

Micromere fate maps in leech embryos: lineage-specific differences in rates of cell proliferation

Constance M. Smith* and David A. Weisblat

Department of Molecular and Cell Biology, 385 LSA, University of California, Berkeley, CA 94720, USA

*Present address: Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA

SUMMARY

Stereotyped early cleavages in glossiphoniid leech embryos yield 25 micromeres, along with 3 macromeres and 10 teloblasts. The micromeres generate prostomial tissues and also give rise to most of the squamous epithelium of a provisional integument that spreads epibolically from the animal pole, covering the rest of the embryo during germinal plate formation.

We systematically injected individual micromeres with fluorescent cell lineage tracers at the time of their birth and quantitatively mapped the contributions of all these cells to the late stage 7 embryo, a time in development that is early in the epibolic expansion. At this time, micromere derivatives comprise two types of cells: squamous epithelial (superficial) cells that cover the germinal bands and the region of the animal cap between the germinal bands; and

underlying (deep) cells that are confined to the distal ends of the germinal bands and in the area between their distal ends. We find that individual micromeres contribute clones of deep and/or superficial progeny that are stereotyped with respect to both numbers and types of cells in the clone and the domains that they occupy. The N teloblasts also contribute cells to the squamous epithelium.

We find significant differences in the rate of cell proliferation between different micromere clones. These differences appear to reflect lineage-specific traits, since there is little or no regulation of cell number after ablation of individual micromeres.

Key words: cell lineage, cell ablation, fate map, leech embryo, micromere, annelid

INTRODUCTION

Glossiphoniid leeches, like other annelids, develop via a stereotyped sequence of early embryonic cleavages, leading to the formation of blastomeres that can be reliably identified from embryo to embryo (Whitman, 1878). Upon the completion of cleavage [i.e. at the end of stage 6 (Fernandez, 1980; Stent et al., 1992)], the zygote has given rise to three large blastomeres (*macromeres*), five bilateral pairs of medium-sized stem cells (*teloblasts*) and also 25 *micromeres*, a group of small cells lying near the animal pole in prospective anterior and dorsal territories of the embryo (Sandig and Dohle, 1988; Bissen and Weisblat, 1989).

Most cell lineage analyses of glossiphoniid leech development to date have focused on the teloblasts, which are the precursors of segmental tissues (for reviews, see Shankland, 1991; Stent et al., 1992). Micromeres, in contrast, contribute definitive progeny to the supraesophageal ganglion and other prostomial tissues (Weisblat et al., 1984; Ramirez and Weisblat, unpublished data) and also to a squamous epithelium that is part of the *provisional integument*, a temporary body wall for the embryo during stages 8-10 (Weisblat et al., 1984; Ho and Weisblat, 1987; Ramirez and Weisblat, unpublished data). The small size of the micromeres renders them more difficult than the teloblasts to work with and, consequently, the tissues constituting their definitive progeny are less well understood. Prior

to the work of Sandig and Dohle (1988) not all micromeres were known and consequently, as will be demonstrated here, others were misidentified. In the work presented here, we have used microinjected cell lineage tracers in combination with other techniques to map quantitatively the fates of all the micromeres in terms of their contributions to the embryo during early epibolic expansion in embryos of *Helobdella robusta*.

We find that each micromere contributes predictable numbers of cells in stereotyped domains. Individual micromeres contribute progeny to the squamous epithelium (superficial cells) or to cells lying beneath the surface of the epithelium (deep cells) or to both. We observe significant micromere-specific differences in the size of descendant clones that cannot be accounted for by differences in the age of the clones. No regulation of cell number was observed within either the entire epithelium or individual clones in response to ablation of selected micromeres. Thus we conclude that the observed lineage-specific differences in cell proliferation reflect cell-autonomous traits. Detailed knowledge of the origins of the squamous epithelium is useful in investigating the role of this tissue in epiboly (Smith et al., unpublished data).

