Cellular mechanisms of epiboly in leech embryos

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SUMMARY

Gastrulation in leech embryos is dominated by the epibolic movements of two tissues: germinal bands, composed of segmental precursor cells, and an overlying epithelium that is part of a provisional integument. During gastrulation, the germinal bands move over the surface of the embryo and coalesce along the prospective ventral midline. Concurrently, the epithelium spreads to cover the embryo. We have begun to analyze the mechanisms involved in gastrulation in the leech by assessing the independent contributions of the epithelium and the germinal bands to these cell movements. Here we describe cellular events during epiboly in normal embryos and in embryos perturbed by either reducing the number of cells in the epithelium, or by preventing the formation of the germinal bands, or both. These experiments indicate that both the germinal bands and the epithelium are able to undergo epibolic movements independently, although each is required for the other to behave as in control embryos.

Key words: epiboly, gastrulation, leech, annelid

INTRODUCTION

Gastrulation is a fundamental process in animal development, during which cells rearrange to form distinct germ layers and begin to elaborate the adult body plan (Trinkaus, 1984a). To understand gastrulation, one must analyze the contributions of specific cells and tissues to these rearrangements. Gastrulation entails the *internalization* of mesodermal and/or endodermal precursors by involution, invagination, delamination and/or ingression of precursor cells. It often also involves *epiboly*, the spreading of an epithelium, usually of ectodermal cells, over the embryo (Trinkaus, 1984a). The relative contributions of these two processes vary widely among species. For example, internalization is prominent during the gastrulation of anuran amphibians but is difficult to observe in teleost fish (Wood and Timmermans, 1988). In these vertebrates, epiboly begins prior to internalization (Keller, 1980). Among invertebrates, echinoderms exhibit prominent internalization but little or no epiboly. In contrast, epibolic movements dominate gastrulation in mollusks and annelids, but begin only after the modest cell movements constituting internalization.

The variability in gastrulation processes between taxa raises questions as to how similar they are mechanistically. To resolve these issues, it will be necessary to analyze and compare these processes in a variety of organisms. Toward this end, we have begun to analyze gastrulation processes in glossiphoniid leeches (phylum Annelida).

In glossiphoniid leeches, internalization occurs after the seventh cleavage in the D cell line (stage 4c; about 13 hours after zygote deposition in *Helobdella robusta*; Fig. 1A), when the two mesodermal precursors (*M teloblasts*) are born and sink into the large underlying endodermal precursors

(*macromeres A‴, B‴* and *C‴*). Epiboly becomes apparent during late stage 7, about 60 hours after zygote deposition, and involves coordinated movements by two tissues, the *germinal bands* and the *provisional epithelium* (Fig. 1B).

The germinal bands comprise bilaterally paired, parallel arrays of segmental founder cells (*primary blast cells*) and their progeny. The primary blast cells arise during stages 5-8 through the repeated, highly unequal divisions of the pair of mesodermal stem cells (the aforementioned *M teloblasts*) and four bilateral pairs of ectodermal stem cells (*N, O/P, O/P* and *Q teloblasts*). Each teloblast produces a column (*bandlet*) of primary blast cells. The five bandlets on each side come together to form a germinal band in which the ectodermal bandlets are on the surface and the mesodermal bandlet is underneath. By the end of stage 7, the germinal bands extend over the surface of the three yolk-filled macromeres in prospective dorsal territory near the animal pole. During stage 8, the germinal bands elongate further and move ventrovegetally, with the n bandlets (derived from the N teloblasts) at the leading edge. The germinal bands gradually coalesce along the prospective ventral midline of the embryo into the *germinal plate*. Cells continue to proliferate within the germinal plate during stages 9-10, forming definitive segmental tissues, while the lateral edges of the germinal plate expand dorsolaterally and eventually coalesce at the dorsal midline, completing the tubular body plan.

The provisional epithelium covers the germinal bands and the prospective dorsal territory that separates them during stages 7-8. The provisional epithelium is derived from a set of 25 *micromeres* produced during cleavage and is part of the *provisional integument*, a temporary covering for the embryo that also contains some underlying muscle fibers derived from the

M teloblast lineage (Weisblat et al., 1984; Ho and Weisblat, 1987; Smith and Weisblat, 1994). Coincident with the movements of the germinal bands, the provisional epithelium spreads over the embryo such that the leading edges of the two tissues remain close to one another. By the end of stage 8, germinal plate formation is complete and the provisional epithelium has covered the entire embryo. The spreading of the provisional epithelium during stages 7-8 is a clear example of epiboly. The movements of the germinal bands taken alone do not constitute epiboly because they move over the embryo without leaving a continuous sheet of cells behind them. Thus, we will use the term *epibolic movement(s)* to denote the movements of the germinal bands taken alone.

At the onset of epiboly, the leech embryo comprises five major cell types: blast cells and the teloblasts from which they arise, provisional epithelium and deep cells derived from the micromeres, and macromeres (Fig. 1). Of these, the blast cells, the provisional epithelium and the macromeres are clearly involved in epiboly. The two other types of cells, the teloblasts and deep micromere derivatives, do not appear to be actively involved in this process.

To examine the contributions of the provisional epithelium and the germinal bands to these morphogenetic movements, we have described cellular events during epiboly in normal and experimentally perturbed embryos. Our findings indicate that both the germinal bands and the provisional epithelium are able to move independently of each other, although each is required for the other to behave as in control embryos.

MATERIALS AND METHODS

Embryos

Helobdella robusta embryos were obtained from a laboratory colony and cultured following standard procedures (Smith and Weisblat, 1994). The developmental staging system (Stent et al., 1992) is based on that of Fernandez (1980); the cell nomenclature is that introduced by those workers and others (e.g., Bissen and Weisblat, 1989).

Selected blastomeres were pressure injected with a mixture of 60 mg/ml Texas Red-conjugated dextran amine (TRDA; Molecular Probes) and 0.8% Fast Green in 0.2 M KCl using standard procedures (Smith and Weisblat, 1994). Injected embryos were fixed in 0.8% formaldehyde in 0.1 M sodium cacodylic acid (pH 7.4) for 30 minutes at room temperature, after developing for either 44, 58, or 72 hours from the time when one half the clutch was at stage 6a (N teloblast formation; Fig. 1A). Superficial cells in the provisional epithelium were then visualized by silver staining (Smith and Weisblat, 1994). Embryos were rehydrated, then stained by incubation with 1 µg/ml Hoechst 33258 in 0.1 M Tris-HCl buffer (pH 7.4) for 30 minutes at room temperature to label their nuclei and mounted in 70% glycerol/0.1 M Tris-HCl (pH 9.4) for whole-mount viewing on a Zeiss Axiophot

microscope. Embryos to be examined in section were embedded in glycol methacrylate (JB-4; Polysciences, Inc.), then sectioned at 14 µm with a glass knife on a Sorvall microtome (MT2-B).

