# An Investigation of the Specification of Unequal Cleavages in Leech Embryos

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Leech embryos develop via stereotyped cell divisions, many of which are unequal. The first division generates identifiable cells, blastomeres AB and CD, which normally follow distinct developmental pathways. When these two cells are dissociated and cultured in isolation, their fates remain distinct and are reminiscent of normal development, but their typical cleavage patterns are disrupted; cell AB undergoes relatively few cell divisions, giving rise to a variable number of macromeres and micromeres, while cell CD cleaves many times, usually forming a poorly organized set of macromeres, embryonic stem cells (teloblasts), and micromeres. We have investigated the hypothesis that the abnormal cleavage pattern of isolated CD blastomeres is due to removal of mechanical constraints normally imposed by cell AB. We find that when cell CD is constrained *in vitro* to mimic its *in vivo* shape, it cleaves more normally. © 1992 Academic Press, Inc.

## INTRODUCTION

Cell divisions that are unequal in terms of the fates of the two daughter cells are intrinsic to the development of most organisms. In some cases, inequalities in fates or in the developmental potential of sister cells has been correlated with inequalities in cell volume (for example, Whitman, 1878; Horstadius, 1939; Deppe *et al.*, 1978; Sulston *et al.*, 1983; Dan *et al.*, 1983; Storey, 1989; Sternberg *et al.*, 1987) or in the inheritance of a cytoplasmic or cortical component of the mother cell (for example, Conklin, 1905; Boveri, 1910; Spemann, 1938; Shimizu, 1982a, b; Milhausen and Agabian, 1983; Gober *et al.*, 1991; Astrow *et al.*, 1987; Johnson *et al.*, 1988; Nelson and Weisblat, 1991, 1992). The range of possible mechanisms for achieving unequal cell divisions includes both intrinsic factors, such as cytoskeletal organization (Conklin, 1917; Meshcheryakov and Beloussov, 1975; Kawamura, 1977; Dan *et al.*, 1983; Dan and Ito, 1984; Dan and Tanaka, 1990; Schroeder, 1987; Hill and Strome, 1988, 1990), and external factors, including inductive cell-cell interactions (Schierenberg, 1986; Johnson and Ziomek, 1981; Ziomek and Johnson, 1980; Sulston and White, 1980; Boring, 1989) and mechanical constraints (Morrill *et al.*, 1973; Meshcheryakov, 1976, 1976, 1978a,b; 1978; Meshcheryakov and Veryasova, 1979; Laufer *et al.*, 1980; for review see Freeman, 1983).

One way to distinguish between the influences of external and intrinsic factors on cell fate is to compare the fate of a cell *in situ* with that which it follows *in vitro*. Embryos of the glossiphoniid leech *Helobdella triserialis* are well-suited for such experiments because their early development comprises a stereotyped sequence of cell divisions which may be equal or unequal in terms of the daughter cells' volumes, subsequent fates, and inheritance of a domain of yolk-deficient cytoplasm called teloplasm. The inheritance of teloplasm has been correlated with the developmental potential of the cell (Astrow *et al.*, 1987; Nelson and Weisblat, 1991, 1992), such that cells inheriting teloplasm give rise to the stem cells (teloblasts) that generate the segmental tissue of the leech.

In normal development the first cleavage division produces daughter cells, AB and CD, that are unequal by three criteria. Cell CD is larger than cell AB, it inherits essentially all of the teloplasm, and it is the sole precursor of the teloblasts. Experiments described here investigate the second cleavage division of cells AB and CD, with attention to the same three criteria. Normally cell AB cleaves equally, while cell CD cleaves unequally and the larger daughter cell, D, inherits all of the teloplasm and is the teloblast precursor. We find that, if either cell AB or CD is cultured in isolation, their normal cleavage patterns are disrupted; in CD isolates the teloplasm is inherited by both sister cells. Nevertheless, both AB and CD retain some aspects of their distinct normal fates. Furthermore, if isolated AB and CD cells are reaggregated within 1 hr, they are able to resume normal development.

When AB and CD are separated, not only is the possibility for inductive interactions between the cells lost, but also the mechanical constraints that affect their shape in normal development. To distinguish between inductive and mechanical influences on the unequal division of cell CD, we have sought to test the hypothesis that cell CD requires its *in vivo* shape in order to cleave

correctly. We find that when isolated CD cells are artificially constrained *in vitro* to mimic their normal shape, they cleave, and the teloplasm is segregated, more normally. This suggests that mechanical interactions between cells AB and CD may have an important role during the second cleavage division.

## MATERIALS AND METHODS

### Embryos

Embryos of the glossiphoniid leech, *H. trisenalis*, were obtained from a laboratory breeding colony, kept at 23°C as previously described by Blair and Weisblat (1984), and staged according to Fernandez (1980). The nomenclature for the cells is as described by Bissen and Weisblat (1989). Macromeres and teloblasts are designated by an upper case letter. Micromeres are designated by a lower case letter corresponding to the parent blastomere (Ho and Weisblat, 1987); the names of the first micromere and large blastomere descended from a particular cell are followed by one prime ('); the names of the second set are followed by two primes ("), and so forth.

### Operations

All embryonic manipulations were carried out in HL medium (Blair and Weisblat, 1984). To allow the isolation of blastomeres, the vitelline membranes of leech embryos were removed during the first cell cycle, prior to teloplasm formation. The vitelline membrane was first weakened by a 6-min incubation at room temperature in 0.3% bacterial protease (type XIV, Sigma; Astrow *et al.*, 1989). The embryos were washed in HL medium and their vitelline membranes were immediately removed using electrolytically sharpened tungsten needles. The embryos were then transferred to a petri dish containing HL medium and a bed of 0.75% agarose (FMC Bioproducts) to prevent them from adhering to the dish.

At first cleavage the daughter cells AB and CD were dissociated by the application of gentle pressure for approximately 30 sec on the cleavage furrow with a thin (30-μm-diameter) glass fiber. The separated cells were then cultured individually in HL medium at 23°C for 60–65 hr, by which time the germinal bands in sibling control embryos were starting to coalesce to form the germinal plate. We shall refer to such experimentally manipulated embryos as having developed to control stage 8 (Fig. 1D). The specimens were then fixed in 3.7% formaldehyde in 0.1 *M* Tris–HCl, pH 7.4, for 1 hr at 23°C and rinsed in Tris, and then the nuclei were stained by
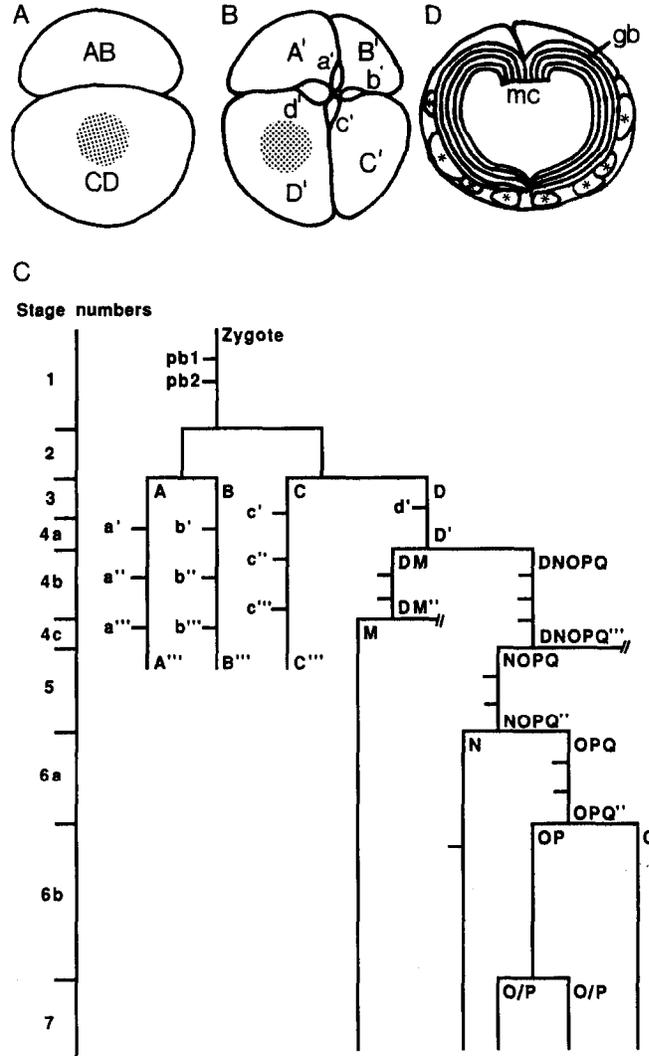