MATERIALS AND METHODS

Embryos

Helobdella robusta embryos were obtained from a laboratory colony

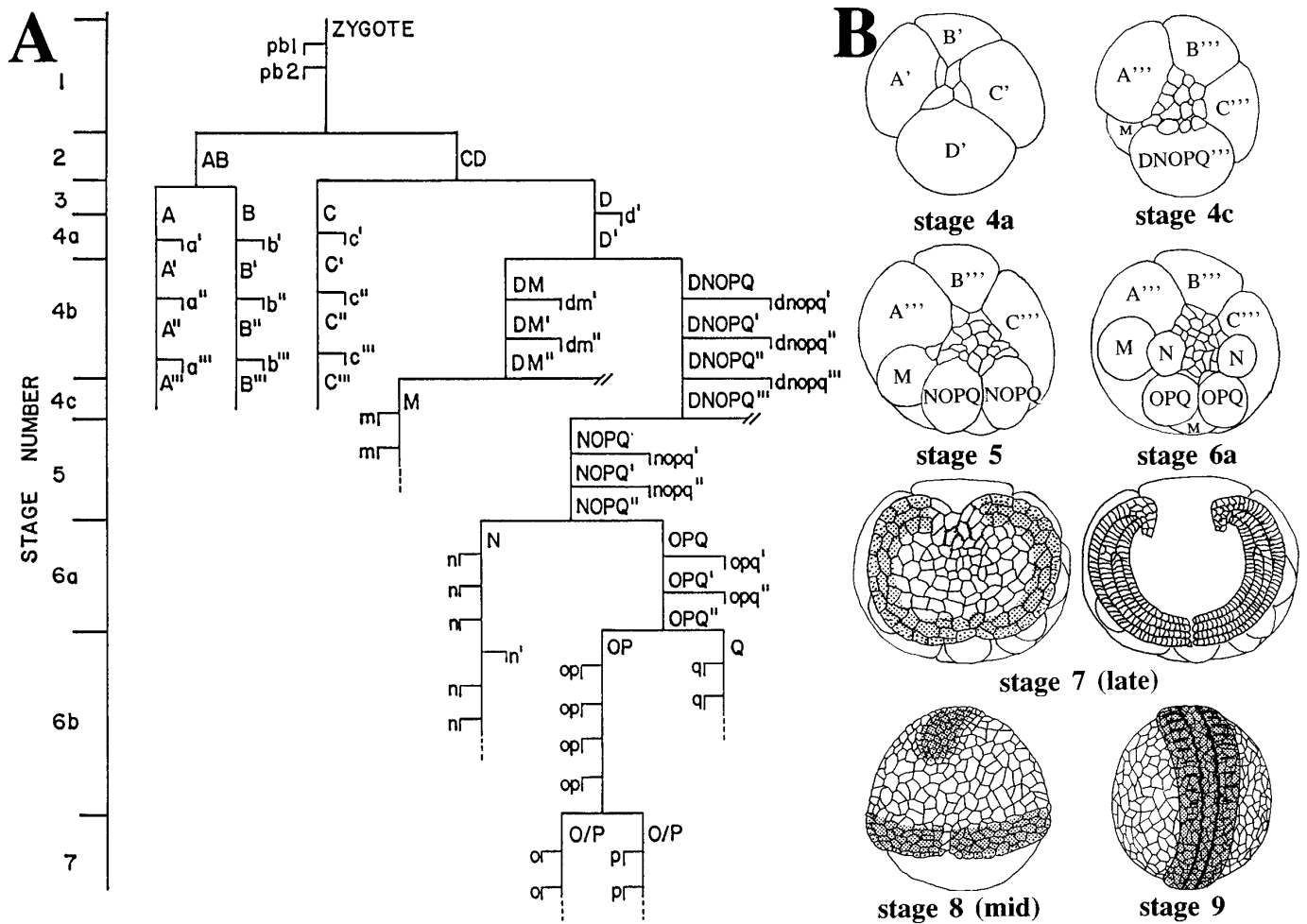


Fig. 1. Summary of glossiphoniid leech development. (A) Cell lineage diagram showing the production of micromeres during stages 1 through 7. (B) Illustrations of selected stages of embryonic development. All view the animal pole and prospective dorsal surface, except for the stage 9 embryo. (Stages 4a-6a) Macromeres, proteloblasts and teloblasts are labeled; the smaller contours in the center of each panel represent the distribution of micromeres. [For precise depictions of micromeres, see Sandig and Dohle (1988).] (Late stage 7 (stage 6a + 44 hours, the endpoint for the experiments reported here)) By this time, each teloblast has initiated a series of several dozen highly unequal divisions to produce a coherent column (*bandlet*) of segmental founder cells (*primary blast cells*). On each side, the five bandlets come together in parallel arrays called *germinal bands* which lie under the provisional epithelium derived from the micromeres. The left drawing depicts the squamous epithelium as it would appear after silver staining; location of the underlying germinal bands are indicated by stippling. The right drawing depicts the germinal bands as they would appear without the overlying epithelium and other micromere derivatives. (Stage 8 (mid)) As more blast cells are budded off by the teloblasts, the germinal bands lengthen and move across the surface of the embryo, gradually coalescing along the future ventral midline into a structure called the *germinal plate*. This is accompanied by epiboly of the sheet of squamous epithelium. The anterior end of the germinal plate (stippling) is visible at the top of the embryo; left and right germinal bands (stippling) lie at its equator, beneath the leading edge of the epithelium. Segmental tissues arise from the proliferation and differentiation of cells within the germinal plate. (Stage 9 (ventral view)) By this point, the germinal plate is complete and segmental tissues are forming, including the segmental ganglia of the ventral nerve cord (heavy outline). The edges of the germinal plate gradually expand dorsolaterally and eventually meet at the dorsal midline (end of stage 10), forming the tube that makes up the body of the animal. During this process, definitive epidermis arises from the germinal plate and also expands dorsolaterally at the expense of the provisional epithelium. Approximate diameter of an embryo is 400 μm .

maintained at 15°C in a 1% dilution of artificial sea water (Instant Ocean). Isolated embryos were cultured at 23°C in standard HL saline (4.8 mM NaCl, 1.2 mM KCl, 2.0 mM MgCl₂, 8.0 mM CaCl₂ and 1.0 mM maleic acid, pH 6.6) which was changed daily. The developmental staging system (Stent et al., 1992) is based on that of Fernandez (1980) and the cell nomenclature is that adopted by those workers and others as a less cumbersome alternative to that of Wilson (1892). Accordingly, macromeres and teloblasts are designated by uppercase letters; micromeres are designated by the lowercase letter(s) corresponding to the parent blastomere. The names of the first

