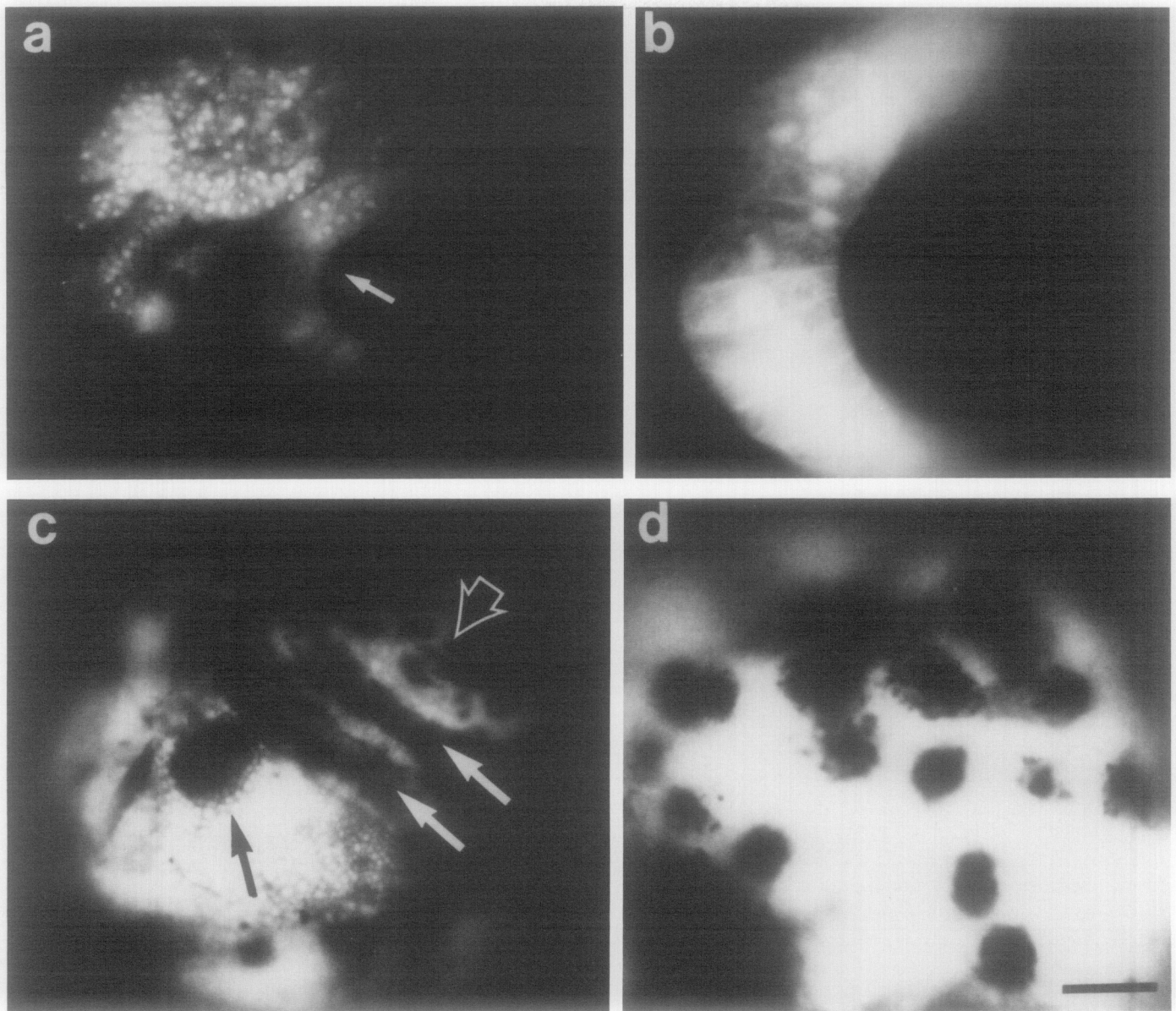


FIG. 7. Progeny of cell CM have properties characteristic of normal mesodermal cell lines. Photomicrographs of centrifuged, class C = D embryos fixed at stage 7. (a) Hoechst 33258 fluorescence reveals many cells, including part of one bandlet (arrow) derived from a CM cell that was also injected with RDA (see b). (b) Higher power view, under rhodamine fluorescence, of the bandlet indicated in (a). Primary blast cells towards the presumptive anterior end of the bandlet (top) have divided laterally, forming a column that is two cells wide. (c and d) Embryo in which cell CM had been injected with HRP. (c) One HRP-labeled teloblast (black arrow), two labeled bandlets (white arrows), and other labeled non-bandlet cells (open arrow) are visible. (d) Higher magnification view of the non-bandlet cells reveals that they are similar in morphology to the larval integument cells normally generated by cell DM. Scale bar, 50 μm in (a and c); 15 μm in (b and d).