FIG. 1. A summary of early leech development. (A) Two-cell embryo. The first cell division is unequal, cell CD is larger than AB and inherits the teloplasm (stippling). (B) In the third round of cell division each blastomere divides highly unequally to produce micromeres a'–d' and macromers A'–D'. (C) A lineage tree summarizing the early cell divisions including formation of the polar bodies (pb1 and pb2), macromeres (A–A''', B–B''', C–C''', and D–D'), teloblasts (M, N, O/P, O/P, and Q) and micromeres (a'–a''', b'–b''', c'–c'''; other micromeres are represented by short horizontal lines). The lineage of only one of the two sets of the teloblasts is shown. Embryonic stages are indicated on the left of the diagram. The blast cells are not shown. (D) Early stage 8 embryo showing the normal arrangement of teloblasts (*), bilaterally symmetrical germinal bands (gb), and micromere cap (mc). The micromere-derived epithelium also covers the germinal bands, which are beginning to coalesce at the anterior end of the embryo.

incubation in 10 μg/ml Hoechst 33258 (Sigma) for 30 min. The specimens were cleared in 70% glycerol or dehydrated through a graded ethanol series and then cleared in a solution of benzyl benzoate and benzyl alcohol (3:2). Cleared embryos were examined using a fluo-

rescence microscope (Zeiss) equipped with the appropriate filters.

### Naming Blastomeres in Experimentally Manipulated Embryos

Astrow and colleagues (1987) have defined cells C and D in experimentally manipulated embryos according to teloplasm content; the daughter of cell CD that inherits the larger volume of teloplasm was defined as cell D, irrespective of the relative volumes of the two cells. Here, we have followed this convention, but refer to these cells in experimentally manipulated embryos as "C" and "D," to distinguish them from their counterparts in unperturbed embryos.

### Injections

Blastomeres were injected with lineage tracers according to the method of Weisblat *et al.* (1984). The lineage tracers fluorescein dextran amine (FDA) or tetramethylrhodamine dextran amine (RDA; Gimlich and Braun, 1985; Stuart *et al.*, 1990) were the gift of Duncan Stuart or were obtained from Molecular Probes.

### Silver Staining

The boundaries of superficial cells in cultured AB and CD isolates were revealed by staining with silver methenamine at control stage 8 according to the method of Arnolds (1979), except that the isolates were briefly rinsed in water, and not fixed, before being introduced into the silver methenamine solution.

### Casting of Embryo-Sized Wells in Agarose

Embryo-sized agarose wells were made to facilitate reaggregation of, or to examine the effects of mechanical deformations on, isolated blastomeres. The wells were cast by placing spare embryos into a thin layer of molten 0.75% agarose (approximately two-thirds the depth of the embryo) in HL medium in a 35-mm petri dish. After the agarose had solidified, the petri dish was filled with HL medium; the embryos were then gently removed with an insect pin and discarded.

### Morphometric Analysis

To make quantitative comparisons between the divisions of CD blastomeres under various conditions, embryos or isolates which had just completed their second cleavage division were fixed in 3.7% formaldehyde in 0.1 $M$ Tris-HCl, pH 7.4, for 1 hr at 23°C. The specimens were rinsed, dehydrated through a graded ethanol series, and embedded in Historesin (LKB) according to the

manufacturer's instructions. Serial 10-$\mu$m sections were cut, mounted on microscope slides, and then viewed via DIC optics with a video camera. The cursor of a digitizing tablet was superimposed electronically upon the video image of the section and was used to outline each cell and the area of yolk-deficient cytoplasm within it (Nelson and Weisblat, 1991). Two domains of yolk-deficient cytoplasm were observed, the teloplasm (see Fig. 2) and the region of yolk-deficient cytoplasm that surrounds the nucleus, the perinuclearplasm (Whitman, 1878; Schleip, 1914; Fernandez, 1980; Fernandez and Olea, 1982; see Fig. 2a). The volumes of perinuclearplasm and teloplasm were combined because the boundaries between them are often ambiguous (see Figs. 2b and 2c). No correction was made for the volume of the nucleus itself because it is small compared to the perinuclearplasm volumes.

The digitizing tablet was also part of a computer graphics workstation (Polycad 10; Cubicomp Corp.) which was then used to calculate the outlined areas. These were converted to volume estimates by correcting for the thickness of the sections. For an initial test embryo, volumes estimated using every section were in good agreement with those obtained looking at every fifth section. Therefore, the data presented here were obtained by examining every fifth section. To avoid the possibility of subconscious bias, samples were coded so that the experimental history of a given preparation was not known until after the morphometric analysis was completed.

To obtain a quantitative measure of the degree of inequality of cell volumes and teloplasm distribution between the sister cells of a given division, the ratios of the total volumes of the cells and the volumes of the teloplasm within them were calculated. Thus, a ratio of 1 indicates an equal division. The ratios of cell volumes and of teloplasm volumes obtained were compared by analysis of variance.

### RESULTS

#### Summary of Early Leech Development

The early stages of *Helobdella* development consist of a sequence of stereotyped cell divisions resulting in three classes of blastomeres: macromeres, micromeres, and teloblasts. These blastomeres are individually identifiable according to their size, position, the order in which they arise; by the differential distribution of teloplasm (yolk-deficient cytoplasm); and by their developmental fates.

The first cell division of the *Helobdella* embryo is unequal; cell CD is larger than cell AB and inherits essen-
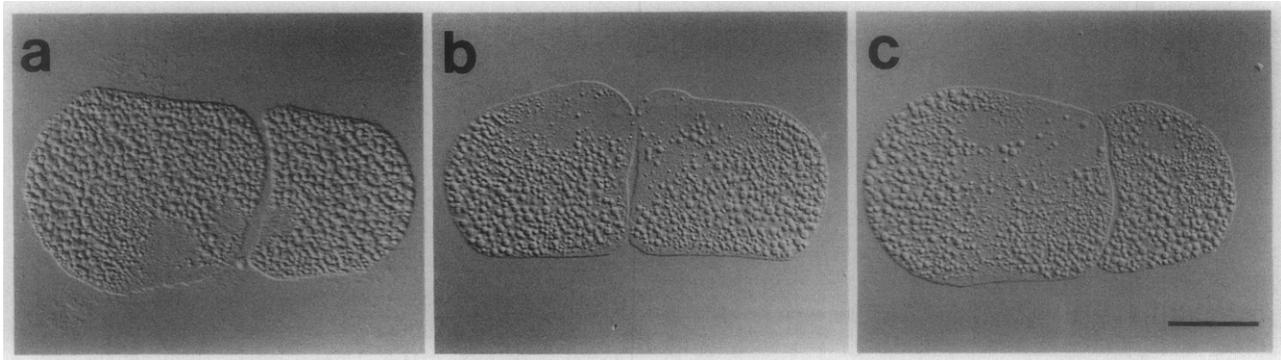
FIG. 2. Yolk and yolk-deficient domains can be readily distinguished in sectioned embryos. Photomicrographs, using DIC optics, of sections through (a) cells C and D of a normal embryo and through cells "C" and "D" of (b) free and (c) constrained CD isolates. Domains of yolk cytoplasm are coarsely granular in appearance, while those of yolk-deficient cytoplasm (teloplasm and perinuclearplasm) are finely granular. Scale bar, 100 µm.

tially all of the teloplasm (Fig. 1A). In the next round of cell division, cell AB divides equally while cell CD cleaves unequally; again, most of the teloplasm is inherited by the larger cell, D.

Blastomeres A, B, and C each go on to produce three micromeres (a'-a''', b'-b''', c'-c''') and three macromeres (A''', B''', C''') during the next 14 hr at 23°C (Bissen and Weisblat, 1989). The macromeres do not divide further and are ultimately internalized, forming the gut contents of the juvenile leech. Cell D also produces a micromere (d') and macromere (D') at third cleavage (Fig. 1B); however, cell D' then undergoes a unique and stereotyped pattern of cell cleavages to form 5 bilateral pairs of teloblasts and 15 micromeres (stages 4a-6; Fig. 1C; Bissen and Weisblat, 1989). Specifically, at fourth cleavage, cell D' divides more or less equally, with an oblique, equatorial cleavage plane, to form cells DNOPQ and DM. DM is the mesodermal precursor blastomere; from it arises a bilateral pair of teloblasts (M) and two micromeres. DNOPQ is the ectodermal precursor blastomere; from it arise four bilateral pairs of teloblasts (N, O/P, O/P, and Q) and 13 micromeres (Bissen and Weisblat, 1989).

