A Provisional Epithelium in Leech Embryo: Cellular Origins and Influence on a Developmental Equivalence Group

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Segmental tissues of glossiphoniid leeches arise from rostrocaudally arrayed columns (bandlets) of segmental founder cells (primary m, n, o, p, and q blast cells) which undergo stereotyped sublineages to generate identifiable subsets of definitive progeny. The bandlets lie at the surface of the embryo beneath the squamous epithelium of a transient embryonic covering called the provisional integument. This "provisional epithelium" derives from micromeres produced during the early cleavage divisions. Previous experiments have shown that the primary o and p blast cells constitute an equivalence group, i.e., are initially developmentally equipotent and undergo hierarchical interactions which cause them to assume distinct O and P fates. Here, we examine the role of the provisional epithelium in determining the fates of the underlying o and p blast cells. Experiments entailing the microinjection of individual micromeres with cell lineage tracers show that, at stages 7–8 of normal development, the epithelium comprises coherent and relatively stereotyped domains derived from particular micromeres. Upon photoablating specific domains of epithelium labeled with photosensitizing lineage tracer, the normal assignment of O fates is disturbed; o blast cells divide symmetrically (as p blast cells do) and some supernumerary definitive progeny expressing P fates arise within the O lineage. We therefore conclude that the epithelium is essential for generation and/or reception of signal(s) by which the o and p blast cells' normally determine their fates. Finally, a new tracer substance, biotinylated fixable dextran (BFD), is described which was essential for this study by virtue of its superior resistance to photobleaching and which offers several other advantages as well. © 1987 Academic Press, Inc.

INTRODUCTION

That seemingly equivalent cells often have different fates is an essential feature of development. A central question in such cases is whether such differences reflect (i) cryptic differences in inherited developmental potential or (ii) responses of a unitary cell type to subtle environmental differences. Addressing this question in the nematode embryo, Kimble *et al.* (1979) defined an *equivalence group* as a group of cells of common developmental potential which normally take on different fates through a hierarchical and (typically) position-dependent interaction. Analogous phenomena have been observed in leech (Weisblat and Blair, 1984; Shankland and Weisblat, 1984) and in insect embryos (Kuwada and Goodman, 1985; Doe and Goodman, 1985a,b). In this paper we report that an embryonic epithelium covering the cells of an equivalence group previously described in the embryo of the glossiphoniid leech *Helobdella triserialis* is necessary for the normal hierarchical interactions among those cells.

The overall outline of leech embryogensis, including a staging system, has been described elsewhere (Stent *et al.*, 1982; Weisblat *et al.*, 1984) and is summarized in Fig. 1. Each segment of the leech comprises five distinct kinship groups of cells, (M, N, O, P, and Q) (Kramer and Weisblat, 1985) produced by stereotyped sublineages from seven classes of segmental founder cells (m, nf, ns, o, p, qf, and qs primary *blast cells*) (Weisblat and Shankland, 1985) arrayed in five bilateral pairs of rostrocaudally arrayed *bandlets* (m, n, o, p, and q). During stages 7–8, the five bandlets on each side of the early embryo come together in left and right *germinal bands*. In each germinal band, the ectodermal bandlets (n, o, p, and q) lie superficially in alphabetical order, with the q bandlets closest to the animal pole of the embryo. The rostral ends of the germinal bands meet at the future head. The primary blast cells are the progeny of *teloblasts*, large yolk-filled stem cells which themselves arise by stereotyped, holoblastic cleavages from the egg. In each bandlet, the blast cells are strictly birth ranked, with older cells at the rostral end. The germinal bands are separated by a group of cells of mixed origins, collectively referred to at first (during stages 7–8) as the *micromere cap* and later (stages 8–11) as the *provisional integument* (Weisblat *et al.*, 1984). Part of this tissue is a squamous epithelium which covers the germinal bands and the area between them. During stage 8 the germinal bands move across the surface of the embryo, eventually coalescing along the ventral midline into a structure called the *germinal plate*, from which the segmental tissues of the leech arise. Coincident with this, the micromere cap ex-

pands from the animal pole of the embryo, covering the embryo with a squamous epithelium and underlying contractile fibers. This tissue, now called the provisional integument, constitutes the body wall of the embryo, pending the generation of definitive body wall by the proliferation of cells in the germinal plate.

There are five bilateral pairs of teloblasts: one mesodermal pair (M) and four ectodermal pairs (N, O/P, O/P, and Q). Previous work (Weisblat and Blair, 1984; Shankland and Weisblat, 1984) has shown that primary blast cells derived from the ipsilateral O/P teloblasts constitute an "equivalence group." Blast cells from each pair of O/P teloblasts are initially equipotent and can take either O or P fate. That O/P-derived bandlet which occupies the position closer to the animal pole normally assumes the P fate, while the distal O/P-derived bandlet assumes the O fate. The P fate is primary to the O fate; if the O/P teloblast generating the p bandlet (defined as the "generative" P teloblast) is ablated, or if p blast cells are selectively ablated within the germinal band, the o blast cells which are now without p blast cell neighbors in the germinal band transfate to generate cells of the P kinship group, instead of their normal O kinship group progeny. Ablating the generative O (or the N or Q) teloblast has little effect on the fate of the p blast cells' progeny, save for minor changes in location (Blair and Weisblat, 1982, 1984).

Primary blast cells in the o and p bandlets can be distinguished from one another not only by position, but also by the symmetry of their first mitoses (Zackson, 1984). Cells in the p bandlet divide to produce anterior and posterior daughters (relative to the long axis of the bandlet) of about equal size, soon after they enter the germinal band; primary o blast cells divide at about the same time, but with an asymmetric anaphase (Settle and Weisblat, in preparation), generating one larger (anterior) and one smaller (posterior) daughter cell. In spite of this initial difference, the o bandlet cells are not yet "committed" to the O fate (i.e., unresponsive to ablation of the adjacent p bandlet). Rather, commitment is a stepwise process which requires the presence of the p bandlet during the early mitoses in the incipient o blast cell clone (Shankland and Weisblat, 1984; Shankland, 1985; Shankland and Stent, 1986).

By what cue(s) do the o and p bandlets sense the polarity of the embryo in normal development? Experiments deleting the other germinal bandlets (namely, n, m, and q) have shown that the o and p blast cells are not using nearest neighbor interactions with other blast cells as cues for determining their fates (Zackson, 1984). Another potential cue is the micromere cap, which spreads like an ice cap ventrocaudally across the surface of the embryo from its origins at and just dorsal to the animal pole. In this paper, we address the question of whether or not the epithelium of the micromere cap provides positional cues for the underlying blast cells.

Previously it was shown that certain micromeres contribute progeny to the provisional integument of the stage 10 embryo and also to two definitive structures, the supraesophageal ganglion and prostomial ectoderm of the stage 10 embryo (Weisblat et al., 1984). However, that study was incomplete, and, for this study it was necessary to know the composition of the epithelium at stages 7-8 rather than stage 10. Therefore, we have carried out a more complete lineage analysis by directly labeling most of the micromeres produced during stages 4a-6b, and mapping their progeny in the micromere cap at stage 8. We find that identified micromeres contribute progeny to the stage 8 provisional integument in stereotypic and reproducible patterns. With the elucidation of a micromere "fate map," we have been able to assess the role of the epithelium of the provisional integument (hereinafter referred to as the provisional epithelium) in determining cell fates within the underlying bandlets and especially in mediating cell interactions within the O-P equivalence group. We have photoablated selected portions of the stage 8 provisional epithelium and studied the effect on the initial mitoses and the definitive progeny of the underlying primary blast cells. Ablation of the provisional epithelium over o and p bandlets interfered with normal determinative interactions within the O-P equivalence group so that o blast cells and some of their progeny express P fates even in the presence of the p bandlet.

MATERIALS AND METHODS

Embryos

Embryos of the glossiphoniid leech *H. triserialis* were obtained from a laboratory breeding colony maintained at 23°C in 1% artificial seawater on physid snails from a commerical supplier. Isolated embryos were cultured at 23°C in HL saline (Blair and Weisblat, 1984). The system of embryonic staging and cell lineage nomenclature is summarized in Weisblat et al. (1984). In particular, micromeres are designated by the lowercase letter(s) corresponding to the blastomere of origin, with a prime (') to distinguish them from primary blast cells (Fernandez, 1980).

Epithelial Mapping

Embryos were oriented and immobilized as before (Weisblat et al., 1978, 1980). Selected cells were microinjected via glass microelectrodes containing a mixture of 40 mg/ml HRP (Sigma, type IX) and 0.1-0.4% fast green in 0.2 M KCl. Injected embryos were allowed to develop to early stage 8, at which time they were fixed overnight

at 4°C in 2% formaldehyde, 2.5% glutaraldehyde, and 1 mM CaCl$_2$ in 0.05 M sodium cacodylate buffer (pH 7.4). After washing in cacodylate buffer and then in an 8% sucrose solution, the embryos were reacted with H$_2$O$_2$ in the presence of benzidine to yield a black reaction product. The embryos were then dehydrated through graded alcohols and mounted in Epon.