Preparation of embryos with reduced provisional epithelium

To reduce the number of cells present in the epithelium, the five micromeres that contribute exclusively to the provisional epithelium (dnopq‴, both opq″ and both n′; Fig. 1A; Smith and Weisblat, 1994)
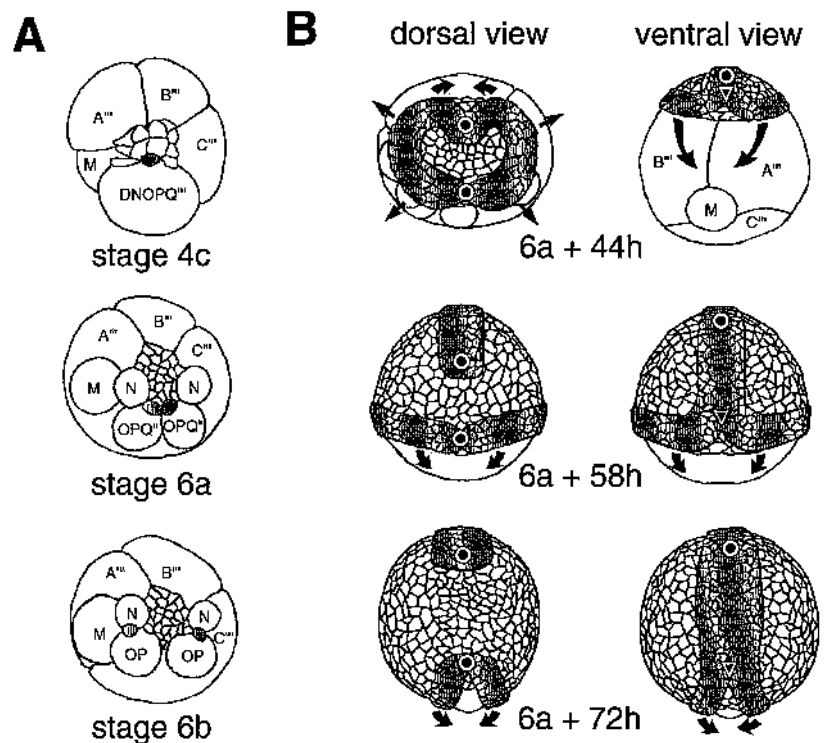

Fig. 1. Depiction of selected stages in leech development. (A) Schematic drawings of *Helobdella* embryos at three stages during cleavage, viewed from the animal pole. Blastomeres relevant to these experiments are labeled, including macromeres (A‴, B‴, C‴), proteloblasts (DNOPQ‴, OPQ″, OP) and teloblasts (M, N); not all teloblasts are visible in these views. The smaller outlines in the center of each embryo represent the distribution of micromeres and their epithelial progeny. The five micromeres that contribute exclusively epithelial progeny are shaded (dnopq‴ in stage 4c; both opq″ micromeres in stage 6a; both n′ micromeres in stage 6b). (B) Schematic representation of the coordinated movements of the germinal bands (stippling) and epithelium (small outlines) during epiboly. The left column presents embryos viewed from the prospective dorsal aspect, which corresponds roughly to the animal pole views shown in A. The right column presents embryos viewed from prospective mid-ventral region. Anterior is up in all panels. The movements of the germinal bands and epithelium are indicated by arrows. In early epiboly (stage 6a + 44 hours), the germinal bands extend over the surface of the embryo in prospective dorsal territory near the animal pole. By mid stage 8 (stage 6a + 58 hours), the germinal bands have elongated, moved across the surface of the embryo and begun coalescing along the future ventral midline to form the germinal plate. By late stage 8 (stage 6a + 72 hours), coalescence of the germinal bands is almost complete. Concurrent with the germinal band movements, the edge of the epithelium spreads so that its margin remains in close proximity with the leading edge of the germinal bands. Thus, by the end of epiboly, the epithelium covers the entire embryo. Circles mark fixed reference points: those in the left column mark the rostral end of the germinal plate and the caudal ends of the germinal bands; those in the right column mark the rostral end of the germinal plate. Triangles mark the caudal progression of germinal band coalescence along the ventral midline during stage 8. Embryo diameter, approximately 400 µm.

were killed shortly after birth by injecting them with a mixture of 30 mg/ml fluorescein-conjugated dextran amine (FDA; Molecular Probes) and 0.4% Fast Green in 0.2 M KCl until they were observed to lyse. To mark the leading edge of the germinal bands in these and control embryos, both N teloblasts were injected shortly after birth with the standard mixture of TRDA and Fast Green. Embryos were then fixed and silver stained at the endpoints described above. After fixation, these experimental embryos were screened to ensure that they contained no cells labelled with FDA, the lineage tracer used for the ablations.

Preparation of embryos with cleavage-arrested teloblasts or proteloblasts

To prevent the formation of ectodermal bandlets, mesodermal bandlets or entire germinal bands, teloblasts and/or proteloblasts were injected with a mixture of 10.8 µg/ml of the A chain of lectin ricin (Sigma), 7.5 mg/ml FDA and 0.4% Fast Green in 0.2 M KCl. In mammalian cells, the ricin A chain modifies the 28 S rRNA, inactivating the ribosomes and inhibiting protein synthesis (Endo and Tsurugi, 1988). In leech embryos, the ricin-injected cells cease cleaving but do not lyse, which minimizes the effect of the perturbation on the geometry of the embryos (Nelson and Weisblat, 1992).

The following cells were injected with ricin: DM″ (the M teloblast precursor), both N teloblasts and both OPQ″ (the O/P and Q teloblast precursors; Fig. 1A). When teloblasts precursors were injected rather than teloblasts, it was to minimize the number of injections required. Cells were injected with ricin only after the completion of cytokinesis associated with the birth of any micromeres they produce, to ensure that all provisional epithelium precursors were present in the resulting embryos. Therefore, DM″ cells were injected after the birth of dnopq‴, since it occurs one hour after the birth of the dm″ micromere; OPQ″ cells were injected when they separate to cleave to OP and Q; and N teloblasts were injected one hour after the birth of their micromeres, n′. To aid the comparison between experimental and control embryos, selected micromeres (opq″$_L$ or dnopq‴), proteloblasts (DM″) or both N teloblasts were injected shortly after birth with the standard mixture of TRDA and Fast Green. Embryos were then fixed and silver stained at the endpoints described above. Ricin-injected cells sometimes underwent one further cleavage but never produced any blast cells, as judged by the distribution of the co-injected FDA lineage tracer and by the Hoechst staining.

In separate experiments, the macromeres, A‴, B‴ and C‴, were injected with ricin (data not shown). The perturbations in gastrulation in those embryos is different than those seen when the germinal band precursors are injected. Thus, we concluded that the developmental perturbations observed when teloblasts and proteloblasts are injected with ricin is not due to a general, toxic effect of the drug.