Teloblasts are embryonic stem cells that give rise to all the segmental tissues. Each teloblast undergoes a series of highly unequal cleavages (stages 5-8) creating a coherent column or bandlet of blast cells. The five bandlets on each side of the embryo come together in parallel arrays called the left and right germinal bands (stage 7; Fig. 1D). The germinal bands and the area between them is covered by a provisional embryonic integument which consists of embryonic epithelium derived from the micromeres together with underlying contractile fibers of mesodermal origin. Within the bandlets the blast cells undergo stereotyped divisions such that each bandlet has a characteristic sequence of

large and small cells which are identified by their position and by the size of their nuclei (Zackson, 1984). Through a series of morphogenetic movements during stages 7-8, the germinal bands become aligned along the ventral midline of the embryo forming a sheet of cells called the germinal plate from which the definitive segmental tissues arise during stages 9-11. The movements of the germinal bands are accompanied by expansion, or epiboly, of the overlying embryonic epithelium. Thus, by the time germinal plate formation is completed, the embryo is sheathed in epithelial cells.

*Morphometric Analysis of Inequality of Second Division in Normal Development*

The unequal cleavage of cell CD was examined quantitatively by morphometric analysis of embryos fixed and sectioned just after completion of the second cleavage division. Yolk and yolk-deficient cytoplasm can be distinguished readily when viewed with DIC optics (Fig. 2a). The cell volumes and volumes of yolk-deficient cytoplasm (teloplasm plus perinuclearplasm) for cells C and D from 80 intact embryos are shown in Figs. 3a and 3b, respectively. A broad distribution of cell and teloplasm volumes was obtained, which was not significantly altered by normalizing the volume of the yolk-deficient cytoplasm by plotting it as a fraction of the total cell volume (Fig. 3c). In general, larger volumes of yolk-deficient cytoplasm in C correlated with larger volumes in cell D (Fig. 4A). However, the distribution of volumes of yolk-deficient cytoplasms in control embryos showed a completely bimodal distribution; every D blastomere contained more yolk-deficient cytoplasm than any C blastomere (Fig. 3b). In contrast, there was some overlap between distributions of cell volumes of control C
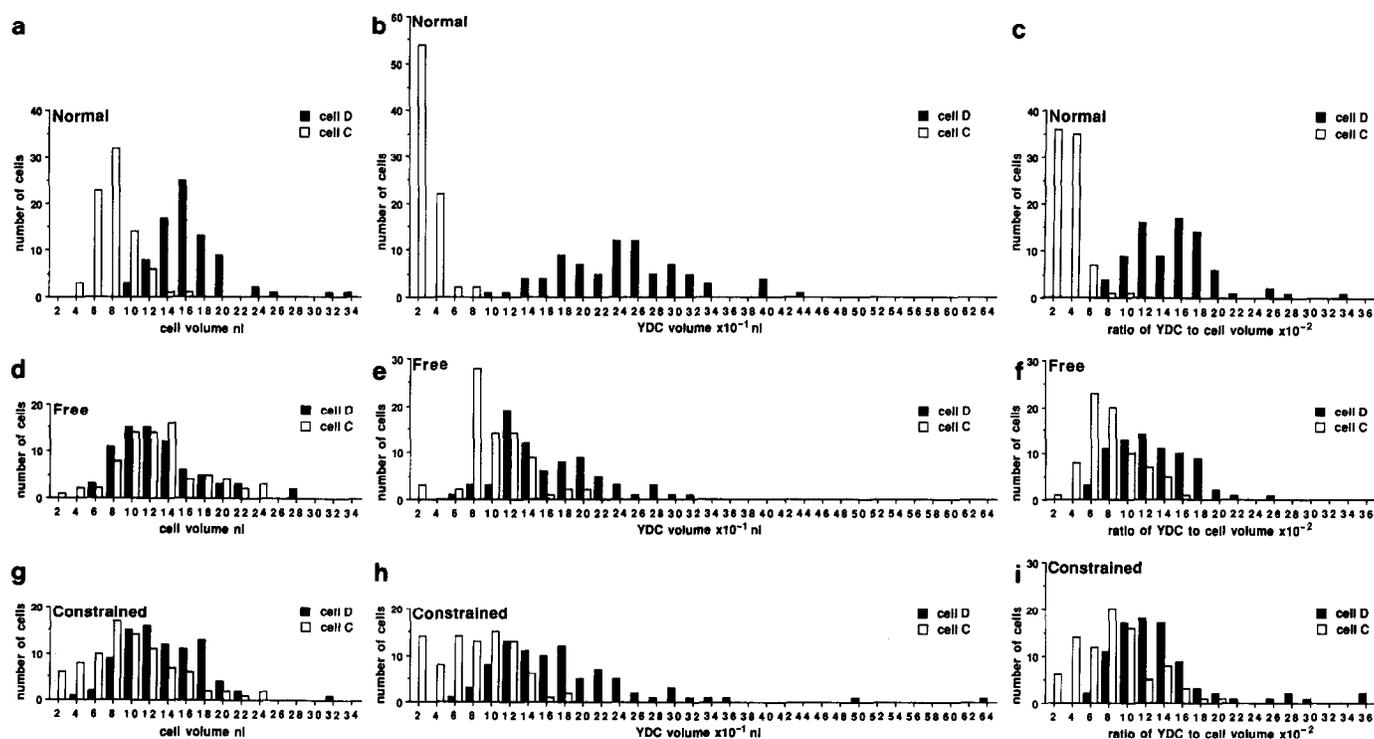
FIG. 3. Morphometric analysis of cells C and D measured from normal embryos and cells "C" and "D" of CD isolates; histograms show the distribution of volumes measured for whole cells (a, d, and g) and for yolk-deficient cytoplasm (YDC; b, e, and h), and for the ratio of YDC to total cell volume (c, f, and i). Values on the *x* axis note the maximum of a particular bin and are represented by open bars for cells C or "C" and by closed bars for cells D or "D". Distributions are shown for control embryos (a-c), free CD isolates (d-f), and constrained CD isolates (g-i).

and D blastomeres (Fig. 3a). Nonetheless, within each embryo, the volume of cell C was less than that of cell D.

The average cell and yolk-deficient cytoplasm volumes calculated for cells A, B, C, and D from the same set of fixed and sectioned control embryos are shown in Table 1. These data confirm qualitative observations that cell D is normally the largest cell and contains almost all of the teloplasm and that teloplasm is occasionally inherited by cells A, B, and C, although only in small quantities. Much or all of the volume of yolk-deficient cytoplasm in blastomeres A, B, and C is perinuclear-plasm rather than teloplasm (Fernandez, 1980; Fernandez and Olea, 1982). The greater volume of teloplasm in cell D does not fully account for the difference in size between it and cell C, however.

*Embryos Develop Normally in the Absence of a Vitelline Membrane*

The vitelline membranes of leech embryos were removed during the first cell cycle and the embryos were cultured in HL medium on a bed of 0.75% agarose at 23°C (see Materials and Methods). Under these conditions the first unequal cleavage was normal; cell CD in-

herited essentially all of the teloplasm and was larger than cell AB. Immediately following this cleavage division the daughter cells appeared more rounded than usual (compare Fig. 5a with 5d). However, within 1 hr the cells flattened against one another (compare Fig. 5b with 5e). Thus, by the time the embryo began its next round of cleavage divisions the cells were approximately normal in shape (compare Fig. 5c with 5f).