oplasm in *Glossiphonia* failed because the centrifuged embryos stopped cleaving (Schleip, 1914). We have found that centrifugation induces novel cleavage patterns and causes a redistribution of cellular proteins in *Helobdella*. The critical difference between Schleip's experiments and ours could be the species of leech studied, but we suspect that a more fundamental difference is the extent of centrifugation. Although Schleip did not report the gravitational force he used, the fact that his embryos

were stratified into three distinct layers (lipid, clear cytoplasm, and yolk) as in our high-speed centrifugations, suggests that the force was stronger than the 350*g* used here, or was applied for too long a time (15–30 min vs 5 min), or both.

Several lines of evidence from our work strengthen the hypothesis that the inheritance of teloplasm may be important in causing cell D, but not cells A, B, or C, to make teloblasts in normal development.

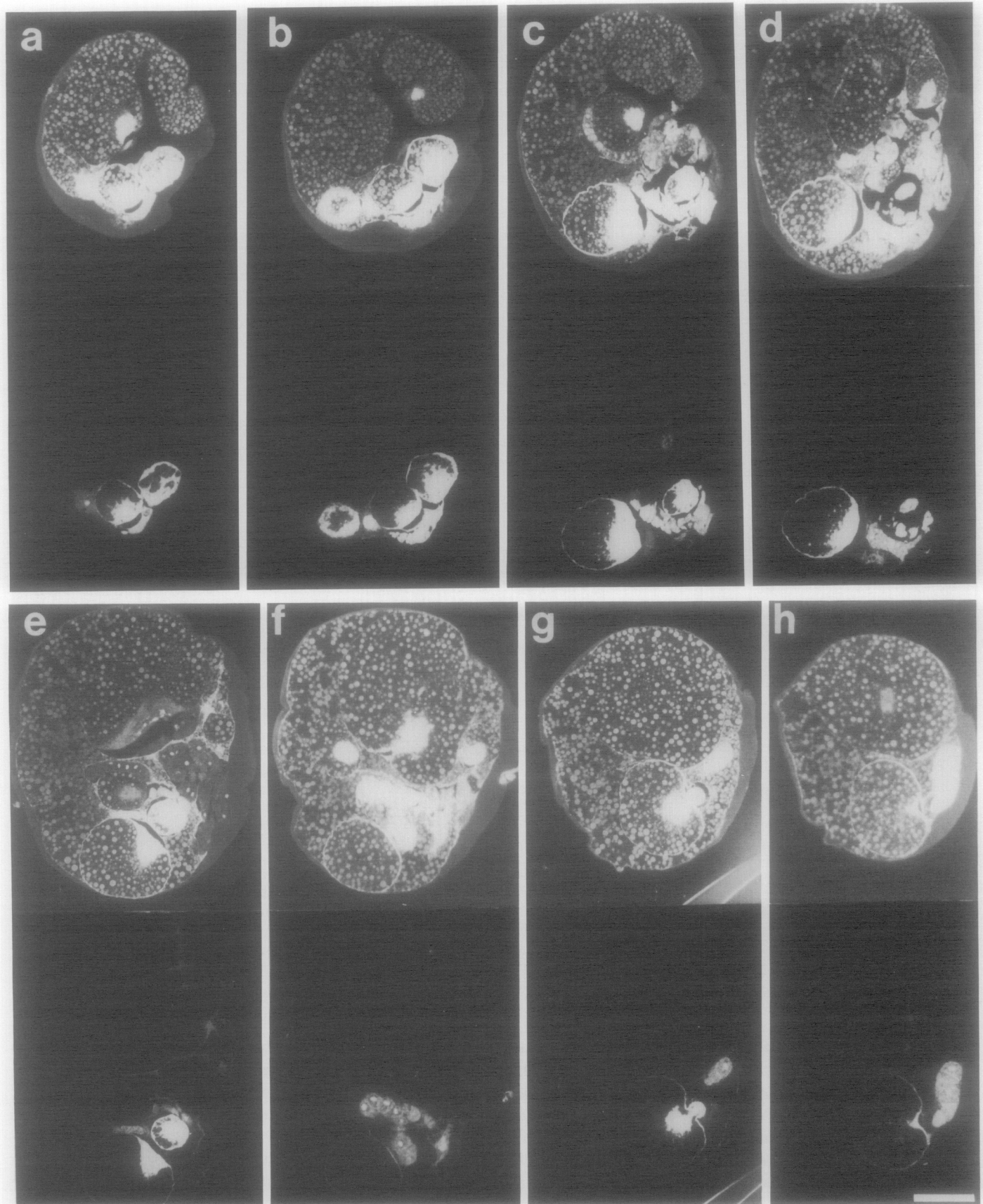


FIG. 8. Progeny of cell CNOPQ in a class C = D embryo. Fluorescence photomicrographs of eight $4\ \mu\text{m}$ thick sections from a serial series through an embryo in which cell CNOPQ had been injected with FDA two days prior to fixation. Top of each panel: Hoechst 33258 fluorescence reveals that a total of 17 teloblasts arose in this embryo, not all of which appear in the sections shown here. Bottom of each panel: FDA fluorescence reveals the seven teloblasts and their handlets derived from CNOPQ. Scale bar, $100\ \mu\text{m}$.

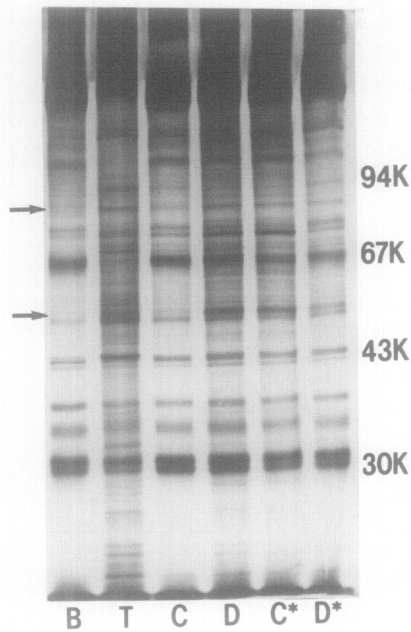


FIG. 9. Silver stained, one-dimensional polyacrylamide gel of extracts of isolated C and D cells (C and D), C and D cells from embryos centrifuged at low speed and falling into class C = D (C* and D*), plus yolk-deficient, top (T) and yolk-rich, bottom (B) fractions of embryos centrifuged at high speed. Note that D contains a higher proportion of proteins found in the centripetal (T) fraction of centrifuged embryos than does C, and that even low-speed centrifugation redistributes these proteins so that C* and D* contain similar proportions of all detectable proteins. Arrows indicate protein bands, at about 50 and 80 kDa, that are greatly enriched in lanes T, D, and C*, relative to lanes B and C.