Synthesis of Biotinylated Fixable Dextran (BFD)

A modification of the method of Gimlich and Braun (1985) was used to conjugate lysine and lysinated biotin (Biocytin, Sigma) to cyanogen bromide-activated dextran carriers (average MW 10,000, Sigma). To 10 ml of a 5 M dextran solution, adjusted to pH 10.8 with HCl, 15 mg (0.14 mmole) of solid cyanogen bromide was added at room temperature. The pH of the reaction mixture was maintained at 10.8 by the addition of 0.01-ml aliquots of 1 N sodium hydroxide. After about 20 min, the rate of decline of pH slowed, indicating the end of the reaction. The pH was then adjusted to 8.4 with HCl; lysine (9 mg, 0.05 mmole) and biocytin (28 mg, 0.075 mmole) were added. This reaction mixture was stirred overnight at 4°C, then dialyzed (Spectrum Medical; MW cutoff 3200) for 2 days against three changes of distilled water. The resultant solution of substituted dextran was lyophilized and reconstituted at 100 mg/ml in 0.2 N KCl at pH 7.0. A test for free amino groups (Habeeb, 1966), showed an average of 1.3 lysine residues per dextran chain.

Biotinylated fixable dextran (BFD) is microinjected in the same manner as the other lineage tracers we employ, mixed with fast green prior to use, and pressure microinjected into single cells from glass micropipets formed on a horizontal electrode puller (Industrial Science Associates).

To visualize BFD-containing cells, the cultured, injected embryos are fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.0, and washed at 4°C for 12 to 24 hr in 0.8% Triton X-100 in 1 × PBS. In this study, the fixed embryos were then incubated overnight at 4°C in Avidin-HRP complex (ABC, Vector Laboratories), then washed and reacted with H$_2$O$_2$ in the presence of 0.5 mg/ml diaminobenzidine tetrahydrochloride to yield a brown reaction product. The embryos were cleared in 70% glycerine, slit open dorsally to remove the gut, and flattened between two coverslips for viewing under Nomarski optics.

One of the advantages of BFD is that it can be visualized in the labeled tissue with various avidin-conjugated fluorphores or enzymes, including fluorescein, rhodamine, and alkaline phosphatase, as well as HRP. A second advantage, of critical importance in this study, is that BFD in primary blast cells was unaffected by

intense illumination (485 nm) needed to photolesion overlying fluorescein-labeled epithelial cells. Under these conditions, HRP was largely inactivated and thus useless as a lineage tracer.

Epithelial Ablation

Micromeres contributing progeny to the area of interest in the early stage 8 embryo were injected (stage 6a) with fluoresceinated, lysinated dextran (FDA) (Gimlich and Braun, 1985). For experiments in which the final progeny of an ipsilateral O/P teloblast were to be determined, that teloblast was injected with BFD very early in stage 7. The embryos were cultured to stage 8 and the FDA-labeled epithelial progeny of the injected micromere(s) were photoablated with a 485 nm laser beam (Lexel, Model 65). Directed through the epifluorescence optics of a Zeiss microscope equipped with a 40× or 25× plan neofluar objective, the focused beam illuminated a spot roughly 25 or 50% the area of an epidermal cell, respectively; scattered light of relatively high intensity emanated from the spot. The translation stage of the microscope was used to move the embryo so that all selected target cells were exposed to the beam in turn. Using these techniques, only the FDA-containing cells are killed, presumably by the action of singlet oxygen (Shankland, 1984).

To study the initial mitoses of primary blast cells, embryos were cultured for 24–36 hr after photolesioning, then fixed overnight at 4°C in 4% parafomaldehyde in PBS, rinsed in Tris-buffered saline (TBS, pH 7.0), stained with the fluorescent DNA stain Hoechst 33258 (1–3 μg/ml in TBS), and cleared in 70% glycerol with 4% n-propyl gallate added to retard fluorescent bleaching (Giloh and Sedat, 1982).

To determine the final progeny of o and p blast cells, photolesioned, BFD-labeled embryos were allowed to develop to late stage 10 (about 4–5 days after photolesioning). The embryos are then fixed and processed as described above to visualize the patterns of BFD-containing cells.

Fate Mapping of the Micromere Lineages

The primary quartet. The first four micromere cells come from unequal divisions of the large macromeres A, B, C, and D and are designated as a', b', c', and d'. In the stage 8 embryo, the progeny from these four micromeres lie in a strip above and within the forming germinal plate (Figs. 2A and 3A). Presumably it is these deeper cells (Fig. 4A), which contribute progeny in the stage 10 larva to the supraesophageal ganglion and prostomial epidermis, as was previously reported for
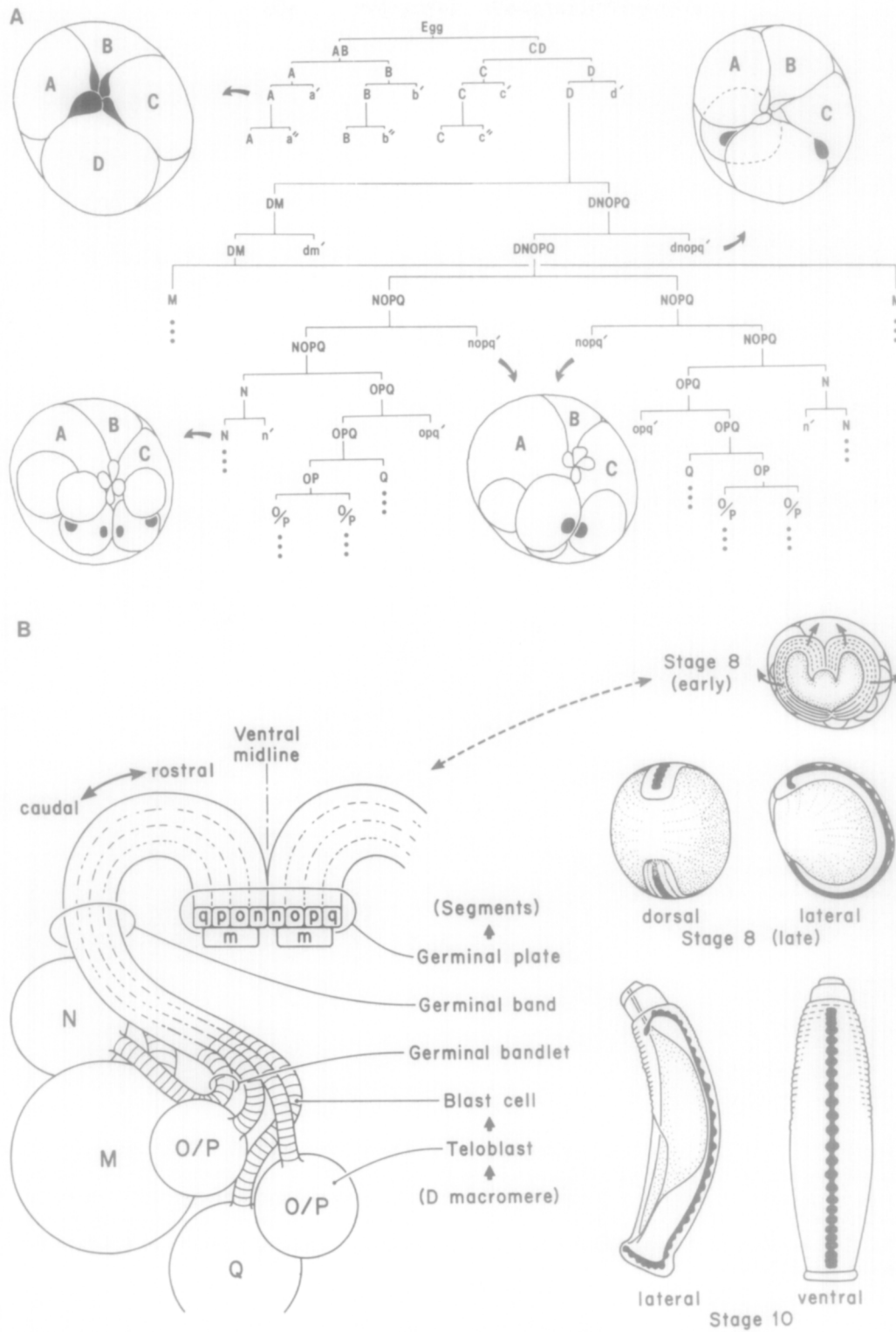
FIG. 1. Summary of *Helobdella* development. (A) Lineage tree showing the early divisions, including the generation of micromeres; four embryos are depicted as well, showing the positions of the blastomeres at four different stages (indicated by arrows) and the most recently born micromeres for each stage (in black). (B) Left. Drawing showing the relationship of the teloblasts and the blast cell bandlets which coalesce to form the germinal band and germinal plate. Note that sister O/P teloblasts generate bandlets which are distinguished as o and p on the basis of their relative positions in the germinal band. The blast cells derived from sister O/P teloblasts interact in a position-dependent, hierarchical manner and thus constitute a developmental equivalence group. Right. Representations of embryos at later stages of development. The definitive progeny of teloblasts injected with lineage tracers are identified in stage 10 embryos, which have almost the complete complement of adult structures.
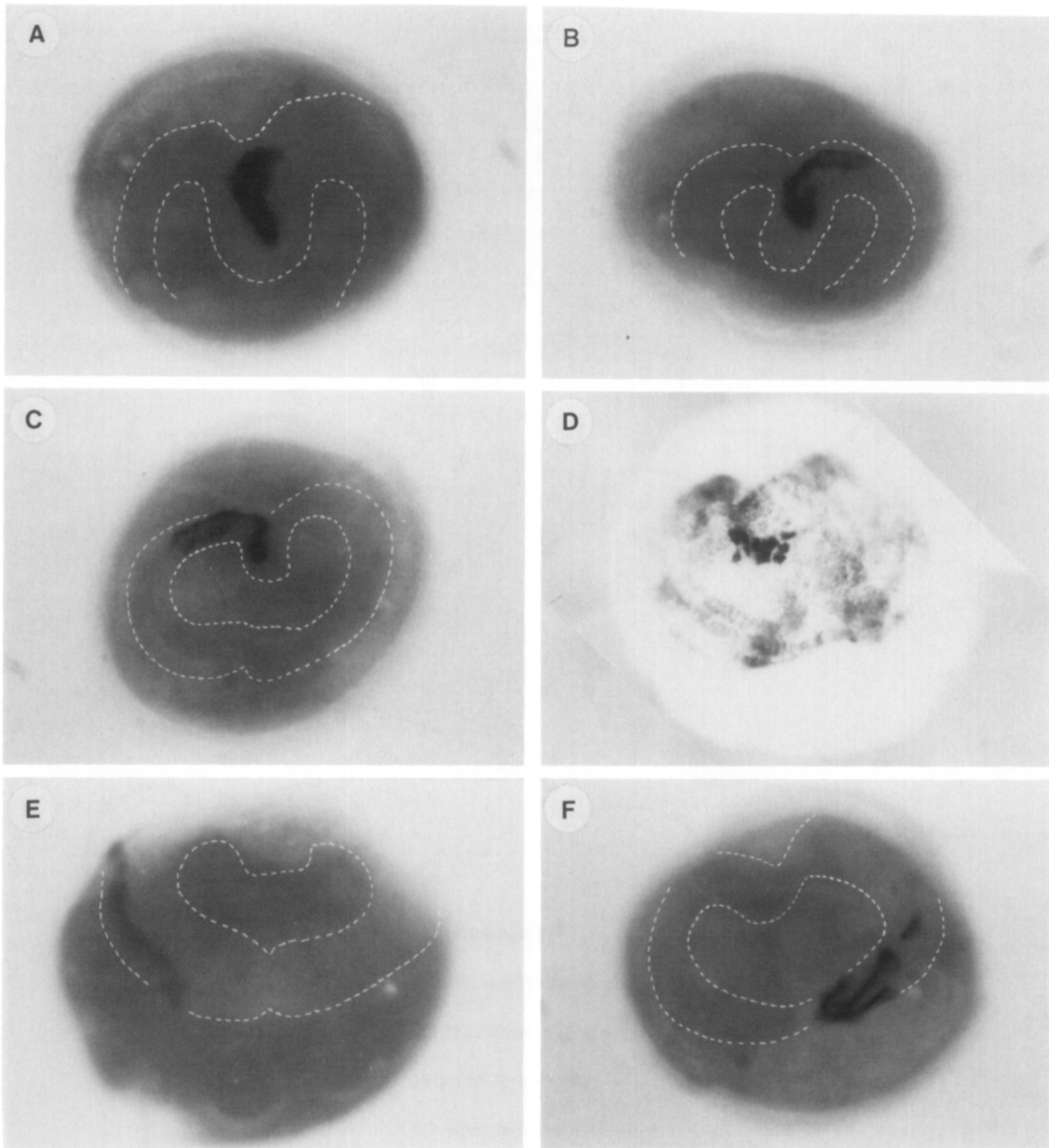
FIG. 2. Different micromeres contribute to different regions of the stage 8 provisional epithelium. Each embryo contains the HRP-labeled clone of an individual micromere injected with HRP at stages 4-6. (A) Progeny of a first quartet micromere. (B) Progeny of a dm' micromere. (C) Progeny of a dnopq' micromere. (D) Progeny of a nopq' micromere. (E) Progeny of an n' micromere. (F) Progeny of an opq' micromere. Dotted white lines in all panels except (D) show the borders of the germinal bands and the germinal plate [compare with drawing of stage 8 (early) embryo in Fig. 1]. The embryo in (D) was both epi- and transilluminated; it appears bright due to autofluorescence. Diameter of an individual embryo is approximately 500 μm.