Embryos cultured in the absence of a vitelline membrane developed normally and in synchrony with sibling control embryos with intact vitelline membranes. In the absence of a vitelline membrane the mortality rate of the embryos increased; in one series of experiments, 64% (14/22 cases) of embryos with intact vitelline membranes survived to become juvenile leeches (stage 10) whereas only 25% (28/102 cases) of embryos lacking vitelline membranes survived to this stage. The major cause of death appeared to be bacterial infection. However, in another series of experiments, 70% (78/112 cases) of devitellinized embryos survived to stage 8 (the stage at which the germinal bands begin to coalesce to form the germinal plate). A similar proportion of control embryos cultured within their vitelline membranes (17/22 cases; 77%) also survived to stage 8.
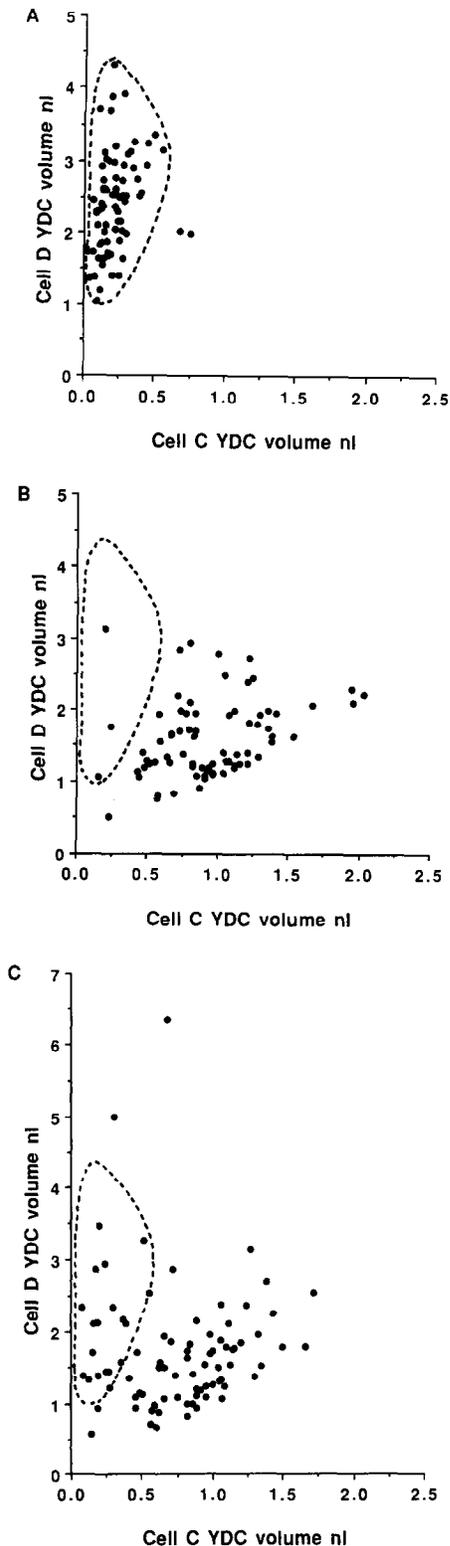
FIG. 4. Scatter plots showing the correlation between the volumes of yolk-deficient cytoplasm in sibling C and D blastomeres in (a) control embryos, (b) free CD isolates, and (c) constrained CD isolates. The dotted line indicates a domain that encompasses 98% of control embryos.

TABLE 1
CELL AND YOLK-DEFICIENT CYTOPLASM VOLUMES OF CELLS
IN INTACT EMBRYOS

| Cell | Cell volume (nl) ± standard deviation | Yolk-deficient cytoplasm volume (nl) ± standard deviation |
|---|---|---|
| A | 8.75 ± 2.93 | 0.21 ± 0.18 |
| B | 7.80 ± 2.25 | 0.16 ± 0.11 |
| C | 9.19 ± 2.11 | 0.22 ± 0.13 |
| D | 17.42 ± 3.88 | 2.39 ± 0.69 |

*Isolated Blastomeres Exhibit Abnormal Cleavages, but Some Aspects of Normal Fates Are Preserved*

Since the unequal first cleavage of devitellinized embryos was normal, AB and CD blastomeres could be identified unambiguously, even after they were disaggregated and had rounded up (Fig. 6). The fates of isolated AB and CD blastomeres are described separately below.

*Cell AB.* During normal development cell AB, the smaller daughter cell of the first cleavage division divides equally and its daughter cells, A and B, each produce three micromeres in the next 14 hr of development (at 23°C; Bissen and Weisblat, 1989). When cell AB was cultured in isolation, however, it exhibited more variability. After overnight culture, isolated AB cells had generated either one (46/124 cases; Fig. 7a), two (44/124 cases; Fig. 7b), three (32/124 cases; Fig. 7c), or four (2/124 cases; Fig. 7d) macromeres. Several micromeres were also generated from each AB isolate; however, the precise numbers of micromeres derived from the isolates was not determined. It is, therefore, possible that the extra macromeres are produced at the expense of micromeres.

The micromeres produced by AB isolates did not undergo epiboly but remained restricted to a compact cluster of cells (Figs. 7a–7e). In those AB isolates with three or four macromeres the micromeres formed at the junction between cleavage furrows (Figs. 7c and 7d). The localization of the micromeres was clearly observed when the isolates were stained with the nuclear stain Hoechst 33258 (Fig. 7e). The failure of the micromeres and their derivatives to spread was consistent with the observation (Ho and Weisblat, 1987) that in early stage 8 embryos the A- and B-derived micromeres are restricted to a strip above and within the anterior germinal plate and have not participated in the epiboly of the provisional epithelium.

*Cell CD.* When CD, the larger daughter cell of the first cell division, was cultured in isolation it also cleaved atypically. Isolated CD cells usually made their next di-
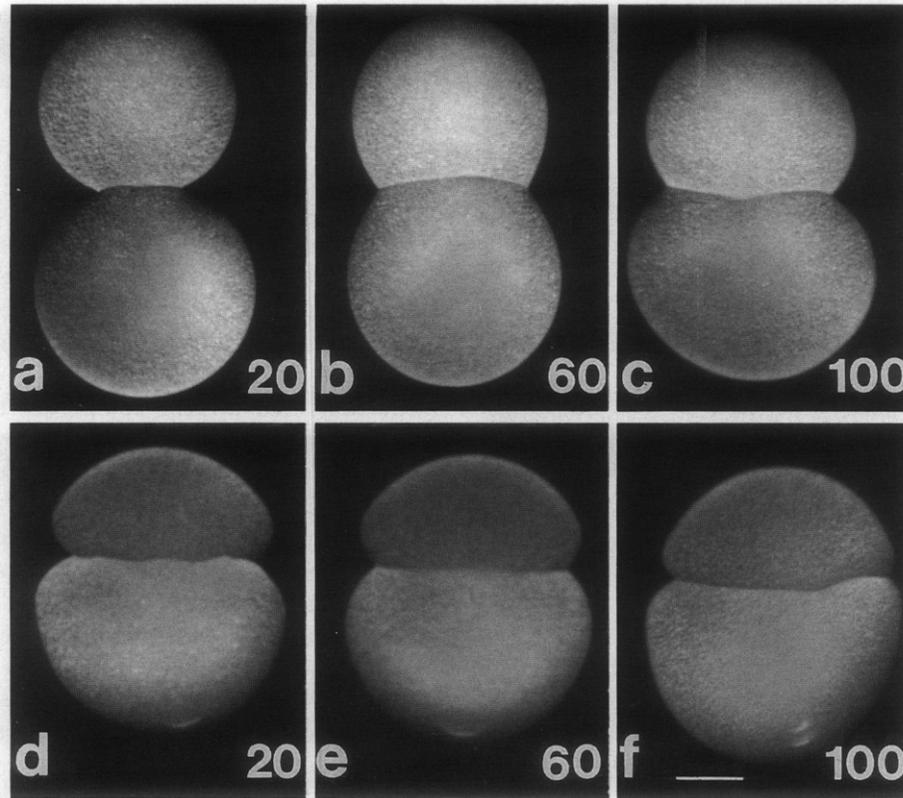
FIG. 5. Cell shape changes in devitellinized (a–c) and control (d–f) embryos between first and second cleavage (stage 2). The elapsed time, in minutes, since the onset of first cleavage is indicated in the bottom right of each panel. Cell AB is the upper cell in each embryo. (a) In the absence of the vitelline membrane AB and CD round up during cleavage. (b) As the cell cycle proceeds, however, the cells flatten against one another, so that by the time CD begins to cleave (c) the cells have largely assumed their normal shapes. (d–f) In embryos with intact vitelline membranes, the cells remain flattened against one another throughout stage 2. Scale bar, 100 μm.

vision (corresponding to second cleavage) in synchrony with sibling control embryos, but tended to divide equally (78/109 cases). Moreover, each daughter cell appeared to inherit teloplasm (72/109 cases; Fig. 8a). Unlike C and D cells in control embryos, there no longer appeared to be a strict correlation between relative cell volumes and relative inheritance of teloplasm by cells "C" and "D" in isolates.