micromere and large blastomere descended from a particular cell are followed by one prime ('), names of the second set are followed by two primes (''), and so on (Fig. 1A). Table 1 compares the names of micromeres according to the standard and revised systems.

Cell lineage tracing and ablation

Micromeres (ca 20 μm in diameter at birth) and N teloblasts (ca 100 μm) were pressure injected with a mixture of either 75 mg/ml fluorescein-conjugated dextran amine FDA; Molecular Probes) or 60 mg/ml Texas Red-conjugated dextran amine (TRDA; Molecular

Table 1. Designations of micromeres*

Bissen and Weisblat (1989)†	Sandig and Dohle (1988)
d'	1d
c'	1c
a'	1a
b'	1b
c''	2c
dnopq'	2d ^I
dm'	3d
a''	2a
b''	2b
dnopq''	2d ^{2I}
dm''	4D
c'''	3c
a'''	3a
b'''	3b
dnopq'''	2d ^{22I}
nopq'	t ^I
nopq''	t ^{II}
opq'	opq ^I
opq''	opq ^{II}
n'	n ^{IV}

*Micromeres are listed in the order they appear during cleavage.

†Terminology used in the manuscript.

Probes) in 0.2 N KCl. The injection mixture also contained 1% fast green to allow for visual monitoring of the quantity of the injection. Injected embryos were allowed to continue to develop, then fixed 44 hours after the time when one half the clutch was at stage 6a (N teloblast formation).

Individual micromeres were injected in each case with the exception of opq', which is difficult to inject because it is usually obscured by the nopq'' micromere. Thus, in approximately half of the

embryos used for counting progeny of opq', the contribution of micromere opq' was inferred by comparing the pattern of cells arising from cell OPQ with that arising from cell OPQ'', using the subtractive method of Zackson (1982). For this purpose, the OPQ blastomere was injected with TRDA and the OPQ'' blastomere with FDA; the progeny of opq' were then identified as those cells in the resultant embryo that were at the distal end of the germinal band and that contained only TRDA. Although it would seem preferable to make the second injection into the OPQ' blastomere, so that only the opq' would contain a single lineage tracer (rather than both the opq' and opq'' clones as in the protocol used here), timing the second injection was problematic because of the difficulty in determining when the opq' micromere has been born. Moreover, the progeny of the opq'' micromere can readily be distinguished by their location over the proximal part of the germinal band, as determined by direct injection of the opq'' micromere.

To verify the identity of certain micromeres, the parent blastomere was injected with lineage tracer before the birth of the micromere. Then either (1) the embryo was fixed shortly after the birth of the micromere, silver stained to outline the superficial cells, and then examined for the presence of tracer in the micromere and/or (2) the putative descendant micromere was injected with a different lineage tracer and the progeny of this micromere were examined for the presence of both lineage tracers.