Morphometry

A graphics workstation (Cubicomp) was used to analyze images of control and experimental embryos otained using a compound microscope (Zeiss Axiophot). Parameters analyzed include: (1) the number of epithelial cells in the micromere cap, (2) the apical surface area of epithelial cells, (3) the aspect ratio of epithelial cells at the margin of the micromere cap and (4) the length of epithelial cells along the margin. Each measurement in each embryo was taken four times and the results were averaged.

The average apical surface area of epithelial cells was examined by

measuring the total surface area of 10 adjoining cells. For consistency, cells were measured in comparable areas of the micromere caps of experimental and control embryos, namely a roughly rectangular region having one side coincident with the margin of the micromere cap. In control embryos, this region coincides approximately with the domain of the opq″$_L$ micromere clone and comprises epithelial cells lying primarily over the germinal bands.

The aspect ratios of marginal epithelial cells reported below were computed by dividing the projected length along the axis perpendicular to the epithelial margin by the projected length along the axis parallel to the margin for each cell. To eliminate the possibility of bias when choosing cells to measure, we measured all the marginal cells in the opq″$_L$ clone; these cells are typical of marginal cells in the micromere cap as a whole. The opq″ clone was also used to measure the length of marginal extent of epithelial cells in normal embryos and in embryos lacking germinal bands. Not all the opq″-derived cells could be measured in the experimental embryos, however, because of their highly irregular epithelial margins.

RESULTS

Epiboly in normal embryos

To generate a more detailed description of cellular events during normal epiboly, the epithelium and the germinal bands were examined in embryos fixed at late stage 7 (stage 6a + 44 hours), mid stage 8 (stage 6a + 58 hours) and late stage 8 (stage 6a + 72 hours). These time points were chosen because they correspond to early, middle and late epiboly, respectively (Fig. 1B). Our findings are described below.

Epithelial cells divide and change shape during epiboly

The area covered by the epithelium increases continuously during epiboly. Our results indicate that the increase in the size of the epithelium is due to both an increase in the number of epithelial cells and to an increase in the surface area of a subset of cells within the epithelium during epiboly.

Inspection of silver-stained embryos confirmed that the number of epithelial cells increases during epiboly. While it was not technically feasible to count all the epithelial cells during mid and late epiboly, examining individual micromere clones marked with lineage tracer revealed that much of the increase in the epithelial cell population during the first half of epiboly can be accounted for by mitoses of epithelial cells themselves. For example, the dnopq‴ and opq″ micromere clones contribute exclusively epithelial cells to the embryo during late stage 7 and throughout stage 8. The opq″$_L$ clone contains, on average, 15.7 cells at late stage 7 (Table 1, Fig. 3A), 44.0 cells at mid stage 8 (Table 1, Fig. 3B) and 49.2 cells at late stage 8 (Table 1, Fig. 3C). The dnopq‴ micromere clone contains, on average, 11.2 cells at late stage 7, 18.1 cells at mid stage 8 and 18.4 cells at late stage 8 (Table 1). The insignificant increase in cell number in the dnopq‴ and opq″ clones

Table 1. Micromere clone size

Micromere	Stage 6a + 44 hours		Stage 6a + 58 hours		Stage 6a + 72 hours	
	Avg. no. cells ± s.d.	n	Avg. no. cells ± s.d.	n	Avg. no. cells ± s.d.	n
dnopq‴ in normal embryos	11.2±1.7	19	18.1±2.2	16	18.4±2.1	10
opq″$_L$ in normal embryos	15.7±1.9	6	44.0±3.6	5	49.2±4.6	6
opq″$_L$ in ricin-injected embryos	11.6±2.5	10	34.0±4.0	6	29.4±9.3	10

Table 2. Shape changes of opq″L-derived marginal epithelial cells during epiboly

	Normal embryos				Ricin-injected embryos			
	Distance on margin in μm		Aspect ratio		Distance on margin in m		Aspect ratio	
	Avg. ± s.d.	n	Avg. ± s.d.	n	Avg. ± s.d.	n	Avg. ± s.d	n
Stage 6a + 44 hours	22.3±11.4	20	0.86±0.36	20	26.2±12.0	20	0.84±0.28	31
Stage 6a + 58 hours	21.4±6.1	30	0.84±0.31	30	23.3±11.8	24	1.13±0.59	29
Stage 6a + 72 hours	7.8±5.3	27	2.64±0.87	27	16.3±12.4	18	1.32±0.66	45

during the second half of epiboly suggests that the increase in the size of the epithelium during that period is not due to an increase in epithelial cell number. We cannot, however, exclude the possibility that the epithelial cell population increases during late epiboly due to contributions from source(s) other than those studied here.

The size of the epithelium also increases because some epithelial cells increase in surface area. This increase in individual cell area does not occur uniformly, however. Epithelial cells lying behind the germinal bands increase dramatically in surface area during epiboly, while the surface area of epithelial cells located over the germinal bands remains constant (Figs 2, 3B, 4B,C). The increase in surface area by individual cells may account for much or all of the increase in epithelial size during the second half of epiboly.

In contrast to the continuous increase in the surface area of the epithelium during epiboly, its marginal circumference must first increase and then decrease as the margin passes from the animal pole to the equator and thence to the vegetal pole (Fig. 1B). Given that the marginal cells present a smooth contour at all stages (Figs 3A,B, 4A-C) and that the average surface area of epithelial cells lying over the germinal bands does not change during epiboly (Fig. 2), the changes in the epithelial circumference could be achieved by changing either the shape and/or the number of cells at the margin of the epithelium. Our results indicate that these two processes contribute differentially during early and late epiboly.
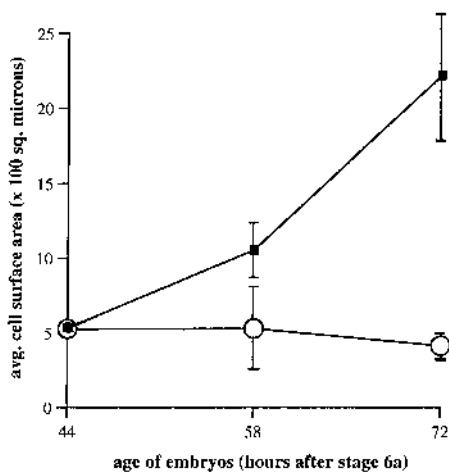
At late stage 7 and mid stage 8, the marginal epithelial cells are roughly rectangular, with their long axes parallel to the edge of the margin (Figs 3A,B, 4A,B). There is no significant difference in the aspect ratio (see Materials and Methods) of the cells between these stages (Table 2) and the average length of these cells along the margin remains relatively constant (Table 2). From this it follows that the expansion of the epithelial perimeter during the first half of epiboly must be accomplished by increasing the number of cells at the margin and this increase was observed. For example, the average number of marginal opq″L progeny increased from an average of 3.3 (s.d.=1.0; n=6) at late stage 7 to 6.0 (s.d.=0.7; n=5) at mid stage 8.