To confirm these qualitative observations, another series of 75 CD isolates was fixed and sectioned for morphometric analysis just after they had divided (Fig. 2b). In contrast to the intact embryos discussed above (Figs. 3a–3c and 4a; which were siblings of the embryos used in this experiment), the CD isolates exhibit an almost coextensive distribution of the cell volumes of "C" and "D" blastomeres (Fig. 3d). Furthermore, the distributions of yolk-deficient cytoplasm for the two sets of cells, while still skewed, also overlapped extensively, whether plotted independently (Fig. 3e) or as a fraction of total cell volume (Fig. 3f). Only 4% (3/75; Fig. 4B) of the isolates showed a distribution of yolk-deficient cytoplasm comparable to that found in the control embryos. Moreover,

within pairs of sister blastomeres, the "D" blastomere did not invariably possess the greater cell volume. Cell pairs were observed in which cell "D" clearly inherited most of the teloplasm and yet was smaller than its sister cell. Thus, the link between cell volume and inheritance of teloplasm at second cleavage seen in control embryos was uncoupled in CD isolates.

The next division of CD isolates, which corresponds to third cleavage, was also abnormal, although it occurred in synchrony with the division of control embryos. This division is normally highly unequal and gives rise to micromeres (c' and d') and macromeres (C' and D'). In CD isolates, however, usually one or both daughter cells cleaved approximately equally (39/58 cases; Fig. 8b). Further divisions of CD isolates were variable and were not monitored precisely. However, teloblast-like cells were seen after overnight culture (Fig. 8c). Furthermore, when sibling control embryos had reached stage 8, CD isolates appeared to contain macromeres, teloblasts, epithelium, and bandlets (Fig. 8d). Epithelia and bandlets could be seen more clearly when the isolates were stained with Hoechst 33258 (Figs. 8e and 8f) or when a
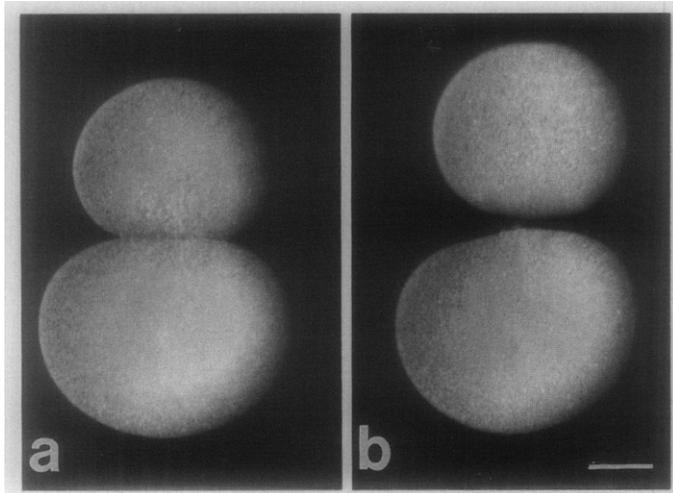
FIG. 6. Dissociation of AB and CD blastomeres at the first cleavage division. (a) Devitellinized embryos at the two-cell stage. (b) Dissociated AB (upper) and CD (lower) blastomeres. Scale bar, 100 μm.

putative teloblast derived after overnight culture of a CD isolate was microinjected with a fluorescent lineage tracer (Fig. 8g).

Although most CD isolates did form bandlets (115/150 cases), these bandlets usually contained fewer blast cells than those in control embryos and were disorganized, extending apparently randomly over the surface of the isolate (Fig. 8e). In some cases (24/150), however, the bandlets were aligned with one another into structures resembling germinal bands (Fig. 8f). In rare cases (14/150) the pattern of large and small nuclei in a portion of a bandlet was reminiscent of that associated with a particular cell lineage. However, the bandlets formed by typical CD isolates were difficult to identify because they were disorganized, making it only possible to trace them for short distances. To investigate the identity of bandlets in CD isolates more conclusively, putative teloblasts derived after overnight culture of a CD isolate

were microinjected with a fluorescent lineage tracer. When sibling control embryos reached control stage 8 the injected isolates were fixed, stained with Hoechst 33258, and examined (Fig. 8g). None of the bandlets examined by this procedure ($n = 43$) appeared to possess a completely normal pattern of nuclei.

Previous studies (Astrow et al., 1987) have demonstrated that teloblast formation is correlated with the inheritance of teloplasm. As described above, CD isolates tended to cleave equally with both cells inheriting teloplasm. To test whether both daughter cells would be capable of making teloblasts, CD isolates were cultured until they had almost completed their first division, that is, when sibling control embryos are cleaving to form cells A, B, C, and D. At this time the daughter cells, "C" and "D," were separated and cultured in isolation until sibling control embryos reached stage 8. These secondary isolates were then stained with Hoechst 33258 and examined. As expected, sibling "C" and "D" isolates both produced teloblasts and bandlets in a majority (6/11) of the cases examined (Figs. 8i and 8j). In the remaining five cases, the development of one or both daughter cells appeared to be arrested shortly after teloblast formation.

In contrast to the progeny derived from micromeres in AB isolates, those arising from micromeres in CD isolates appeared to undergo epiboly. To confirm this, CD isolates at sibling control stage 8 were incubated in a silver methenamine stain, the reaction product of which outlines superficial cells, and then counterstained with Hoechst 33258. An epithelial layer of cells overlying the bandlets was revealed (Fig. 8h). As in control embryos, the epithelium covered only the bandlets and the areas between them and did not extend over the macromere-like cells.

The results of the preceding experiments showed that when AB and CD were separated they expressed many aspects of their normal, distinct fates, although their
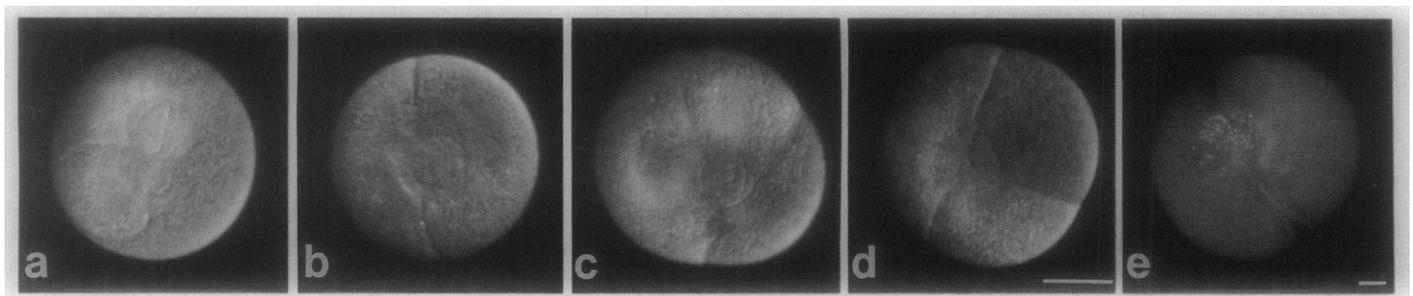


FIG. 7. The development of AB isolates cultured for 24 hr contain (a) one, (b) two, (c) three, or (d) four macromeres and several micromeres. (e) An AB isolate that had generated two macromeres was fixed, stained with Hoechst 33258, then flattened slightly with a coverslip. The micromeres do not undergo epiboly. Nuclei are restricted to the cleavage furrow between the macromeres. Scale bars, 100 μm; scale bar in d applies to frames a–d.

cleavage patterns became both abnormal and variable. One interpretation for these results is that cells AB and CD are capable of extensive, independent development and that the abnormal and variable developmental patterns observed in isolates result entirely from damage incurred during the isolation procedure. A second possibility is that blastomere isolation interrupts interaction(s) between cells AB and CD necessary for normal development. Such interaction(s) could be via some sort of biochemical signaling (i.e., an inductive interaction) or via shape constraints imposed by each cell upon the other (i.e., a mechanical interaction). In the experiments reported below, we sought to assess the relative contributions of damage, interrupted inductive interactions, and interrupted mechanical interactions to the abnormal and variable development of isolated CD blastomeres.