The ages of the micromere clones given in Table 2 represent the average interval between the birth of the micromere in question and the endpoint used in these experiments (44 hours after stage 6a). The times of birth, relative to egg deposition, for the micromeres were determined by observing 6-9 fairly synchronous embryos from 3-6 clutches every 10-15 minutes until most or all of the embryos had made a particular micromere. The observed time to cleavage for the various clutches was averaged and rounded off to the nearest 5 minutes; the age of descendant clones was measured relative to that average time.

Table 2. Micromere contribution to the late stage 7 embryo

Micromere	Ave. no. epithelial cells*·†	n	Ave. no. deep cells*·‡	n	Ave. no. total progeny*·§	n	Clonal age in hours
d'	9.6±3.1	10¶	(20.1)	—	29.7±4.2	6	54.5
c'	10.4±1.8	10	(16.1)	—	26.5±3.7	4	54.2
a'	9.0±1.3	9	(11.3)	—	20.3±1.5	7	53.8
b'	7.9±2.7	15¶	(14.7)	—	21.1±2.0	7	53.8
c''	0	—	6.5±1.4	10¶	(6.5)	—	52.2
dnopq'	9.9±1.4	9¶	(5.1)	—	15.0±2.7	6	51.8
dm'	0	—	10.6±1.3	8¶	(10.6)	—	51.7
a''	0	—	4.8±1.0	4	(4.8)	—	51.7
b''	0	—	5.8±0.5	4	(5.8)	—	51.7
dnopq''	12.9±3.4	14¶	(5.1)	—	18.0±2.0	5¶	50.5
c'''	0	—	5.3±0.5	7	(5.3)	—	50.2
dm''	0	—	15.0±2.5	4	(15.0)	—	50.2
a'''	0	—	5.8±1.0	6¶	(5.8)	—	49.7
b'''	0	—	6.7±0.6	3¶	(6.7)	—	49.7
dnopq'''	7.6±0.7	8	0	—	(7.6)	—	49.2
nopq'***	6.0±1.6	16¶	2.5±1.0	4¶	(8.5)	—	46.7
nopq''***	5.5±1.7	14¶	1.9±0.5	11	(7.4)	—	45.6
opq'***	3.7±1.8	18¶	(7.5)	—	11.2±1.7	8	43.0
opq''***	16.8±3.5	13	0	—	(16.8)	—	41.8
n'***	7.7±1.5	23	0	—	(7.7)	—	39.6

*Averages are given ± s.d.

†The sum of the average number of epithelial cells contributed by each micromere clone and the average number of epithelial cells contributed by each n bandlet (7.8±2.3; n=8) to this stage is 162.3. The average number of epithelial cells in the embryo at this stage as determined by counting the number of cells in silver-stained micromere caps is 157.2 (s.d.=13.8; n=91).

‡The deep cell averages which have s.d.s were obtained as described in Materials and Methods. The deep cell averages in parentheses were calculated by subtracting the average number of epithelial cells from the average number of total progeny for that micromere.

§The total progeny averages which have s.d.s were obtained as described in Materials and Methods. The total progeny averages in parentheses were calculated by summing the average number of epithelial and deep cells.

¶This data was obtained from more than one clutch of embryos.

**No difference was observed between the contribution of each bilateral homologue.

Selected micromeres were killed shortly after birth by overinjecting with lineage tracer until they lysed. After fixation, embryos were screened to ensure they contained no cells labeled with the lineage tracer used for the ablation.

Microscopy

Embryos to be viewed by epifluorescence microscopy (Zeiss Axiophot) were fixed overnight at 4°C in 3.7% formaldehyde in 0.1 M Tris-HCl buffer (pH 7.4), rinsed twice in 0.1 M Tris-HCl buffer, stained for 90 minutes at 4°C with 5 µg/ml Hoechst 33258 in 0.1 M Tris-HCl buffer, washed in 0.1 M Tris-HCl buffer, dehydrated in an ethanol series, and cleared in methyl salicylate and viewed as whole mounts.