1. Redistributing yolk-deficient teloplasm can result in embryos in which cells C and D both cleave to make teloblasts. The probability and nature of the fate change can be predicted by the relative proportions of the clear cytoplasm partitioned into cells C and D. Conversely, the relative size of cells C and D have no predictive value in this regard. Together, these results suggest that it is the clear cytoplasm and not the amount of membrane, yolk cytoplasm, or cortical cytoplasm that is important.

2. The degree of centrifugation sufficient to change the cleavage patterns is also sufficient to induce the redistribution of cellular proteins associated with the teloplasm and to abolish protein differences between cells C and D.

3. The only visible differences between cells C and D are their size and their teloplasm content. The differences in protein composition we detected between cells C and D are accounted for by the differences between clear and yolk cytoplasm. (However, the zone of clear cytoplasm which is obtained by centrifugation may contain not only native teloplasm but also clear cytoplasm, normally found between yolk granules, that has been extruded centripetally. Therefore, protein differences

between cells C and D cannot be attributed solely to teloplasm.)

Properties of Determinative Factor(s)

Whether or not the factor(s) which normally determine the subsequent cleavages of the D macromere are located in the teloplasm, the experiments reported here raise several points of interest regarding this determinative process.

1. A priori, determinative factor(s) might act stoichiometrically, such that 10 teloblasts and no more would be produced from the D macromere. In that case, we might expect that redistributing the factor(s) would instruct both cell C and cell D to make teloblasts, but only up to a total of 10 between them. But in our experiments, supernumerary teloblasts and bandlets were observed, suggesting either that the determinative factors act *permissively*, eliciting a cleavage subroutine inherent in each macromere, or that stoichiometrically acting factor(s) are normally overabundant and that other factors serve to limit the production of teloblasts by each macromere.