progeny of the primary quartet. This primary quartet occasionally contributes a few cells to the provisional epithelium between the germinal bands (not shown).

The secondary trio. At least one additional round of cells is produced by the A, B, and C macromeres, and are designated as micromeres a", b", and c". Macromere D cleaves more or less equally at this time; thus the cell

that would, in a purely spiral cleavage be micromere d", is DNOPQ, precursor of the ectodermal teloblasts. That macromeres A, B, and C undergo further rounds of kar-yokinesis is indicated by the presence of syncytial yolk nuclei in these cells later in development (Weisblat *et al.*, 1984). It is not possible to see or inject the secondary trio directly because they lie beneath the primary quar-
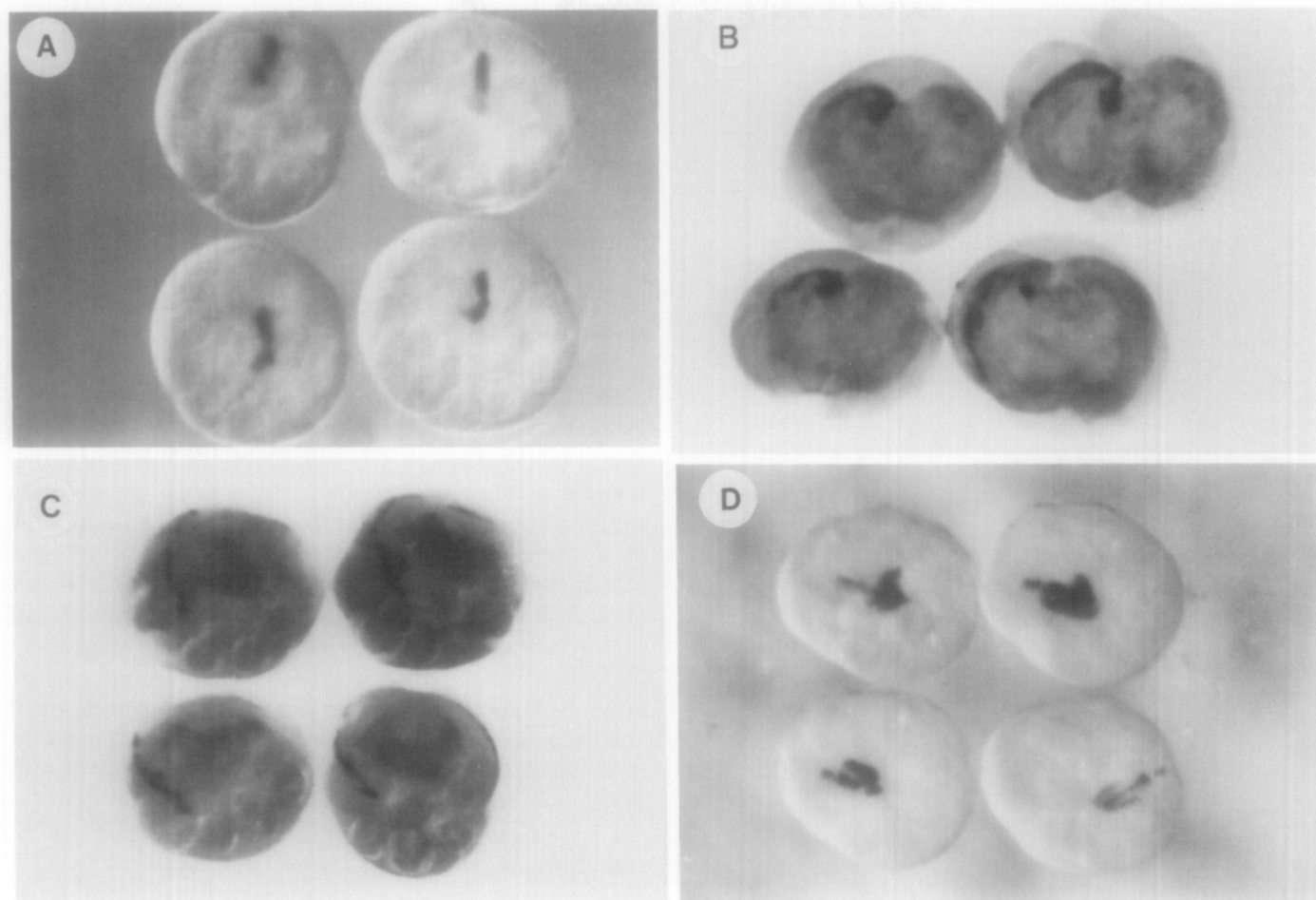
FIG. 3. Spatial stereotypy of micromere clones. HRP-labeled progeny of various micromeres in stage 8 leech embryos. Four replicate embryos are shown in each panel. (A) Progeny of single first quartet micromeres. (B) Progeny of dm' micromeres. (C) Progeny of left n' micromeres. (D) Progeny of three left opq' micromeres and one right opq' micromere (lower right). Embryos are oriented as in Fig. 2. Diameter of a leech embryo at this stage is 500 μm.