### Reaggregation of Cells AB and CD

To test the notion that the variable and abnormal cleavages of isolated AB and CD blastomeres result from damage incurred during the isolation procedure, we investigated the viability of reaggregated embryos. For this purpose, daughter cells AB and CD were dissociated during the first cleavage division as described above, except that in this experiment care was taken to keep each cell with its original partner because siblings within a batch of leech embryos do not cleave synchronously. Sibling AB and CD blastomeres were then recombined within 1 hr of their isolation in embryo-sized wells cast in agarose (see Materials and Methods). Cell AB was inserted into one end of a well and then cell CD was pushed into the well with it. The cells were carefully oriented so that they assumed their original positions with respect to one another (Figs. 9a–9c). To assess the success of these reconstitution experiments, the embryos were removed from the wells after the next cleavage (at the four-cell stage) and further cleavages were monitored by direct observation.

In 26/79 cases, the recombined cells annealed to form a coherent embryo and underwent apparently normal second and third cleavages, to form macromeres A'–D' and the primary quartet of micromeres a'–d' (Figs. 9d–9f). During experiments in which the later divisions of such recombinants were monitored 5/10 embryos cleaved to form cells DNOPQ and DM (Figs. 9g–9i). Of these 5, 2 cleaved further to form 2 M teloblasts (Figs. 9j–9l) and 2 NOPQ cells (the precursor cell for teloblasts N, O/P, O/P and Q; Figs. 9m–9o). Furthermore, one recombinant embryo developed normally until one of its OP teloblasts had divided, at which point cleavages stopped (not shown). Of the remaining 53/79 cases, 16

divided so that the cleavage furrows of cells AB and CD were perpendicular to one another, probably due to the inaccuracy of the orientation of blastomeres within the wells, and in 37 cases either one or both cells failed to cleave. Such recombinants were not followed further.

Due to the technical difficulty of these experiments and the overriding interest in observing their cleavage pattern beyond the four-cell stage, no morphometric analysis was carried out on recombinant embryos. Nonetheless, from these results we conclude that experimental trauma does not account for all the observed abnormalities of isolated CD blastomeres, especially since the success rate for the reaggregation experiment must be greatly reduced because of the additional trauma inflicted while recombining the blastomeres. Thus, we conclude that inductive and/or mechanical interactions between cells AB and CD are important for normal development.

It is difficult to distinguish the relative contributions of inductive and mechanical interactions between cells without isolating the cells mechanically as well. However, when cell AB is poisoned by microinjection at the two-cell stage with ricin A chain (a potent cytotoxic protein that in a variety of cell types has been shown to inactivate ribosomes catalytically; Endo *et al.*, 1987; Endo and Tsurugi, 1987; Youle *et al.*, 1979; Fulton *et al.*, 1986; Vitetta, 1986) cell CD is able to undergo its normal cleavage pattern even though AB fails to divide (B. H. Nelson and D. Lans, personal communication). This suggests that a biochemical inductive event may not be necessary for the interaction between cells AB and CD in normal development. Moreover, since cell AB remains intact after injection with ricin (D. Lans, personal communication; Nelson and Weisblat, 1992) it continues to deform cell CD in these experiments. Thus, the following series of experiments was carried out to assess the importance of mechanical deformation in influencing the cleavage pattern of cell CD.

### Deforming Cell CD in Vitro Using a Synthetic Bead in Place of Cell AB

In this series of experiments, isolated CD cells were gently pushed into agarose wells containing single Sephadex beads (G-100, Pharmacia) of about the same size as, and oriented to occupy the normal position of, cell AB (100 $\mu$m diameter; Fig. 10a). CD isolates manipulated in this way will be referred to as constrained CD isolates while unmanipulated CD isolates will be called free CD isolates. Cell volumes and the teloplasm content of the daughter cells of constrained and free CD isolates and intact sibling controls were compared qualitatively and by morphometric analysis of sectioned specimens.
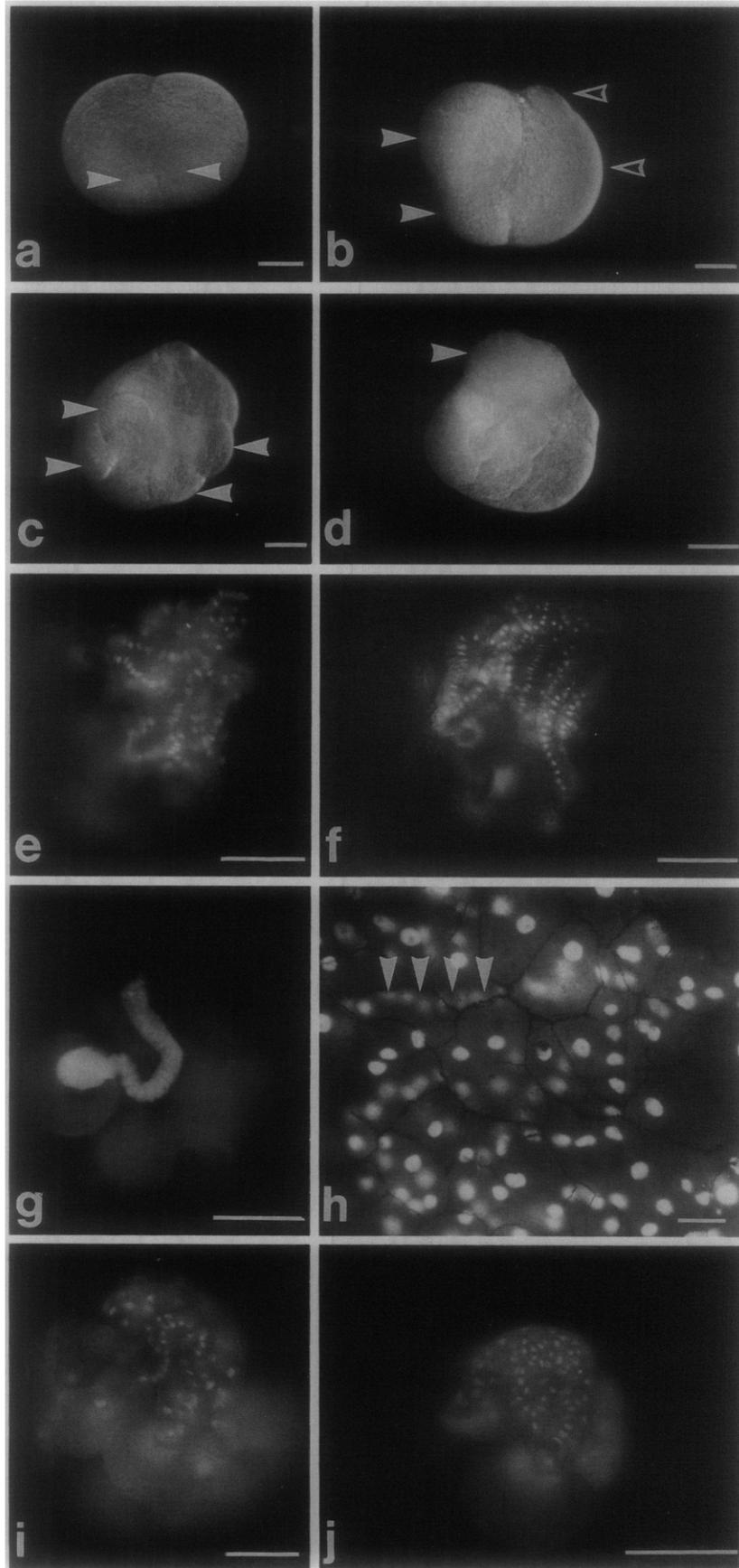
In one series of experiments, 31/40 (78%) of constrained CD isolates cleaved unequally and 35/40 (88%) showed an unequal teloplasm distribution as judged by visual inspection, although in most cases both cells appeared to inherit some teloplasm (Fig. 10b). By contrast, free CD isolates cleaved unequally and the teloplasm was unequally distributed in only 3/20 cases (15%). In one of these experiments, further cleavages of the isolates were monitored. At the next cell division constrained CD isolates formed apparently normal micromeres in 7/12 cases (Fig. 10c) while only 2/8 of the free CD isolates formed micromeres. Further cleavages of all the free CD isolates were typically abnormal. However, 7/12 constrained CD isolates cleaved to form DNOPQ and DM cells, and 5/12 completed their next division, forming two M teloblasts. All sibling control embryos, including those cultured within or in the absence of their vitelline membranes, developed normally.