After mid stage 8, the perimeter of the micromere cap decreases in length as the leading edge approaches the vegetal pole. This second half of epiboly is accompanied by marked changes in shape of the marginal epithelial cells. By late stage 8, these cells have elongated along the axis perpendicular to the marginal edge (Figs 3C, 4C) and their average length on the margin is much reduced (Table 2). Consequently, their average aspect ratio has increased more than threefold (Table 2). The decreasing epithelial perimeter at late stage 8 may also reflect a decrease in the number of marginal cells; the average number of opq″L-derived cells located on the margin decreased slightly, from 6.0 at mid stage 8 to 4.5 (s.d.=1.9; n=6) at late stage 8.

The edges of the provisional epithelium and the germinal bands are in close proximity during epiboly

Both the epithelium and the germinal bands undergo epibolic movements. If one of these tissues is moving passively as a result of its attachment to the other, one would expect that the leading edge of the actively moving cells would be consistently ahead of the edge of those moving passively. Thus, to begin to identify the source of the motive force for these movements, the relative position of the leading edges of the germinal bands and the epithelium were examined in embryos fixed during early, mid and late epiboly. The n bandlets of these embryos were labelled with lineage tracer to outline the leading edge of the germinal bands and the embryos were silver stained to outline the provisional epithelium.

At late stage 7, the germinal bands have extended to nearly their full length and are about to begin coalescing into the germinal plate. At this time, the two n bandlets are separated by an average of 52 μm at their distal ends (s.d.=20; n=4). The area between their distal ends is covered by the epithelium; otherwise, at this time, the leading edge of the provisional epithelium and the germinal bands are nearly coincident, except that the middle portions of the germinal bands occasionally extend 4-20 μm beyond the epithelium (Fig. 5A). (The width of the n bandlet is approximately 20-30 μm.) In this

Fig. 2. Epithelial cell surface area during epiboly. Plot of the average apical surface area of epithelial cells located over the germinal bands in control embryos (open circles) and sibling embryos in which the number of epithelial cells had been reduced as a result of micromere ablation (squares). Data for each time point was obtained from 4-11 embryos. Vertical bars indicate the standard deviation (Those for the first time point are less than the height of the symbols).

region, the leading portion of the germinal bands are covered by flaps of macromere membrane (Fig. 6).

By mid stage 8, the germinal plate is half formed and the epithelium covers approximately one half the embryo. As at late stage 7, the leading edges of the epithelium and the germinal bands are nearly coincident over most of the length of the germinal bands. Occasionally the epithelium extends 4-12 µm beyond the edge of the germinal bands at their proximal ends. The epithelium may also spread across the area separating the distal ends of the germinal bands.

At late stage 8, the germinal plate is nearly complete and the provisional epithelium covers the entire embryo except for a small area at the vegetal pole (Figs 4C, 5C). In contrast to the situation at early and mid epiboly, at this stage the epithelium always extends 8-40 µm beyond the edge of the germinal bands throughout their length.

Epiboly in experimentally perturbed embryos

From our observations of normal epiboly, it seemed possible that both the epithelium and the germinal bands were capable of moving actively. The margin of the epithelium is clearly ahead of the leading edges of the germinal bands at late stage 8, suggesting that the germinal bands do not provide the driving force for the epithelial movements of late epiboly. The situation is not so clear at earlier stages, however, since the two edges are nearly coincident, with each tissue being slightly ahead of the other in different regions. To further investigate the question of whether the provisional epithelium and the germinal bands can move independently of one another, we examined epibolic movements in experimentally perturbed embryos.

Epiboly occurs in embryos lacking germinal bands

To test whether the germinal bands are necessary for the provisional epithelium to undergo epiboly, the germinal bands were prevented from forming by injecting their precursors with ricin A chain after the birth of any micromeres they produce (see Materials and Methods for details); this insures that all the precursors to the provisional epithelium are born. Epithelia of embryos lacking germinal bands did undergo epiboly but differed in several ways from controls. Detailed studies of selected epithelial clones in experimental and control embryos were made by injecting the parent micromere with lineage tracer.

At stage 6a + 44 hours, the embryonic surface area covered by provisional epithelium in experimental embryos (n=10) is about 80% of that in controls (n=6) (Fig. 4A,D). In many respects, these epithelia resemble those in control embryos. For example, they contain an average of 149.1 cells (s.d.=17.1; n=7), approximately the same as in controls (137.1; s.d.=9.4; n=6); individual dnopq''' and opq''$_L$ clones also make approximately the normal number of cells (Table 1). The number and aspect ratios of marginal cells in the opq''$_L$ clone also did not differ significantly between experimental and control embryos (Table 2). Thus, the reduction in epithelial coverage results either from a reduction in the apical surface area of individual epithelial cells in these embryos and/or from a folding of the epithelial surface. In some cases, the epithelium of experimental embryos was clearly thrown into folds (Fig. 7) that are never seen in control embryos, suggesting that the epithelium has increased in area, but that the epithelial margin has failed
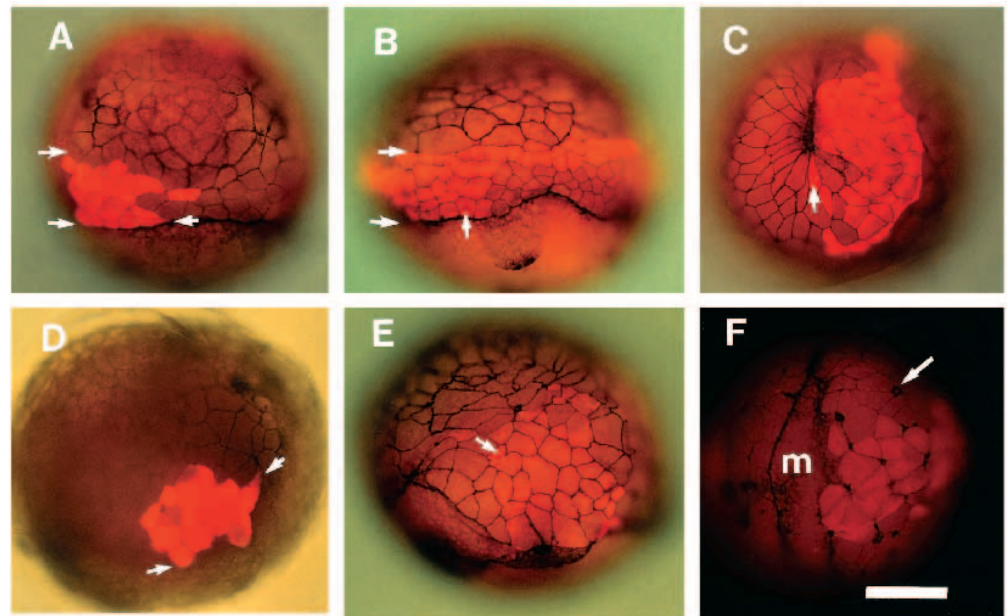