tet, but their presence has been confirmed in fixed, dissected, or sectioned embryos (S. Settle, personal communication; our unpublished results). Progeny of the secondary trio (and any other cells made by the macromeres after the primary quartet) were selectively labeled by injecting the parent macromere within 2 hr after formation of the primary quartet. In stage 8, no progeny of the a", b", and c" micromeres were detected in the provisional epithelium. Instead, their progeny were largely, if not entirely, deep cells, which, in stage 10, seemed to contribute to the esophagus (not shown).

dm' and dnopq'. Shortly after the birth of the mesodermal (DM) and ectodermal (DNOPQ) blastomeres from cell D, these cells produce one micromere each, dm' and dnopq', respectively. [For other annelid embryos, it has been reported that three micromeres arise from the homolog of cell DNOPQ (cell 2d^{222}, in Anderson, 1973).] Micromere dnopq' arises relatively close to the animal

pole, in the furrow separating C from DNOPQ, while dm' arises from lower and deeper in the embryo, in the furrow separating DM from DNOPQ (Fig. 1); later, both micromeres migrate to a position underneath the quartet and trio at the animal pole of the embryo. At stage 8, the progeny from these two micromeres, dm' on the left and dnopq' on the right, extend from the nascent germinal plate and cover the anterior ends of the germinal bands in a stage 8 embryo (Figs. 2B, C, and 3B). At stage 10, some of their progeny survive within the definitive epidermis. It is interesting that dm' and dnopq', arising in different sites in the embryo and from precursors whose fates are also different, propagate as right and left homologs and also that dm' gives rise to ectodermal structures, whereas its parent blastomere DM otherwise exclusively gives rise to mesodermal structures.

Right and left nopq'. After DNOPQ cleaves to form the left and right NOPQ cells, another pair of micromeres,
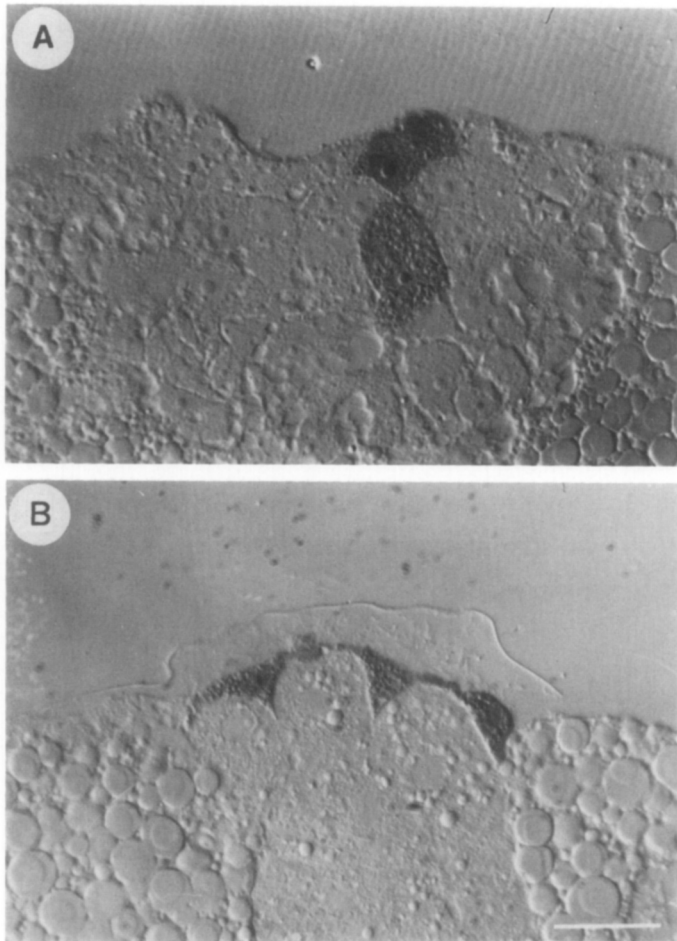
FIG. 4. (A) Section through the germinal plate of a stage 8 embryo showing the superficial and deep progeny cells of an HRP-labeled first quartet micromere. (B) Section through the caudal portion of one germinal band in a stage 8 embryo, showing the labeled progeny of an opq' micromere overlying the O, P, and Q bandlets. At this position the N bandlet has not yet entered the germinal band. The thin wavy line above the epithelium is a remnant of the vitelline membrane. The large round granules flanking the germinal band are yolk particles. Scale bar = 25 μm.

left and right nopq', are born from where the parent cells are in apposition at the recent cleavage furrow (Fig. 1). These cells also migrate to join the cap of micromeres at the animal pole. In the stage 8 embryo, their progeny are distributed symmetrically as clusters of cells just lateral to the future head (Fig. 2D). With the formation of left and right NOPQ cells, the bilateral symmetry of the embryo is unambiguously established; subsequent descriptions of micromere births and fates apply to both sides of the embryo.

Right and left opq'. Each NOPQ divides unequally, yielding an OPQ cell and an N teloblast. Subsequently, the nucleus of OPQ must move medioposteriorly, because the opq' micromere is born near the midline of the em-

bryo, where left and right OPQ cells touch (Fig. 1). As do the other micromeres, opq' migrates to the edge of the micromere cap; the pair of opq' cells is prominent, between the OPQ blastmeres. The progeny of opq' are predominantly squamous in stage 8 (Figs. 2F and 3D). They lie over the caudal end of the germinal band, typically covering blast cells in one or more bandlets from the time they first emerge from the depths of the embryo until after their first mitosis (Fig. 4B). In stage 10, progeny of opq' lie near the midline on the dorsal aspect of the embryo.

Right and left n'. From each N teloblast, a micromere arises between the N teloblast and the OPQ blastomere. Evidence exists suggesting that the N teloblast gives rise to two or three primary blast cells before generating n' and that these premicromere blast cells may give rise to specialized structures at the head of the animal, such as the ventral adhesive spot and the atypical anterior half of the subesophageal ganglion (R.K.H. and J. Braun, unpublished results). In stage 8, progeny of n' are almost exclusively squamous, and lie in a thin strip (usually 1–2 cells wide) at the very edge of the provisional epithelium, overlapping the outer edge of the germinal band and the macromere surface beyond it (Figs. 2E and 3C). In a stage 10 embryo, n' progeny contribute to the epithelium on the dorsal aspect of the leech.

Variability and Deficiency in the Fate Map

In the experiments described above, entailing 15–20 successful injections of each type of micromere, the variability in the pattern obtained for a given type (Fig. 3) was comparable to that observed for the segmentally repeating epidermal patches resulting from tracer injection of teloblasts. This variability resides both in the number of cells and in their relative position within the micromere cap. Such variability notwithstanding, it was possible to produce a map of the cells in the stage 8 provisional integument according to their micromere of origin (Fig. 5). Note that the central region of the epithelium is as yet unaccounted for. This unexplained deficiency may result either from contributions of undetected micromeres or from overly dilute tracer in progeny of cells that were injected or from reductions in the complement of epithelial cells generated by microinjected cells or from a combination of such factors. In any case, with even this partial map, we could predict which micromere(s) needed to be injected to generate domains of tracer-labeled epithelium over the germinal bands at stage 8. By filling chosen micromeres with photosensitizing tracer, specific portions of the provisional epithelium were rendered susceptible to laser ablation (Fig. 6), a necessary condition for testing the effect of
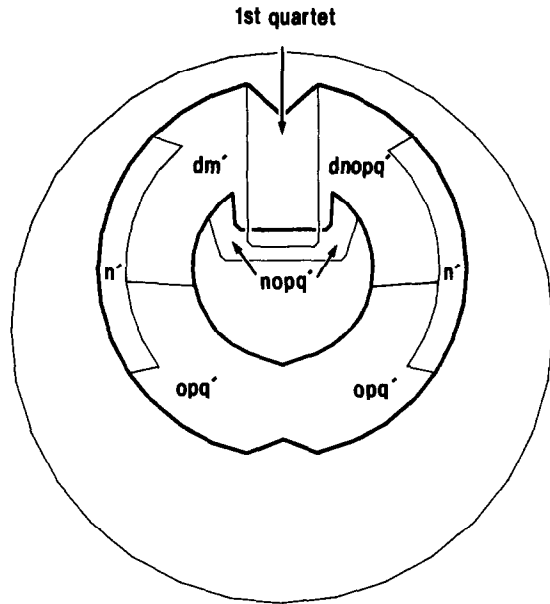
1st quartet

FIG. 5. A schematic view of an early stage 8 embryo (in the same orientation as that shown in Fig. 1 with a map of the provisional epithelium according to micromere of origin. Thick lines represent the outline of germinal bands and the germinal plate at this stage. Thin lines within the germinal bands delineate the idealized boundaries of the domains of micromere progeny. Macromeres, teloblasts, and bandlets are not represented.

epithelial ablations on fate determining interactions within the O–P equivalence group.