In a second series of experiments, additional free and constrained CD isolates were scored qualitatively, as described above, after the second cleavage division of sibling control embryos. They were then fixed and sectioned, along with intact sibling controls, for quantitative morphometric analysis. In accord with the first series of deformation experiments, 49/86 (57%) of the constrained CD isolates and 20/75 (27%) of the free CD isolates appeared to cleave unequally. An unequal teloplasm distribution was observed in 57/86 (66%) of constrained CD isolates and 18/75 (24%) of free CD isolates. The morphometric analysis of the free CD isolates and the control embryos have been described above (Figs. 3a–3f, 4A, and 4B). The distributions of cell and teloplasm volumes of the constrained CD isolates (Figs. 3g–3i, and 4C) were clearly different than the controls. However, a significant restoration of the normal inequality of the second division had occurred in two respects. First, the observed distributions of cell and teloplasm volumes in constrained CD isolates were intermediate between those of homologous cells from control embryos and free isolates; 22% (19/86 Fig. 4C) of the constrained isolates but only 4% (3/75 Fig. 4B) of the free CD isolates showed a comparable distribution of yolk-deficient cytoplasm to that found in the control

embryos (Fig. 4A). Second, among the constrained embryos the coupling between cell size and teloplasm volume was for the most part restored, so that the "D" blastomeres were generally larger than their sister "C" blastomeres. This was not the sole cause of the change in teloplasm distribution, however, since the distribution of the ratios of yolk-deficient cytoplasm to total cell volume in constrained embryos was also changed so as to more closely resemble that of the control embryos (Figs. 3c, 3f, and 3i).

### Deforming Cell CD in Vitro Using a Synthetic Bead Placed Ectopically to the Normal Position of Cell AB

Additional experiments were performed in which isolated CD blastomeres were deformed by beads placed at 90° with respect to the normal position of cell AB (Fig. 11a). The cells were cultured until they had cleaved to form cells "C" and "D" (Fig. 11b) and then scored by visual inspection. In four experiments, the cleavage of 13/16 (81%) of such deformed CD isolates resembled that of cell CD in normal embryos; cell "D" contained appreciably more yolk-deficient cytoplasm and was also larger than cell "C." Similar results were obtained with 14/21 (67%) of sibling embryos constrained with the bead in the normal position of cell AB. In contrast, only 1/21 (5%) of free CD isolates cleaved unequally in these experiments.

### DISCUSSION

In the experiments reported here, we have examined factors influencing unequal cell divisions in early development. We find that development of *H. triserialis* embryos proceeds normally in the absence of the vitelline membrane and that sister blastomeres can be isolated from one another during cleavage. This makes it possible for us to investigate the influence of internal and external factors on subsequent cleavages in isolated blastomeres. We have focused on the unequal division of blastomere CD at second cleavage.

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FIG. 8. The development of CD isolates. (a) The first cleavage division of a typical CD isolate is equal and teloplasm (arrowheads) is inherited by both cells. (b) The second division, which should form macromeres and micromeres, is only slightly unequal in one of the daughter cells (solid arrowheads) and highly unequal in the other (open arrowheads). (c) Putative teloblasts (arrowheads) have formed after overnight culture. (d) After 3 days in culture, a typical isolate contains macromeres, teloblasts, bandlets (arrowhead), and micromeres. (e) The bandlets are more clearly seen when stained with the nuclear stain Hoechst 33258. (f) In this case, the bandlets formed roughly parallel arrays. (g) After 24 hr in culture, a putative teloblast was injected with RDA; after two more days in culture, a labeled bandlet had been produced. (h) Micromeres produced by CD isolates appear to undergo epiboly. All the nuclei have been stained with Hoechst 33258. Cells in the superficial layer, outlined by the black silver methenamine reaction product, lie over the cells of the bandlets (one of which is identified by arrowheads). (i and j) Daughter cells "C" and "D," respectively, separated after the equal cleavage of a CD isolate, both produced bandlets. Scale bars, 100 μm, except in h where it is 20 μm.
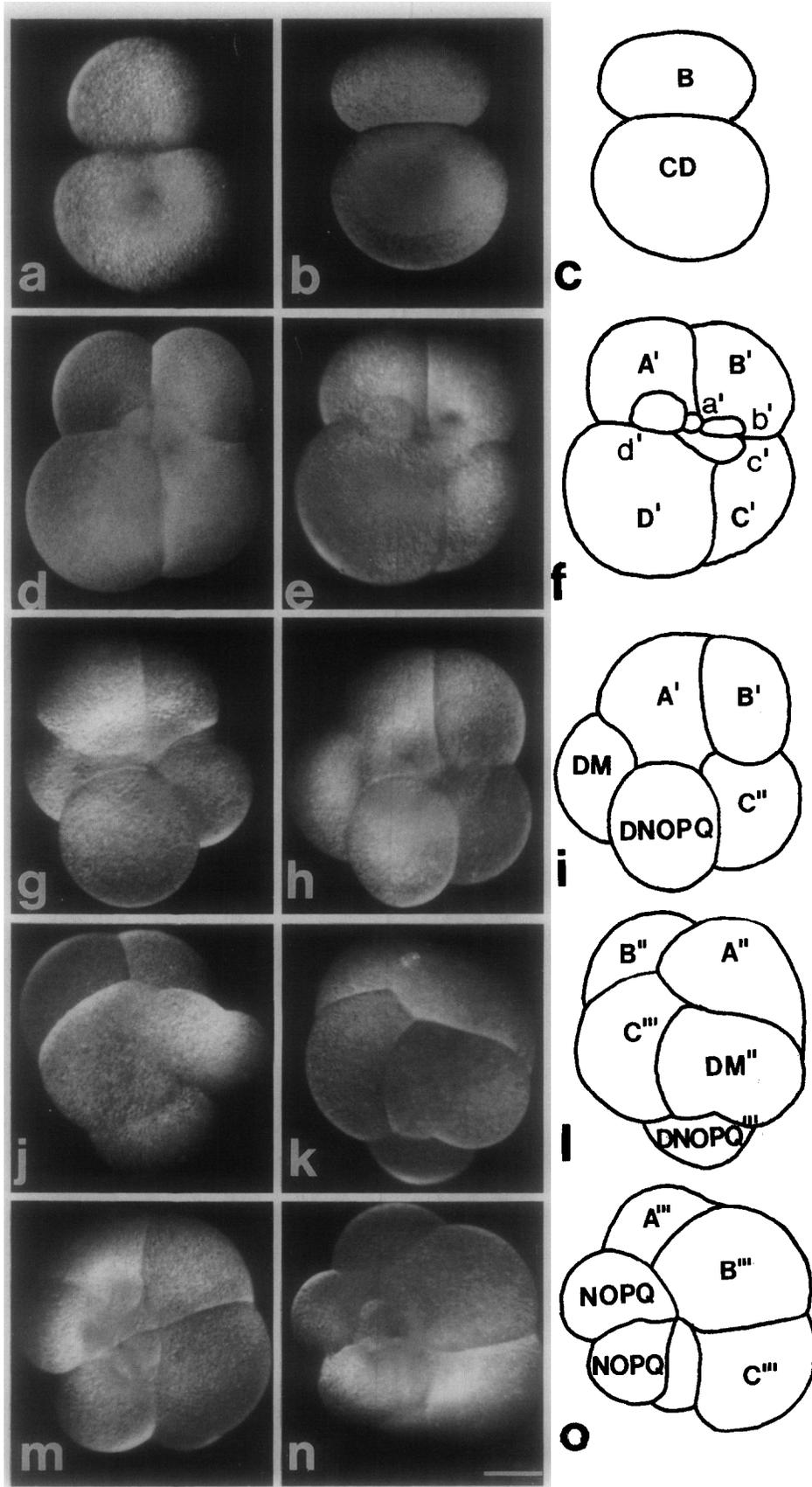
## *Isolated Blastomeres Retain Distinct Developmental Potential*

When blastomeres AB and CD are dissociated, they lose their normal stereotyped cleavages but retain some features of their normal fates. CD isolates form teloblasts and bandlets, although none of the bandlets appear fully normal. Isolated AB cells undergo fewer cell divisions than CD isolates, produce micromeres and macromeres, but do not produce teloblasts or bandlets. This observation is consistent with the notion that the subsequent fates of these two cells are correlated with the presence or absence of teloplasm (Astrow *et al.*, 1987; Nelson and Weisblat, 1991, 1992). Although both AB and CD isolates produced micromeres, epibolic spreading of the micromere-derived epithelia was observed only in CD isolates. This difference may reflect intrinsic differences in the properties of AB- versus CD-derived micromeres; alternatively, it is possible that bandlets are required for epiboly of the micromere derivatives.