Fig. 3. opq''$_L$ micromere progeny in normal embryos and in embryos lacking germinal bands. Double exposure [fluorescence and bright-field (F is fluorescence only)] photomicrographs of embryos that were fixed and silver stained at early, mid and late epiboly, after opq''$_L$ had been injected with TRDA (red) at birth in normal embryos (A-C) and in experimental embryos in which the germinal bands were prevented from forming by injecting their precursors with ricin A (D-F). At stage 6a + 44 hours, comparison of obliquely animal views of normal (A) and experimental (D) embryos reveals marked irregularities in the epithelial margin in the absence of germinal bands; lower arrows in A and arrows in D bracket marginal cells in the opq'' clones. At stage 6a + 58 hours, comparison of roughly equatorial views of normal (B) and experimental (E) embryos reveals abnormally shaped epithelial cells in the absence of germinal bands; e.g., cell in E marked with arrow and its neighbors. At stage 6a + 72 hours, comparison of roughly vegetal views of normal (C) and experimental (F) embryos illustrates the irregular margin and the retardation of epiboly in the absence of germinal bands. While epithelial margins have converged almost to the point of closure in the control (center of panel C), a large, spindle-shaped domain of macromere (m) remains uncovered in the embryo without germinal bands (F); the epithelium of this experimental embryo also exhibits gaps (arrow). In A and B, two arrows at left indicate the approximate width of the germinal band (c.f. Fig. 1B); note that in early epiboly (A), cells have similar surface areas throughout the epithelium whereas, by mid epiboly, cells lying behind the germinal bands are dramatically larger than those lying over the germinal bands. In B and C, note the dramatic change in aspect ratio of typical marginal cells (marked by vertical arrows) from mid to late epiboly. Scale bar, 100 µm.

to move peripherally. Epithelia in these embryos differed from those in controls in other ways as well; individual cells within the epithelium are more variable in shape and the leading edge of the epithelium is often markedly irregular (Figs 3A,D, 4A,D). In addition, some opq″L clones in experimental embryos contained some deep, rather than exclusively superficial, progeny (data not shown).

By stage 6a + 58 hours, the epithelium of experimental (*n*=6) and control (*n*=5) embryos have spread to approximately the same extent (Fig. 4B,E). Folds are no longer seen in the epithelium and all the cells of the opq″L clone are superficial. However, the opq″L clones in experimental embryos are now significantly smaller than in control embryos [34.0 versus 44.0 cells (a≈0.001; test of the difference between the means; Alder and Roessler, 1968; Table 1, Fig. 3B,E)]. The opq″ clone in experimental embryos is also less elongated parallel to the margin than in controls. As with the earlier time point, the leading edge of the epithelium (Fig. 4B,E) and the apical contours of individual epithelial cells (Fig. 3B,E) are irregular in experimental embryos. In addition, the size distribution of individual cells in the epithelium appears more uniform than in controls (Figs 3B,E, 4B,E).

At stage 6a + 72 hours, the epithelium of experimental embryos (*n*=10) covers only slightly less of the embryo than in controls (*n*=6), despite the reduced number of cells in the epithelium relative to controls [e.g., 29.4 versus 49.2 cells in the opq″L clone (a<0.001; Table 1, Figs 3C,F, 4C,F)]. Accordingly, some of the cells in the epithelia of experimental embryos are larger than in controls (Fig. 3C,F); moreover, the average length of their opq″L cells on the margin is 16.3 μm (Table 2), compared to 7.8 μm in controls (Table 2) and the aspect ratio of the marginal cells in these clones (1.32; Table 2) is significantly different (a<0.001) than that of controls (2.64; Table 2). The epithelia in these experimental embryos have the irregular leading edge seen in the earlier time points and frequently appear to contain small gaps, as indicated by the presence of silver-stained contours without nuclei (Fig. 3F). Moreover, some multinucleate cells can be seen in the epithelia of experimental embryos. In addition to the difference in the shape of the epithelial opening between the control and experimental embryos (Figs 3C,F, 4C,F), the epithelium appears to be closing near the prospective head region in experimental embryos, rather than near the teloblasts as in controls.

Early epiboly is retarded in embryos with reduced micromere caps

The above results demonstrate that the epithelial margin can move without the germinal bands. Two observations suggest that the germinal bands may also play an active role in epibolic movements, however, especially in the early part of the process. First, the leading edges of the mid-region of the germinal bands sometimes lead the overlying epithelial margin in normal embryos at late stage 7 and second, the movement of the epithelial margin is retarded in embryos lacking germinal bands.

To further distinguish the morphogenetic roles of the germinal bands and epithelium, the number of cells in the epithelium was reduced by killing the five micromeres which produce only provisional epithelium (i.e., dnopq‴, both n′ and both opq″; Smith and Weisblat, 1994). These micromeres contribute one-third of the epithelial cells at late stage 7. It was not feasible to kill other micromeres because the resultant loss of their deep cell contribution to the micromere cap might independently affect the movements of the germinal bands (B. H. Nelson, personal communication; Smith, 1994).

At stage 6a + 44 hours, the experimental embryos exhibit the expected reductions in cell number. They contain, on average, 101.2 epithelial cells (s.d.=11.8; *n*=6), whereas controls average 161.5 cells (s.d.=11.1; *n*=4). The surface area of individual epithelial cells was the same in experimental and control embryos (Fig. 2), resulting in a total epithelial surface
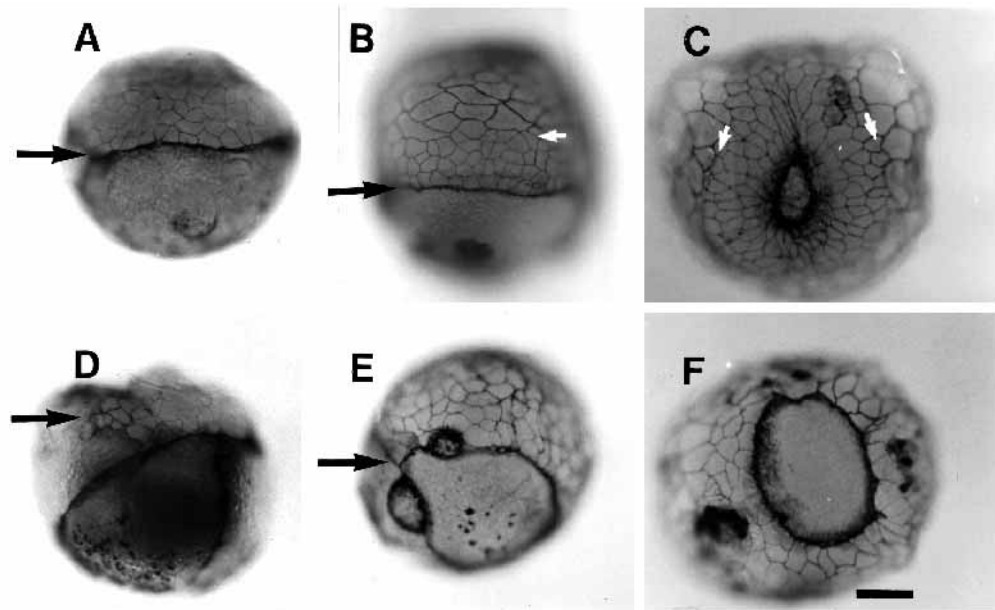