Effect of Epithelial Ablation on Primary Blast Cell Division

In normal development, each of the seven classes of primary blast cells (two each in the n and q bandlets, one each in the m, o, and p bandlets) can be distinguished by the timing, symmetry and spindle orientation of its mitosis (Zackson, 1984). In particular, primary p blast cells divide symmetrically soon after entering the germinal band and with the spindle axis roughly parallel to the long axis of the bandlet. Thus, anterior and posterior daughter cells of about equal size result, designated as p.a and p.p, respectively; primary blast cells in the o bandlet, divide at about the same time but via an asymmetric anaphase (Settle and Weisblat, in preparation) that results in a larger anterior daughter o.a and a smaller posterior daughter o.p. with a markedly compacted nucleus. Thus, upon staining stage 8 embryos with Hoechst 33258, in the segment of germinal band where primary o and p blast cells have undergone their first mitoses, nuclei in the o bandlet appear as a distinct pattern of alternating large diffuse and small bright spots, whereas the nuclei in the adjacent p bandlet ap-

pear as a string of uniformly large, diffuse spots (Zackson, 1984).

To test the hypothesis that the provisional epithelium is involved in normal determinative interactions between o and p blast cells, we examined the division patterns of the o and p blast cells after photolesioning the overlying opq'-derived epithelium. In our experiments, irradiated epithelial cells developed large vacuoles during the course of the illumination; after 2–3 hr they had frequently rounded up and separated from the body of the embryo (Fig. 7). Unlabeled epithelial cells appeared not to be affected by such illumination (Fig. 7), although we could not directly rule out this possibility because of the difficulty in following the fates of unmarked cells. Embryos were photolesioned 24–36 hr (early stage 8) after micromere injection, and were fixed 18–24 hr (mid stage 8) after photolesioning. These ablations did not affect the division pattern of the primary p blast cells but did cause the adjacent o bandlet to undergo a more or less equal division, like p blast cells (Fig. 8). Moreover, observation of slightly older o blast cell clones in the affected region of the germinal band in experimental embryos revealed that both of the o daughter cells di-
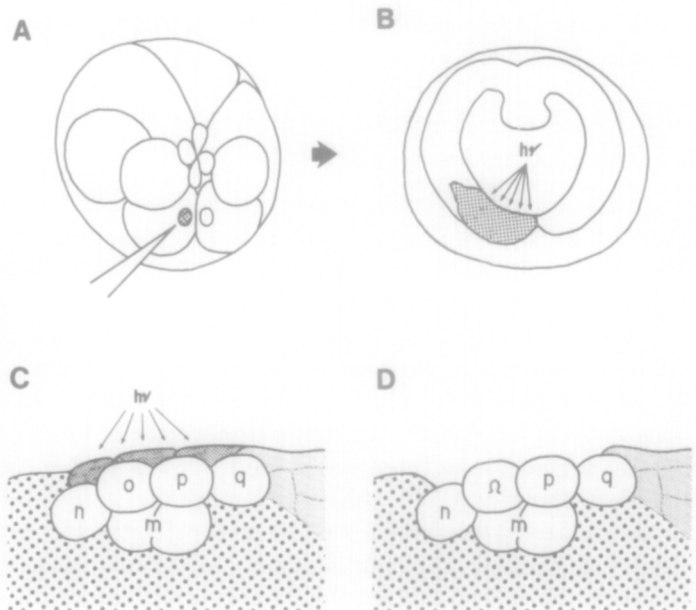

FIG. 6. Diagram outlining the procedure of the ablation experiments. (A) A micromere is injected with a photosensitizing lineage tracer, in this case fluorescein dextran. (B) The embryo develops to stage 8 at which time the labeled progeny from the micromere are ablated by laser irradiation at the fluorescein absorbtion maximum. (C) A cross-sectional view to show the relative positions of the provisional epithelium and the blast cell bandlets. (D) The effects of the ablation are then followed in the O and/or the P blast cells which contain the nonphotosensitive lineage tracer, BFD. The nominal o bandlet is now designated as Ω to emphasize its altered fate.
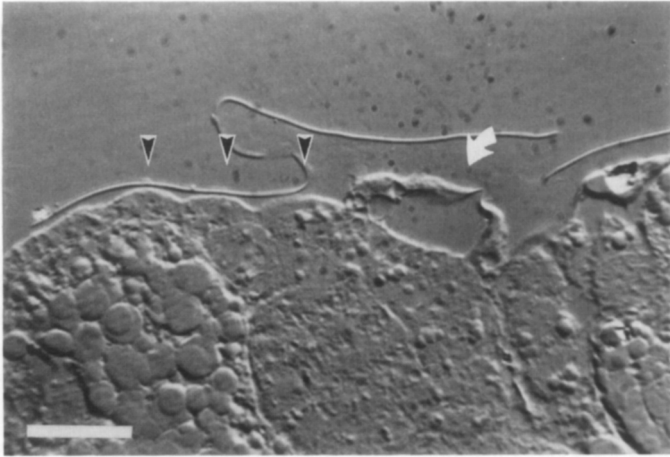
FIG. 7. Photomicrograph of a section through the caudal portion of one germinal band in a stage 8 embryo fixed 2 hr after photolesioning FDA-labeled epithelial progeny of micromere opq'. The orientation of this figure is about the same as for Fig. 4B. In this figure normal, unlabeled epithelial cells can be seen over the yolk and part of the germinal band on the left (black arrowheads). But epithelial cells to the right (white arrow), which have been photolesioned, are swollen, vacuolated, and partially detached from the underlying blast cells, whose morphology appears normal. Scale bar = 25 μm.

vided at least once more, again as in the P sublineage. These effects were not seen in 11 control embryos, in which unlabeled epithelial cells were similarly irradiated.

Effect of Epithelial Ablation on Definitive O and P Progeny

In normal development, the patterns of cells in the O and P kinship groups are quite stereotyped from segment to segment (Shankland and Weisblat, 1984). These patterns are summarized below and shown in Fig. 9.

The O kinship group at stages 10–11 includes three distinct clusters of neurons in the segmental ganglion, a large anterodorsal cluster, plus smaller medial and posteroventral clusters. In the periphery the O kinship group includes one large mediolaterally running strip of 20–25 epidermal cells, the medial epidermal patch and a small lateral patch of 2–4 epidermal cells, the lateral skin dot; 2 cells in the nephridiopore, also known as cell floret 2, the second of six epidermal specializations distributed circumferentially along segmental nerve MA; 2 of the most distal nephridial tubule cells; plus periperal neurons oz1, near the ganglion in the segmental nerve AA, oz2, similarly situated in nerve PP, and LD2, a more distal, dopamine-containing neuron on nerve PP (Stuart et al., in press).

The P kinship group at this stage includes a mediolateral wedge of about 15 neurons and one isolated interneuron pz4 in the segmental ganglion (Shankland and

Weisblat, 1984). In the periphery, the P kinship group includes a large irregular patch of ventral epidermis, complementary to that of the O kinship group (1–2 cells arise from N); several cells in cell floret 2, the nephridiopore rudiment; all of cell florets 1 and 3; plus peripheral neurons pz5, near the ganglion in the AA nerve, pz6 and LD1 (another dopamine-containing neuron) further out along the PP nerve, pz7, near the medial edge of the nephridiopore in the MA nerve, pz8, near the edge of cell floret 3, pz9 associated with the main body of the nephridium and pz10, near the O-derived LD2 in the PP nerve.

To further test the hypothesis that the provisional epithelium is involved in the normal determinative interactions between o and p blast cells, we examined the patterns of the definitive progeny of these cells after ablating overlying epithelial cells. In this set of experiments, both the opq' and the n' micromeres on one side of the embryo were injected with FDA shortly after they were born. A few hours later, one of the ipsilateral O/P teloblasts (i.e., either the generative O or the generative P teloblast) was injected with BFD. After 24–36 hr of subsequent development (to mid stage 8) the FDA-containing progeny of the injected micromere(s) were ablated by laser irradiation at 485 nm. At the time of ablation, the oldest progeny of the injected O/P teloblast had already grown past the area of the ablation and undergone one or more divisions; younger blast cells in the labeled bandlet were directly under the ablation zone or caudal to it and had divided but once or not at all. Photolesioned embryos were allowed to develop to stages 10–11 (4–5 days), then processed to visualize BFD and thus reveal the fates of cells derived from the labeled O/P teloblast.

Of approximately 50 embryos, from three different clutches of eggs, 36 survived these manipulations and developed to stages 10–11, including 31 in which the generative O teloblast had been injected and 5 in which the generative P teloblast had been injected. All of the P-labeled embryos and 12 of the 36 O-labeled embryos exhibited the normal labeling for their respective kinship groups. But the other 19 O-labeled embryos exhibited aberrations of the labeling pattern, frequently evident under the dissecting microscope as a deficiency of epidermal progeny in varying numbers of caudal segments. Eight such embryos were dissected for closer scrutiny with Nomarski optics; the periphery of the labeled segments in these embryos was examined cell by cell. Illumination did not affect the fates of BFD-labeled blast cells in eight embryos with unlabeled provisional epithelia.