2. The cleavages that the C macromere undertook after centrifugation were highly reminiscent of the normal D cleavage pattern. The failure of the centrifuged embryos to develop beyond stage 7 prevented us from determining the definitive fates of their progeny. But the immediate progeny of affected C macromeres, CM and CNOPQ, exhibited traits associated with their D-derived namesakes. In particular, cell CM often formed two teloblasts whose blast cells divided laterally within the bandlet, and also gave rise to non-bandlet cells resembling larval integument cells. These are all properties of the mesodermal precursor blastomere. In contrast, CNOPQ almost always made more than two, and sometimes as many as eight, teloblasts, as would be expected from the ectodermal precursor. The variable and reduced numbers of teloblasts observed in centrifuged embryos could be attributed at least in part to their reduced resistance to the trauma of injection, as evidenced by the reduced survival of injected, centrifuged embryos relative to injected, uncentrifuged controls.

Animal blastomeres (CNOPQ and DNOPQ) in centrifuged embryos have distinct fates from vegetal blastomeres (CM and DM) even though they inherit teloplasm from what appears to be a common pool at the centripetal (animal) pole of the embryo. If separate mesodermal and ectodermal determinants exist, how do they find their way to the appropriate sites in centrifuged embryos? Indeed, how can the prospective CM and DM cells get any teloplasm at all? First, there was ample time for a redistribution of cytoplasm between the second and fourth cleavages in centrifuged embryos, as ev-

idenced by their loss of apparent stratification and return of the center of gravity towards its normal location. Second, the cytoplasm normally undergoes reorganization in cell D between the second and fourth cleavages, such that most of the teloplasm is pooled at the dorsal pole of cell D (B. Holton, unpublished). Thus, even normal embryos redistribute a common pool of teloplasm to cells DM and DNOPQ. The main effects of centrifugation, therefore, are to induce a *premature* pooling of teloplasm, and to distribute it to both cells C and D. Finally, the relatively meridional orientation of the cleavage furrows in cells C and D of centrifuged embryos tends to give all four daughter cells some centripetal cytoplasm.

There are two basic possibilities regarding the assumption of distinct fates by cells CM and CNOPQ. One is that teloplasm contains specific mesodermal and ectodermal determinants which mix and then resegment prior to the fourth cleavage (as in the preceding paragraph). The other is that teloplasm does not specify mesodermal and ectodermal fates, but merely permits further cleavages. In that case, the two daughter cells might be developmentally equipotent, following different fates as the result of positional interactions (e.g., with the micromeres). Alternatively, such permissive factor(s) in teloplasm could interact with factors that remain localized to future animal and vegetal regions even after low-speed centrifugation (e.g., in the cortex or membrane of the egg). In this regard, studies on cytoplasmic segregation in *Tubifex* indicate that cortical polarity, as revealed by actin organization, is retained in eggs centrifuged with forces up to 650g (Shimizu, 1986). Note that these cortical factors need only be localized longitudinally and could uniformly be distributed latitudinally, activated only in macromeres inheriting teloplasm. This model resembles one that accounts for the effects of centrifugation on the induction of the dorsal axis in *Xenopus* (Gerhart *et al.*, 1983).

3. Our techniques were adequate to detect blastomere-specific proteins at levels as low as 1–10 pg/cell (Morrissey, 1981) that might be candidates for determinative substances. The failure to detect any such proteins leaves open three possibilities regarding the nature of a determinant factor: it is a protein present in quantities below our threshold of detectability; it is a specific component other than a protein; it is not a specific molecule at all, but rather the ratio between certain proteins and/or other cellular components. A good test for the latter hypothesis would be to pool the small amounts of clear cytoplasm available from macromeres other than D and test for a cleavage-inducing effect by microinjecting that material into macromere A, B, or C. Definitive cytoplasmic transplants have been achieved in several systems, including *Drosophila* (Anderson and Nusslein-

Volhard 1984), *Paramecium* (Hiwatashi *et al.*, 1980), and ascidians (Deno and Satoh, 1984). Thus far, attempts to transfer cytoplasm between blastomeres of *Helobdella* embryos have proved unsuccessful, due to the high viscosity of the cytoplasm (K. Anderson and S. Astrow, unpublished).

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