Fig. 4. Epiboly in normal embryos and in embryos lacking germinal bands. Bright-field images of silver-stained embryos fixed at stage 6a + 44, 58 or 72 hours in normal embryos (A-C, respectively) and in embryos lacking germinal bands (D-F, respectively). Embryos at the two earlier time points (A,B,D,E) are viewed roughly equatorially with the animal hemisphere up; note the relatively smooth epithelial margin (black arrows) in controls (A,B) relative to embryos lacking germinal bands (D,E). (Silver-stained contours below the epithelial margins outline macromeres and teloblasts.) Embryos at the later time point (C,F) are viewed from the point of convergence of the epithelium (corresponding to the vegetal aspect in C). The larger opening in F relative to C reflects the retardation of epiboly in the absence of germinal bands. Note also, in normal embryos at mid and late epiboly, the marked difference in the size of individual epithelial cells over the germinal bands (between epithelial margin and white arrows) versus those behind the germinal bands. Scale bar, 100 μm.

area in experimental embryos that was one third less extensive (Fig. 5A,B).

The germinal bands undergo significant epibolic movements in these experimental embryos, despite the reduced size of the epithelium. As a consequence, the leading edges of the proximal and middle portions of the germinal bands lie 30-60 µm beyond the leading edge of the micromere cap (Fig. 5B). [The distal ends of the germinal bands remain covered by epithelium, presumably derived from the first blast cells in the n bandlets (Smith and Weisblat, 1994).] Although parts of the proximal and middle germinal bands in these embryos extend well beyond the edge of the epithelium, they do not appear to be exposed to the perivitelline environment because their blast cells are not outlined by the silver stain (Fig. 5B). Examination of sectioned embryos reveals that a thin flap of the macromere extends back over the leading portion of the germinal bands, as if firmly linked to the leading edge of the epithelium (Fig. 6). Partial germinal bands, lacking either the mesodermal or the ectodermal bandlets do not reach beyond the micromere cap (data not shown).

Although the germinal bands in embryos with reduced epithelia undergo marked epibolic movements, they are abnormal in that their distal ends are, on average, 108 µm from each other (s.d.=12; n=6), compared to 52 µm in controls (s.d.=20; n=4). In addition, the germinal bands exhibit abnormal curvature (Fig. 5A,B).

At stage 6a + 58 hours, the germinal bands in both experimental and control embryos have reached the equatorial region of the embryo. Surprisingly, the edges of the germinal bands and the epithelium are once again nearly coincident in experimental embryos, with the epithelium now lagging by at most 20 µm over the proximal portion of the germinal bands. In some experimental embryos, the epithelium even extends beyond the distal ends of the germinal bands. The restoration of the micromere cap area in experimental embryos reflects a compensatory expansion of individual cells rather than a compensatory increase in the number of epithelial cells; the average area of cells in experimental embryos is twice that of those in controls (Fig. 2). These experimental embryos (n=7) are still retarded in their coalescence, having coalesced only two-thirds the distance of control embryos (n=9). Moreover, the proximal ends of the experimental germinal bands are neither as close to each other nor as symmetric as those in the controls.

At stage 6a + 72 hours, the edge of the epithelium typically extends beyond the edge of the germinal bands, in experimental embryos (n=11) as in controls (n=6), although the germinal band coalescence is still retarded relative to controls (Fig. 5C,D). While the leading edges of the germinal bands and the epithelium may be nearly coincident in some parts of embryos with reduced epithelia, elsewhere the epithelium may extend up to 120 µm beyond the edge of the germinal bands, which is even more than in controls. This regulative behavior of the epithelium reflects primarily an increase in apical surface area of individual epithelial cells, rather than an increase in cell number. The apical surface area of 10 adjoining cells is now 5.4 times greater in experimental than in control embryos in comparable areas (Figs 2, 5C,D). It is not technically feasible to count all the cells in the epithelium at late epiboly, but examination of single micromere clones indicates that there is no regulative increase in clonal cell number after micromere ablation either at late stage 7 (Smith and Weisblat, 1994) or

late stage 8. For example, in one experiment, dnopq$'''$ micromeres were injected with lineage tracer at stage 4c (Fig. 1A) and the labeled clones were counted at stage 6a + 72 hours; there was no difference between the size of the clone in control embryos (21.0 cells; s.d.=4.1; n=4) and in siblings in which both opq$''$ micromeres had been ablated at stage 6a (22.5 cells; s.d.=4.0; n=4). Embryos with reduced epithelia can eventually give rise to normal juveniles (F. Z. Huang, personal communication).

DISCUSSION

In the studies presented here, we have begun an analysis of gastrulation in glossiphoniid leeches by examining the epibolic movements of the provisional epithelium and germinal bands in embryos of *Helobdella robusta*. Our results indicate that the provisional epithelium and the germinal bands can move independently of one another, although both tissues are required for the normal execution of the epibolic movements.

Germinal band movements predominate during early epiboly

Three observations suggest that the movements of the germinal bands dominate movements of the provisional epithelium during early epiboly.

(1) In normal embryos, when the epibolizing tissues are still within the animal hemisphere (during late stage 7 and early stage 8), the leading edges of the germinal bands are slightly ahead of the leading edge of the epithelium over much of their length.

(2) In embryos in which the population of epithelial cells is reduced by roughly one-third, the germinal bands still undergo their epibolic movements, such that the distance between the leading edges of the germinal bands and the leading edge of the epithelium is dramatically increased.

(3) In some embryos lacking germinal bands, folds arose in the epithelium during late stage 7. No such folds are seen in experimental embryos later in epiboly or at any time in normal embryos. These observations suggest that, in the absence of the germinal bands, the epithelium still expands, but that the peripheral movements of its margin are retarded, so that folding occurs.

It is possible that the epithelium remaining in embryos with reduced micromere caps induces the germinal bands to move. Nonetheless, it is clear that the force for germinal band movement in these embryos is not provided by the epithelium because the germinal bands move beyond the epithelium, even though this significantly deforms the macromeres. Moreover, embryos lacking germinal bands also exhibit marked irregularities in the leading edge of the epithelium relative to controls. This may indicate that, in normal embryos, the relatively smooth leading edge of the epithelium is due to its being put under tension by the movements of the germinal bands. All of the above observations lead us to conclude that the movements of the germinal bands are more active than those of the epithelium during early epiboly.