Typically (Fig. 10), a distinct transition between the anterior, normal segments and the posterior, affected
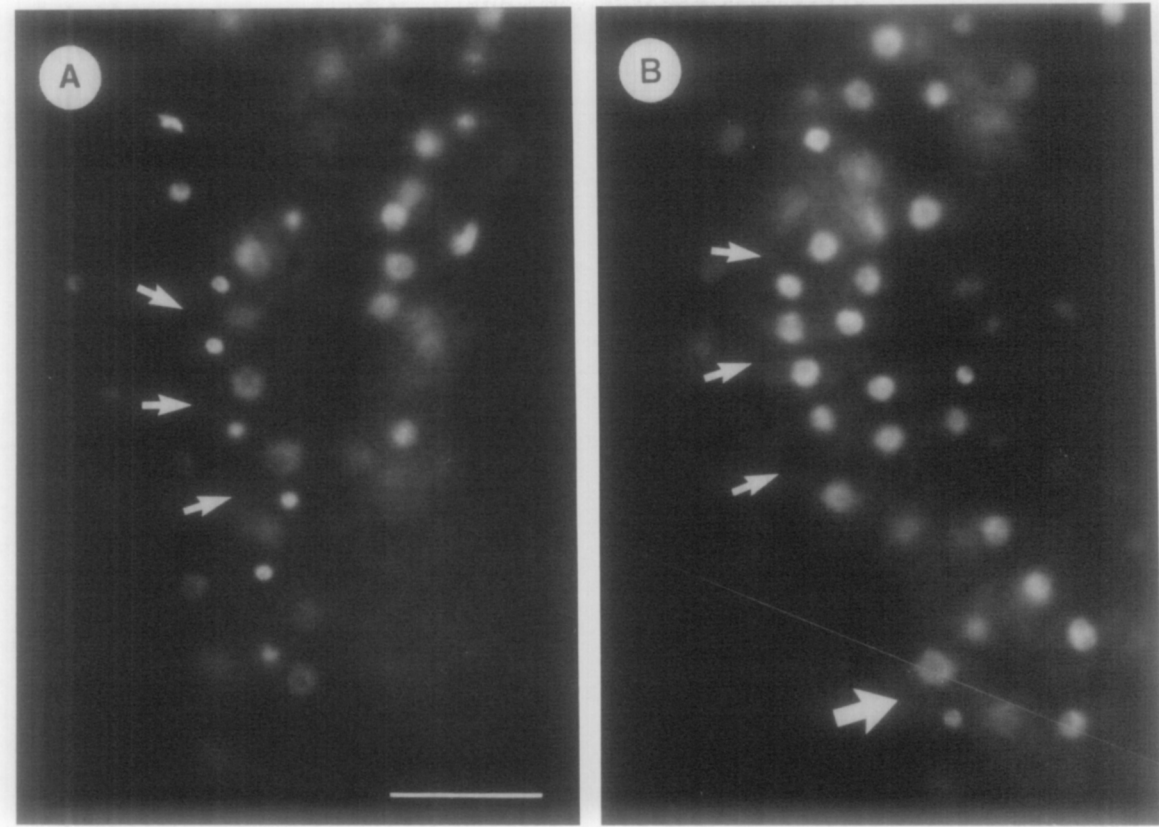
FIG. 8. Epithelial ablation affects the symmetry of blast cell mitoses in the o bandlet. Embryos stained with the fluorescent DNA stain Hoechst 33258 and photographed in an area of the left germinal band where the primary o and p blast cells have already undergone their first mitoses. Only portions of the o and p bandlets are visible. The o bandlet lies to the left in each panel. Small arrows point to daughter cell pairs arising from o blast cell divisions in normal (A) and photoablated embryos (B). (A) In the normal embryo, each o blast cell has divided into one larger cell and a smaller cell with compact nucleus. (B) The o blast cells affected by photoablation have undergone an essentially equal mitosis like that of the adjacent p blast cells. Large arrow at bottom points to a younger blast cell that has resumed the normal pattern of o blast cell division, presumably due to wound healing in the overlying epithelium. Anterior is up, medial to the right, scale bar = 25 μm.

segments was seen. Rostral to the transition, the O pattern was normal. Caudal segments had the normal O pattern in the ganglion, but were abnormal in the periphery. Most obviously, the large medial epidermal patch was often (22 of 66 abnormal segments) missing. All of the other normally derived O pattern elements, including the small lateral skin dot, were present; what truly set these segments apart was the appearance of supernumerary periperal neurons and other cells, most of which were indistinguishable from cells normally seen in the P kinship group. Because the locations and morphologies of these supernumerary neurons appeared as stereotyped as those of the normal O pattern elements, we refer to them in a similar manner with a lower case o (to denote their bandlet of origin) and y (rather than z, to denote this experimentally altered lineage) followed by a number (using the number of the apparent pz homolog when appropriate).

One such neuron, oy7, was located at the medial edge of the nephridiopore, as is pz7, and likewise sent an axon centrally along the MA nerve. In photolesioned embryos in which the P kinship group was labeled with BFD, we could frequently see an unlabeled, supernumerary neuron beside the labeled pz7, presumably oy7. This is evidence that the supernumerary neurons produced by the affected o blast cells duplicated P kinship group homologs rather than replacing them (Fig. 11A). Another periperal neuron, oy8, lay more peripherally along the MA nerve, as does pz8. Lateral to oy8, a cluster of supernumerary, labeled epidermal cells contributed to cell floret 3, a structure normally derived entirely from p blast cell progeny. Finally, in the PP nerve, we frequently found a neuron that had no obvious correspondent in the P kinship group. It could be that this cell was a homolog to pz9 or pz10 that failed to reach its final position, but we have designated it as oy11.

In this study, 66 experimentally affected segments were scored. The pattern described above was the most common result obtained. But there was considerable variability between segments as to the number and
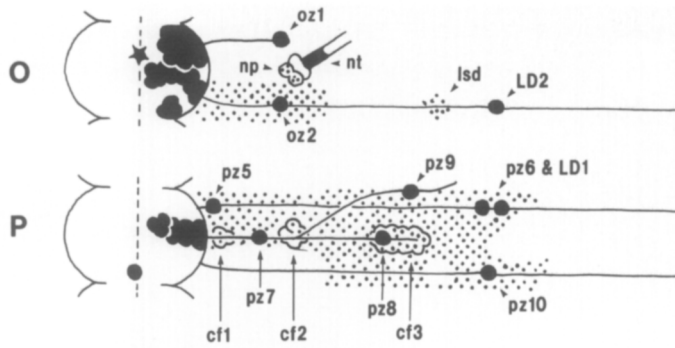
FIG. 9. Schematic representations of the O and P kinship groups in stage 10 embryos. In each panel one segment is shown. Anterior is up and the representation is that of an animal that has been opened along the dorsal midline and laid out flat. One segmental ganglion (and portions of two adjacent ones) are indicated by the large curved outlines. The ventral midline is indicated by the dashed line; dorsal midline lies at the right side of the figure. Stippled areas indicate epidermal domains in each kinship group. Cell bodies of neurons and glia are shown in black, as are afferent axons of peripheral neurons. Lobed shapes within the stippled areas indicate epidermal specializations called cell florets. From anterior to posterior the three major nerves (shown in the P lineage diagram) are designated AA, MA, and PP. cf, cell floret; np, nephridial tubule cells; np, nephridiopore cells; LD, lateral dopamine-containing neuron; lsd, lateral skin dot; oz and pz, identified neurons arising from the o bandlet and p bandlets, respectively.

identity of the supernumerary cells, as summarized in Table 1. The extent of the effect seen in a given segment probably depended on both the extent of the epithelial ablation and the age of the nascent o clones at the time of ablation. For example, embryos in which only the opq'-derived epithelium was lesioned still generated supernumerary neurons homologous to pz neurons (not shown), but in smaller numbers and in fewer segments than when both opq'- and n'-derived epithelium was lesioned. These effects were not seen when micromeres were ablated before they generated progeny; such embryos gave rise to normal O kinship groups. Thus the effect was limited to the local interactions of the overlying epithelium with the underlying blast cells.

Examination of the photolesioned embryos in which the P cell line had been labeled failed to reveal any deviations from the normal pattern, apart from segments in which epidermal progeny had spread over a larger than normal area of body wall, presumably in compensation for the loss of the O-derived medial epidermal patch (Blair and Weisblat, 1984). This fact, together with the observation that (except for the medial epidermal patch) the O kinship group was intact in all affected segments, was further evidence that the supernumerary cells in the affected segments represent duplications of presumptive P kinship group cells, rather than the replacement of missing cells as occurs in response to abla-

tion of the P bandlet. This was confirmed for the case of cell floret 3, the cells of which can be identified by their position and morphology even when they are not labeled. Transverse three micron sections cut through affected segments of O-labeled, photolesioned embryos show unlabeled (presumably normal, P-derived) and labeled (abnormal, O-derived) specialized epidermal cells in cell floret 3, which is normally derived exclusively from p bandlet progeny (Fig. 11B).