## *Cell Shape Contributes to the Normal Unequal Cleavage of Cell CD*

The positions of the cleavage furrows in isolated blastomeres are abnormal and variable. The cleavage of isolated CD blastomeres typically produces daughter cells of approximately equal size and teloplasm content. This is consistent with the observation that both daughters frequently generate teloblasts. One indication of the shift toward an equal second cleavage division in CD isolates is illustrated by the change in the volume of yolk-deficient cytoplasm in "C" blastomeres: 56% inherit more than the largest volume of yolk-deficient cytoplasm found in control C blastomeres. Another indication of the shift toward an equal second division in CD isolates is the decrease of the mean ratio of the volumes of yolk-deficient cytoplasm in daughter cells of CD blastomeres. The mean ratio of the volume of yolk-free cytoplasm of cell D to cell C in control embryos is 15.81 ± 15.40 (mean ± standard deviation) whereas in free CD isolates, the comparable ratio between cells "D" and "C" is only 2.11 ± 1.91. (An equal division would have a ratio of 1.) This difference between free CD isolates and control embryos is highly significant ($P < 0.01$).

The observations that isolated CD cells tend to undergo equal rather than unequal divisions and that normal cleavages are largely restored by recombining the isolated blastomeres suggest that either inductive or mechanical factor(s) arising from cell AB are essential for producing the normal unequal division of the CD blastomere. A role for mechanical factors might at first seem unlikely in specifying an unequal cleavage division because *Helobdella* zygotes develop normally when removed from the confines of the vitelline membrane. However, we found that cells AB and CD adhere and deform one another following cleavage even in the absence of the vitelline membrane. Furthermore, we found that mimicking the natural deformation of CD blastomeres with dextran beads substantially restored the normal inequality of the second cleavage, both in terms of teloplasm distribution and total cell volume.

An important caveat to this conclusion is the possibility that the beads themselves exert an inductive effect on cell CD. This seems unlikely for two reasons. First, CD isolates cultured in contact with beads but not deformed by them undergo equal cleavages. Second, the beads did not adhere to the CD isolates, suggesting that biochemical interactions inherent in adhesion are unlikely to account for the effects of deforming CD isolates with beads.

In most cases sister blastomeres "C" and "D" arising from constrained CD isolates were unequal in size and teloplasm content, and these constrained isolates underwent further cleavages reminiscent of their normal fates, including the formation of micromeres and teloblast precursors. However, in most constrained isolates, the inequality of the second cleavage was not fully restored. The average ratio of the volumes of yolk-deficient cytoplasm between cells "C" and "D" in constrained isolates (4.10 ± 4.94) lay between that of cells "C" and "D" in free CD isolates and that of cells C and D in control embryos and was significantly different ($P < 0.01$) from both. One possibility is that the degree of inequality of the cleavage may be sensitive to the exact timing and extent of the deformation, so that the failure to rescue reflects our inability to exactly mimic the normal deformation. Nevertheless, the results of experiments presented here are consistent with the notion that mechanical interactions between cells AB and CD are necessary for the correct placement of the second cleavage furrow during the development of the leech.

FIG. 9. Development of reaggregated embryos. Left column (a, d, g, j, and m) shows a control embryo that is a sibling of the reaggregated embryo shown in the center column (b, e, h, k, and n). Line drawings of the reaggregated embryo are in the right column (c, f, i, l, and o). All frames are views from the animal pole of the embryo unless otherwise stated. (a, b, and c) Two-cell embryos (stage 2). (d, e, and f) Eight-cell embryos (stage 4a). (g, h and i) Cell D' cleaved to form cells DM and DNOPQ (stage 4b). (j, k and l) Cell DM", viewed from the vegetal pole, is dividing to form 2 M teloblasts (stage 4c). (m, n and o) Cell DNOPQ''' divided to form 2 NOPQ blastomeres (stage 5). Scale bar, 100 μm.
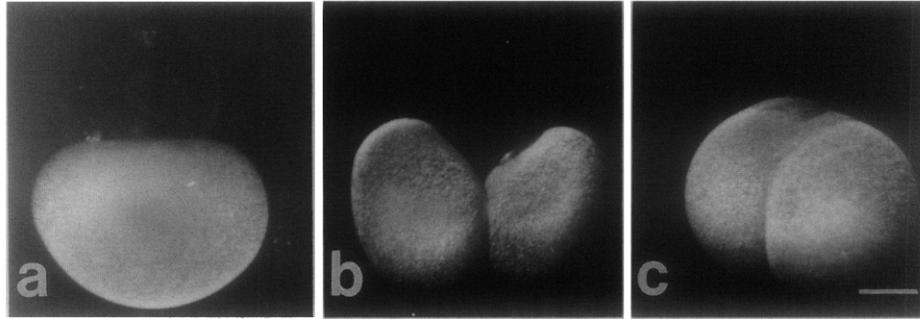
FIG. 10. Development of constrained CD isolates. (a) Cell CD (bottom) constrained in an agarose well with a synthetic bead (top) to mimic its *in vivo* shape (compare to Fig. 5f). (b) A constrained CD isolate cleaved unequally in terms of both cell volume and teloplasm content (compare to Fig. 8a). (c) A constrained CD isolate removed from the well after second cleavage has formed apparently normal c′ and d′ micromeres (compare to Fig. 8b). Scale bar, 100 μm.

## *Relationship of Cell Shape to Cleavage Orientation*

The mechanism(s) by which cell shape influences the orientation and position of the mitotic spindle, and hence the location of the cleavage furrow, remain unclear. One factor known to influence the cleavages of some embryonic cells is the presence of specific microtubule attachment sites in the cell cortex that have been implicated in affecting the movements of nuclei in the vegetal quadrant cells of the eight-cell sea urchin embryo (Dan *et al.*, 1983), the movements of the centrioles which orient the spindle in the $P_1$ cell of the two-cell nematode embryo (Hyman, 1989), and the positioning of the mitotic apparatus during the first cell cleavage of the mollusc embryo (Dan and Ito, 1984). Based on homologies between leech and mollusc embryos, it seems likely that similar cortical sites are present in leech

blastomeres; thus, it is possible that mechanical constraints in leech embryos could influence the equality of cell division by affecting the distribution of cell cytoplasm relative to a mitotic spindle tethered to the cell cortex. Our observation that cell CD cleaves unequally after CD blastomeres were deformed either to mimic their natural mechanical interaction with cell AB or along an orthogonal axis is consistent with this model. However, when leech embryos are gently centrifuged prior to the second cleavage division, the cleavage furrow of cell CD often forms ectopically, sometimes resulting in "C" and "D" cells that are transposed with respect to C and D cells in a normal embryo (Astrow *et al.*, 1987). It is unlikely that such mild centrifugation is sufficient to displace cortical features. It seems, therefore, that such sites are not required for unequal cleavages but may serve to bias the orientation of the cleavage furrow so that it consistently forms in a particular position during normal development.
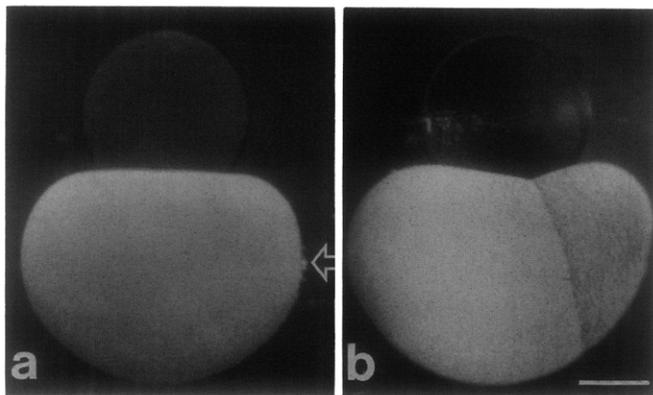
FIG. 11. Cleavage of a CD isolate constrained by a bead placed ectopically to the normal position of cell AB. (a) Photomicrograph of a CD isolate (bottom) constrained in an agarose well by a Sephadex bead (top) at 90° from the normal position of cell AB (open arrowhead). (b) After 2 hr, the cell has cleaved unequally both in terms of cell volume and in terms of teloplasm distribution. Scale bar, 100 μm.

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