The forces that produce the movement of the germinal bands remain to be determined. One possibility is that the blast cells crawl actively over the surface of macromeres. It is already known that bandlets move relative to one another within the

Fig. 5. The positions of the leading edges of the epithelium and germinal bands in normal embryos and in embryos with reduced numbers of epithelial cells: early and late epiboly. Double exposure (fluorescence and bright-field) photomicrographs of silver-stained embryos in which the n bandlets are labelled with TRDA (red). In each panel, the epithelial margin (solid arrow), the n bandlet (pairs of arrowheads) and the general direction of epibolic movements (open arrow) are indicated at representative locations. Posterior dorsal views of stage 6a + 44 hours control (A) and experimental (B) embryos; the leading edges of the epithelium and of the n bandlet are nearly coincident in the control, but the entire n bandlet lies ahead of the epithelial margin in the experimental embryo. No blast cell contours are visible in the n bandlet, however, indicating that it is still covered. Posterior, ventral views of stage 6a + 72 hours control (C) and experimental (D) embryos; in both cases, the epithelial margin is ahead of the n bandlet. Note that the epithelial cells in D are much larger than in C. Scale bar, 100 µm.
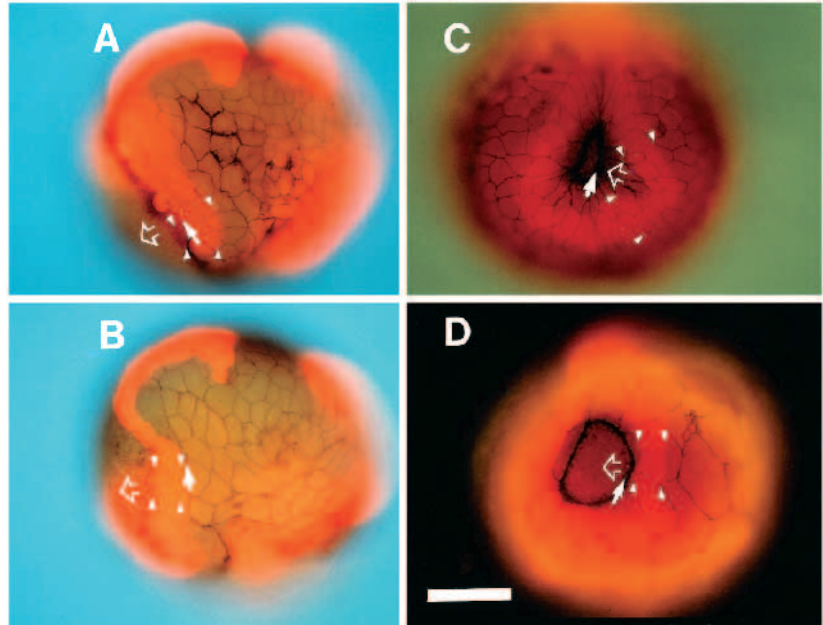
germinal bands. During stage 7, for example, the ectodermal bandlets move distally relative to the mesodermal bandlets (B. H. Nelson, personal communication) and, during stages 7-8, the n and q bandlets move relative to the m, o and p bandlets (Weisblat and Shankland, 1985). Signs of protrusive activity by blast cells within the bandlets have been observed (Zackson, 1983; C. M. Smith, unpublished observations). These protrusions could be involved in movements of the germinal bands over the embryo.

A second possible source of germinal band movements is force resulting from cell divisions and growth (fed by the underlying yolk) within the germinal bands. Blast cells undergo stereotyped cell divisions (Zackson, 1984; Bissen and Weisblat, 1989). The majority of the early mitoses are longitudinally oriented, which gives them the potential to generate a force that could propel the ends of the germinal bands over the embryo. Later in epiboly, when the distal ends of the germinal bands become tethered to one another and the germinal bands continue to increase in length, the resulting forces could buckle the germinal bands so that their middle portions move ventrovegetally.

Epiboly proceeds in the absence of germinal bands

The conclusion that the germinal bands are not required for the epiboly of the provisional epithelium is unambiguous because the epithelium spreads in embryos lacking germinal bands. Moreover, in both normal embryos and experimental embryos in which the margin of the epithelium is initially far behind the leading edge of the germinal bands, the epithelium overtakes the germinal bands as epiboly proceeds. We believe this shows that the germinal band movements cease to dominate those of the epithelium during later epiboly.

The forces driving the movements of the epithelium remain to be determined. Epithelial cells divide during epiboly, but it is unlikely that these divisions contribute significantly to the movement of the epithelial margin since the epithelium in embryos with reduced numbers of epithelial cells can cover

nearly the same surface area as that of controls. It also seems unlikely that the epithelium crawls over the apical surface of the macromeres, since this surface is presumably itself epithelial and lacks an extracellular matrix. Instead, the pronounced deformation of the macromeres in embryos with reduced epithelia suggests that the leading edge of the epi-
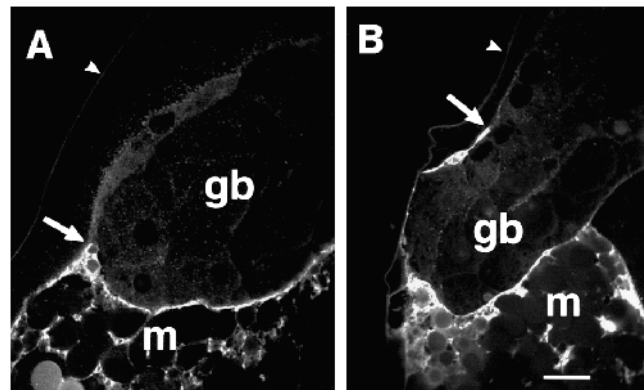

Fig. 6. Boundary between macromere and epithelium, relative to germinal bands, in normal and experimentally perturbed embryos at stage 6a + 44 h. Confocal fluorescence photomicrographs of transverse sections through the left germinal band of a normal embryo (A; cf Fig. 5A) and of an experimental embryo in which the size of the micromere cap was reduced by ablation of five micromeres (B; cf Fig. 5B); the A and B macromeres were labeled with RDA. The cortical cytoplasm of the macromere (m) is brightly labeled; circular contours in the macromeres are yolk platelets. Germinal bands (gb) and overlying epithelium feature cells with granular cytoplasm and prominent nuclei. In each panel, the boundary between the epithelium and the macromere is indicated by a large arrow and the vitelline envelope by a small arrowhead. (A) Normally, the leading edge of the epithelium is close to the edge of the germinal band but, in embryos with reduced epithelia (B), the leading edge of the epithelium is well back of the germinal band, which is covered instead by a thin flap of macromere. Scale bar, 25 µm.

thelium is firmly linked to the macromeres. Thus, one can imagine that forces generated by the cytoskeleton of the macromeres contribute to pulling the epithelium back over the germinal bands later in the epiboly of these experimental embryos. The contribution of the macromeres to movements of the germinal bands and epithelium remains to be fully investigated (see below).