DISCUSSION

The primary conclusion of this paper is that an epithelium covering the germinal bands in the stage 8 embryo is necessary for the interactions that normally cause equipotent cells to follow different fates. This epithelium is derived from micromeres in the early embryo. Previous work has dealt mainly with fate mapping progeny of the teloblasts, which generate the definitive segmental tissues of the leech, and on the interactions between those progeny. We have extended this fate mapping work by examining the contributions of the micromeres, small cells which arise during the cleavage divisions which generate the teloblasts. It was known from previous work that some of these cells, the primary quartet, contribute progeny to the prostomial ectoderm, including neural and epidermal tissues, and to the epithelium of the provisional integument (Weisblat et al., 1984). Here, in a more exhaustive analysis of the progeny of the micromeres, we have found that each identified micromere contributes in a fairly stereotyped and reproducible fashion to the epithelium of the stage 8 provisional integument. Thus a fate map has been derived which relates particular micromeres to particular epithelial domains at stage 8. Two observations from this part of the study are of intrinsic interest: (1) All the micromeres studied, including dm' which arises from the mesodermal precursor DM, generate epidermal progeny. (2) Each micromere born after the first seven (quartet and trio) arises at the animal pole, moves in stereotyped fashion from its site of origin, and joins the first seven. Perhaps the most dramatic examples of this phenomenon are the movements of dm' and dnopq', two micromeres which arise from very different sites in the embryo well separated from the animal pole, to become left and right homologs, as judged by the distribution of their progeny at mid stage 8.

The elucidation of a fate map (Fig. 5) for the provisional integument allowed us to first label and later photolesion selected epithelial domains. These experiments suggested that the provisional epithelium mediates interactions between cells of the underlying O-P equivalence group. Normally, cells in the O/P-derived
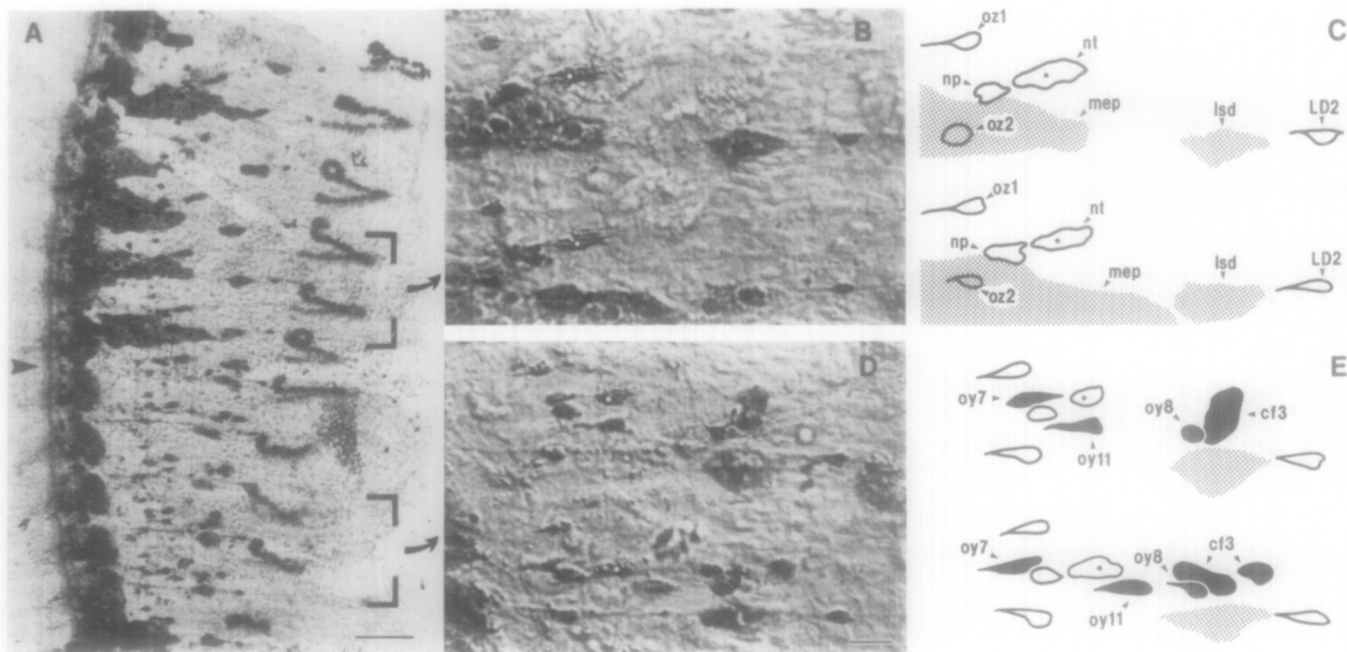
FIG. 10. Epithelial ablation affects the definitive progeny derived from cells in the o bandlet. (A) Twelve midbody segments of a stage 10 embryo in which the progeny of the right o bandlet are labeled with BFD and in which the ipsilateral opq'-derived and n'-derived provisional epithelium was photoablated in stage 8. The embryo was opened along the dorsal midline and flattened; anterior is up. Large arrowhead shows the point behind which the effects of the epithelial ablation were evident. Anterior to this point, the pattern of O-derived cells was normal. Behind this point, the medial epidermal patch (mep) was absent and a greater number of labeled periperal cells were present. Brackets delineate pairs of segments from the normal and the affected areas which are shown at higher magnification in (B) and (D), respectively. Small open arrow points to a segmentally repeated nephridium, which is stained artifactually by virtue of endogenous peroxidase activity. (B) and (C) Higher magnification photograph and drawing of two segments from the normal portion of the embryo shown in (A). The pattern of O kinship group cells is normal (compare with the diagram in Fig. 8). (D) and (E) Higher magnification photograph and drawing of two segments from the affected region of the preparation shown in (A). Except for the mep, the normal complement of O kinship group cells is present (shown in outline and stippling). The affected o bandlet also generated supernumerary peripheral cells, shown in black. Most of these supernumerary cells were homologous to cells normally derived from the P cell line, (i.e., oy7, oy8, and cf3). Occasionally ectopic cells were seen (i.e., oy11) which did not have identifiable P homologs. The small white dots over the nephridial tubule (nt), in (B) and (D), correspond to the small black dots in drawings (C) and (E). Abbreviations are as in Fig. 8. Nomarski optics, scale bar = 50 μm in (A), 10 μm in (B).
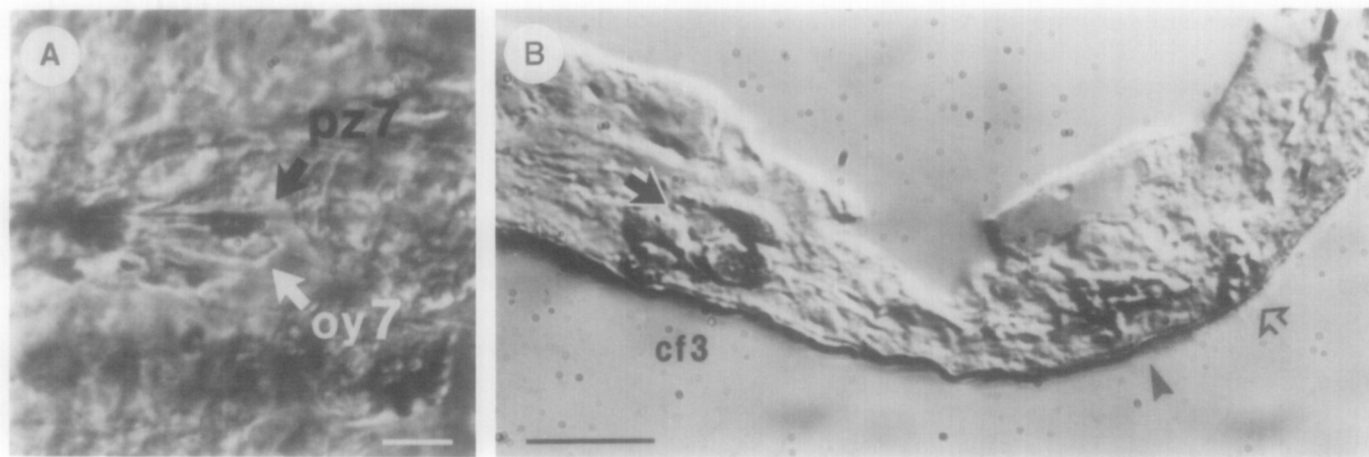

FIG. 11. Supernumerary cells derived from the o bandlet after epithelial ablation duplicate, rather than replace, P kinship group homologs. (A) Photograph of part of an embryo, dissected as in Fig. 9, in which a p bandlet was labeled with BFD and the overlying provisional epithelial cells were ablated. The pattern of labeled P kinship group cells was normal; beside the labeled pz7 neuron shown here is an unlabeled, supernumerary neuron, presumably oy7, the product of an altered o blast cell lineage. Anterior is up, ventral midline to the left. (B) Photograph of a transverse section of the dissected germinal plate of an embryo in which the o bandlet was labeled with BFD and the provisional epithelial cells ablated. Here, cell floret 3 contained BFD-labeled, o-derived cells as well as unlabeled p-derived cells (black arrow). Cell floret 3 normally arises exclusively from the p bandlet. Arrowhead points to labeled nephridial tubule cells; open arrow points to a labeled nephridiopore at the epidermal surface of the body wall; ventral midline is to the right. Nomarski optics, scale bar = 10 μm for both (A) and (B).

TABLE 1

PATTERN ABNORMALITIES IN AFFECTED O LINEAGES

Pattern element[a]	No. of times seen in 66 affected segments	Frequency
mep (missing)	22	0.33
cf1 (labeled)	8	0.12
cf3 (labeled)	54	0.81
oy5 (present)	3	0.05
oy7 (present)	32	0.48
oy8 (present)	52	0.79
oy9 (present)	2	0.03
oy10 (present)	24	0.36
oy11 (present)	28	0.42
other ectopic cells	17	0.26

[1] Abbreviations are as in Fig. 8.

bandlet nearest the animal pole divide more or less equally and assume the dominant P fate. Cells in the O/P-derived bandlet further from the animal pole divide unequally, assuming the O fate, unless "rescued" by ablation of the p bandlet before they become developmentally committed. Disrupting the integrity of the provisional integument affects both the symmetry of the initial mitoses of the o blast cell and their definitive fates. These results permit us to draw further conclusions regarding the interaction between the epithelium and the o and p bandlets:

(1) It is the ablation of the epithelium overlying the bandlets that causes the effects, not the absence of a particular micromere's clone; when an opq' micromere is ablated before generating progeny, the progeny of other micromeres will cover the opq' territory (unpublished observations), presumably by occupying abnormally large domains. Thus, as in the definitive epidermis (Blair and Weisblat, 1984), cells of the provisional epithelium seem to be interchangeable and the fate determining interaction does not depend on special lineage dependent properties of certain micromeres' progeny.

(2) The effect of epithelial ablation is local. The o and p bandlets contralateral to the ablation develop normally. Thus the effect seems to be mediated either by loss of contact or by short range interactions.

(3) The effect of epithelial ablation is graded rather than all or none. In response to ablation of opq' progeny only, primary o blast cells generate fewer definitive P kinship group progeny than with larger ablations, i.e., of the combined n'- and opq'-derived epithelial progeny. But even after these larger ablations, the O kinship groups are almost completely intact in affected segments (the only clear deficit is the absence of the medial epidermal patch in some affected segments). Only certain cells of the P kinship group are generated in these experiments (for instance, homologs to pz6 and LD1 were

never seen), and not all these cells appear even in the most severely affected segments. A likely explanation for this result is that the epithelium re-covers denuded blast cells [spreading as in the definitive epidermis by a combination of mitosis and spreading of existing cells (Blair and Weisblat, 1984)] and that the extent of the effect is dependent on both the length of time for which the nascent o blast cell clones are denuded, and their developmental age at that time.

(4) From what precursor(s) do the supernumerary cells arise? The answer to this question awaits further work, but we suggest the possibility that at least some of them may arise from a sublineage arising from the nominal o.p cell. In the normal, o lineage, this cell is much smaller than o.a; moreover, intact O segmental complements of definitive progeny are obtained from individual o.a blast cells injected with lineage tracers (Shankland and Stent, 1986), suggesting that o.p normally dies. But in the epithelial ablation experiments, the initial mitosis is approximately equal and the nucleus of the posterior daughter cell appears normal. The notion that this cell lives and generates definitive progeny in the lesioned embryos is appealing for two reasons. First, it would be consistent with the observation that the O pattern often appears complete in affected segments, since o.a alone normally generates the entire O complement. And second, the generation of supernumerary P homologs from the *posterior* daughter of the initial blast cell mitosis is in accord with the observation that many of the supernumerary cells that we observe (cf3, oy8, and oy10) are homologous to definitive progeny of p.p, the posterior daughter of the first p blast cell mitosis, (Shankland, personal communication). This model is at best incomplete, however. For example, one of the cells (pz6 or LD1) that is never duplicated is held to arise normally from p.p, and one of the most frequently duplicated cells (pz7) is held to arise normally from p.a (Shankland, personal communication). In addition this model fails to account for the frequent loss of the O-derived patch of medial epidermis.

The difficulty in interpreting our results in terms of the effect of the epithelial lesion on particular cells' fates may stem from the simultaneous influence of the two quasi-independent variables, namely the timing and extent of the epithelial lesion. But a probabilistic analysis of our results suggests that the occurrence and grouping of the supernumerary cells is far from random. For example, if the deviations from the normal O pattern (listed in Table 1) occurred independently in each affected segment, there would be $2^{10} = 1024$ possible classes of segmental patterns, whose predicted frequencies can be calculated from the probability of the presence or absence for each element. From Table 1 then, we would predict that the most common pattern in affected seg-

ments should be the one in which the medial epidermal patch, cell floret 3 and oy8 are present, but no other supernumerary cells, and that this pattern should appear with a frequency of 0.05, or in about 3 of 66 affected segments. In fact, this pattern was obtained in 12 of 66 affected segments ($P < 0.0001$ by test of equal proportions), and was only the second most commonly observed pattern. The most commonly observed pattern (15 of 66 affected segments), in which the medial epidermal patch is absent, and cell floret 3, oy8, oy7, and oy11 are present, has a predicted frequency of only 0.02, or about 1 of 66 affected segments ($P < 0.0001$). Thus, it is likely that certain supernumerary cells occur with linked probabilities in these altered lineages rather than randomly.

These results are in accord with previous observations on linked cell fates and the reciprocal relationships between pattern elements in the O–P equivalence group. First, it was found that cell floret 3 and neuron pz7 appear together in the stepwise transformation of the O pattern to the P pattern after ablation of the p bandlet (Shankland and Weisblat, 1984), just as we found that cf3 and oy7 frequently appear together in segments affected by epithelial ablation. Second, it was found that the appearance of these (cf3 and pz7) P pattern elements in nominal o blast cell clones coincides with the loss of the medial epidermal patch from the O pattern (Shankland, 1987). In the studies on cell commitment, the only variable was the age of the nascent o blast cell clone at the time the adjacent p bandlet was ablated. Thus, it was possible to make relatively precise correlations between the appearance of O pattern elements and the simultaneous disappearance of P pattern elements. In the present study, by contrast, the age of the clone and the extent of the epithelial ablation varied independently; moreover, most affected segments contained supernumerary neurons without any deficit of O pattern elements. Nonetheless, we conclude that the patterns of cells seen after epithelial ablations arise from abnormal, but nonetheless stereotyped lineages deriving from the normal lineages as the result of the experimental perturbation of the embryonic environment.

(5) It has previously been shown that an o blast cell can generate a normal P complement of definitive progeny upon ablation of the adjacent p bandlet, even after undergoing the asymmetric first mitosis that is idiotypic of the O sublineage (Shankland and Weisblat, 1984). Here, conversely, we have shown that a primary o blast cell can generate a normal O complement of definitive progeny even after, in response to the ablation of the provisional epithelium, it has undergone the symmetric first mitosis that is idiotypic of the P sublineage. Presumably, when only the opq′-derived progeny are ablated, the epithelium heals before the nascent clone gives rise to many cells committed to an identifiable P

fate. Moreover, older o clones may reach a point where they are refractory to effects of epithelial ablation as has been found for the response to p bandlet ablation. Thus, it appears that commitment to the P fate may occur in a stepwise process, as has been shown for commitment to the O fate (Shankland and Weisblat, 1984). This conclusion remains tentative, however, in the absence of a more precise correlation between the timing and extent of epithelial deprivation with the fate of individual o blast cell clones.

The definition of the equivalence group was abstracted from results of combined lineage and ablation experiments in the nematode *Caenorhabditis elegans* (Kimble et al., 1979; Sulston and White, 1980). Since then, phenomena satisfying the same formal criteria have been found in the leech, as described here, and in insect (Kuwada and Goodman, 1985). Ironically, recent work on *Caenorhabditis* suggests that the different fates of the cells in the archetypal, vulval equivalence group are determined by graded inductive interactions with another cell called the anchor cell and not among the six equivalent cells at all (Sternberg and Horvitz, 1986). These authors conclude that in the absence of outside influences the equivalent cells would follow the tertiary fate and that cells are stimulated to adopt the primary or secondary fates by diffusible signal(s) from the anchor cell. Our finding in *Helobdella* that the epithelium plays a role in determining cell fates in the O–P equivalence group is consistent both with a model in which there are no interactions among equivalent cells and also with one in which the epithelium *mediates* interactions between o and p blast cells. We are unable to distinguish between these alternatives at present. Similarly, it is not yet possible to be certain whether the O or the P fate constitutes the ground state for these blast cells. A priori, it has been assumed that the symmetric divisions of the p blast cells must represent the unperturbed state, i.e., that the primary fate is the ground state for the O–P equivalence group. This is not necessarily the case, however, granted that membrane or cytoskeletal asymmetries could be inherited by the blast cells from the parent O/P teloblasts that would lead to asymmetric divisions by unperturbed blast cells. Thus, the question of whether or not the phenomena defined as developmental equivalence groups share a common mechanism, as opposed to being operationally equivalent manifestations of different processes, remains unresolved. Nonetheless, the answer to this question is of relevance to discussions of how broadly and at what level to generalize in interpreting developmental mechanisms.

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