All major embryonic cell types contribute to the epibolic movements

The results summarized above indicate that the germinal bands and provisional epithelium can move independently, but these results also indicate that both tissues are required for normal movements. In embryos lacking germinal bands, for example, the point at which the epithelium is destined to close is ectopically located, the epithelial margin is irregular and epiboly is retarded. Moreover, at least some of the micromere clones within these embryos proliferate less rapidly and the apical surface area of individual epithelial cells is increased. Conversely, in embryos with reduced micromere caps, germinal bands move more slowly and exhibit abnormal curvature, and their coalescence is retarded. [The analysis of germinal band movements in such embryos may be further complicated by the observation that mitoses are delayed in primary blast cells that are covered by macromere, rather than epithelium (F. Z. Huang, personal communication).]

Our present experiments have not addressed the possible role of the macromeres in providing motive force for these epibolic movements, but we believe these cells are important. Experiments in which one or more macromeres were injected with ricin showed effects on the epibolic movements that varied depending on the macromere(s) injected (Smith, 1994; C. M. Smith, unpublished observations). Moreover, the increase in the surface area of epithelial cells lying behind the germinal bands during the second half of epiboly suggests that this part of the epithelium is under traction from the macromeres and/or the germinal bands. The fact that the epithelial cells located over the germinal bands do *not* expand during normal epiboly, suggests that the germinal bands normally prevent these cells from being stretched. These results indicate that the macromeres are not just a passive substratum for the epibolic movements. Thus, although epibolic movements in glossiphoniid leeches are relatively simple compared to morphogenesis in some other animals, the forces that produce these movements are complex. No one cell type is sufficient to account for all the observed movements, nor can remaining cell types move normally when one cell type is impaired. Thus, epiboly in leech entails the integration of multiple, semi-independent processes.

Comparisons with other animals

The formation of epibolizing epithelia from the progeny of micromeres produced at the animal pole is a common feature of several protostome phyla, including annelids and molluscs. Assuming that such similarities in gastrulation processes are inherited from a common ancestor, the discovery of underlying mechanis-

tic similarities would not be surprising, but the paucity of work in this area means that few direct comparisons can be made. For example, it has been suggested that epithelial microvilli serve as a source of membrane for epithelial expansion during epiboly in the squid *Loligo* (Arnold, 1961, 1971; Arnold and Williams-Arnold, 1974) and in the marine snail *Ilyanassa* (Craig and Morrill, 1986), but this issue has not been addressed in leech embryos. Craig and Morrill also observed junctional complexes between primary quartet micromeres and the underlying macromeres in the early *Ilyanassa* embryo but not between marginal epithelial cells and macromeres in late epiboly. This latter observation seems at odds with the strong mechanical attachment that we conclude links the marginal epithelial cells to the macromeres in *Helobdella*; this discrepancy may reflect either species-specific or stage-specific differences.

The epibolic cell movements in annelid and mollusc embryos also resemble epiboly in vertebrate gastrulation, especially that of teleost fish, for which an extensive analysis has been achieved (Betchaku and Trinkaus, 1978; Trinkaus, 1984a,b; Keller and Trinkaus, 1987; Warga and Kimmel, 1990). As is to be expected since annelids and chordates are even more distantly related than annelids and molluscs, there are clear differences between gastrulation in leech and fish. For example, during fish epiboly, the blastoderm and enveloping epithelium spread over a single yolk cell, and prospective mesodermal cells segregate from ectoderm by involuting at the margin of the blastoderm. In leech, by contrast, epibolic movements occur over the surface of three yolk cells (the macromeres) and the relative positions of the mesoderm and ectoderm layers are fixed at the onset of epiboly.

However, our results suggest similarities between the epibolic movements in leech and fish. In fish, it has been proposed (Betchaku and Trinkaus, 1978; Trinkaus, 1984b; Strahle and Jesuthasan, 1993; Solnica-Krezel and Driever, 1994) that cytoskeletal elements in cortical cytoplasm of the yolk cell result in vegetally directed forces that pull the attached epithelium. Similarly, Lillie (1902) reported that,
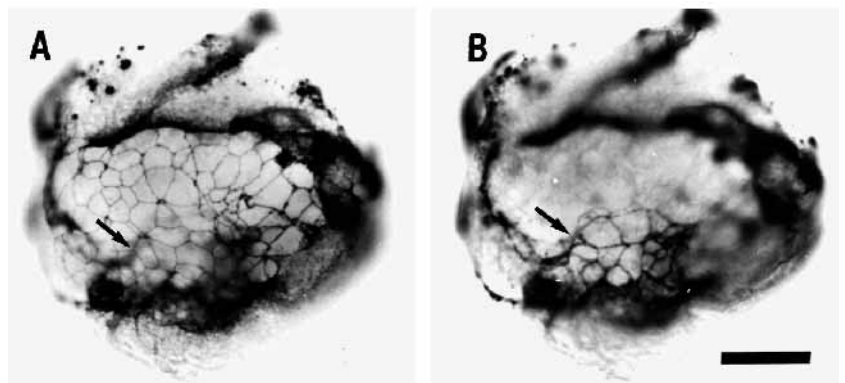

Fig. 7. Epithelial folding in an embryo lacking germinal bands. Photomicrographs showing two different focal planes of the prospective dorsal aspect of a silver-stained, stage 6a + 44 hours embryo reveal two of the three overlapping layers of epithelial cells (arrows) resulting from folding within the micromere cap. (A) Superficial focal plane; arrow indicates epithelial cells overlying the layer seen in B. (B) Intermediate focal plane; arrow indicates the fold leading from this intermediate layer to a yet deeper layer (out of focus). Scale bar, 125 μm.

when cleavage is suppressed in parthenogenetically activated eggs of the polychaete annelid *Chaetopterus pergamenta-ceous*, they exhibit a vegetally directed migration of clear cortical cytoplasm at a time corresponding to epiboly in normal embryos. This migration of cytoplasm seems quite similar to the movements that occur within the cortical cytoplasm in fish. Here, we speculate that the germinal bands and the margin of the epithelium in leech are both connected via transmembrane proteins to the cytoskeleton of the macromeres, which contributes force for their movements.

Thus, the question of whether there are significant homologies between the mechanisms of epiboly in annelids and chordates remains open. In this regard, it is curious that, with respect to epiboly, the gastrulation processes of leech and fish are more similar than those of more closely related organisms, such as fish and sea urchins, or leeches and insects. If epiboly proceeds by similar mechanisms in leech and fish, it may be an example of convergent evolution, i.e. there may be only one way to move an epithelium over an underlying yolk cell. Alternatively, these may be homologous processes, inherited from a common ancestor, in which case the broad spectrum of gastrulation processes observed in other animals are in fact variations on this ancient underlying process.

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