Visualizing and counting superficial cells

Superficial cells in the provisional epithelium were visualized by silver staining, using the method of Arnolds (1979), as modified by Ho (1988). Embryos were fixed in 0.8% formaldehyde in 0.1 M sodium cacodylic acid (pH 7.4) for 30 minutes at room temperature. Embryos were then briefly washed in distilled water to remove excess ions and incubated in the dark for a minimum of 5 minutes in silver methenamine solution [0.1% AgNO₃, 1% hexamethylene tetramine, and 0.25 M boric acid (pH 9.4)]. Then embryos were exposed to strong, white light from two fiber optic lamps (Dolan Jenner Industries) until the superficial cells were outlined by deposition of a dark brown reaction product along the furrows of the cells (about 15 minutes). Embryos were then fixed overnight at 4°C in Carnoy's fixative (6 parts 100% ethanol: 3 parts chloroform: 1 part glacial acetic acid) before being transferred to 100% ethanol for storage.

To count superficial cells in the micromere cap, embryos were rehydrated, cleared in 70% glycerol/0.1 M Tris HCl (pH 9.4), and the image of the embryo was projected onto a video monitor (MTI series 68 video camera and Zeiss Axiophot microscope). Superficial cells in the entire cap were counted using the capabilities of a graphics workstation (Cubicomp). Each specimen was subjected to four separate counts and the results were averaged. The standard deviations obtained for recounting individual micromere caps ranged from 1.0 (range of 141-143 cells) to 9.9 (range of 127-147 cells); the average standard deviation was 3.9 ($n=91$). Three potential sources of systematic error in these counts include (1) missing cells at the edge of micromere cap if it projects out of the horizontal plane, (2) missing cells that present small contours in the silver-stained epithelium or (3) failing to resolve adjacent cells due to suboptimal silverstaining. These errors would all lead to underestimating the actual number of cells.

To facilitate counting of the number of tracer-labeled cells and to obtain clearer photographs, embryos were flattened slightly beneath a coverslip. All epithelial cell counts are reported as the average of all embryos counted \pm the standard deviation between those counts.

Visualizing and counting deep cells

To count the number of deep cells in the clone arising from a given micromere, embryos in which that micromere had been injected with lineage tracer were stained with a mouse monoclonal antibody (provided by D. Stuart) that recognizes leech nuclei following the method of Nelson and Weisblat (1992) with the exception that the antibody staining took place at 4°C. The embryos were then viewed as whole mounts in either 3 or 5 µm optical sections with a scanning confocal laser microscope (BioRad MRC600) and the number of nuclei in tracer-labeled deep cells was determined. (For the *opq'* micromeres, subtractive techniques were used, as described above.) The counts reported are the averages of all embryos counted \pm the standard deviation between those counts. For micromeres whose descendant clones contain superficial cells in direct apposition to deep cells at the endpoint used for these experiments, it was difficult to distinguish reliably between the two types of cells. With these clones, the total number of cells in the clone were counted and the number

of deep cells was determined by subtracting the average number of superficial epithelial cells in the clone from the average number of cells in the entire clone.

RESULTS

Summary of leech development

Fig. 1 summarizes features of leech development that are relevant to the present study. Cell divisions are stereotyped, giving rise to blastomeres that can be identified individually by size, position, the order in which they arise and/or by the segregation of domains of yolk-deficient cytoplasm. During cleavage, three distinct classes of blastomeres are formed, *teloblasts*, *macromeres* and *micromeres*. The five bilateral pairs of teloblasts (designated M, N, O/P, O/P and Q) are progenitors of the segmentally iterated cells in the leech. The three macromeres, A''', B''' and C''', are the largest cells in the stage 6 embryo and provide the substratum upon which the morphogenetic movements of embryogenesis take place. 25 micromeres are produced during stages 4-6 via highly unequal cell divisions (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). They are born in prospective anterior and dorsal territory at the animal pole of the embryo. Nine micromeres arise from the A, B and C quadrants of the embryo. The remaining 16 micromeres, including 6 unpaired cells and 5 bilateral pairs, come from the D quadrant. Thus, for this study, we recognize 20 distinct types of micromeres in the leech embryo. The micromeres and their progeny are sometimes referred to as the micromere cap.

Micromeres contribute tissues to the prostomium of the adult leech (Weisblat et al., 1984; Ramirez and Weisblat, unpublished data). They also give rise to a squamous epithelium that covers the germinal bands (parallel arrays of the teloblast-derived segmental founder cells) and the intervening prospective dorsal territory (see Fig. 1B). Coincident with the circumferential movement of the germinal bands to form the germinal plate, the micromere cap undergoes an epibolic expansion to cover the embryo with a squamous epithelium. [This epithelium and underlying muscle fibers derived from the M lineage constitute the *provisional integument*, which provides a temporary body wall for the embryo during germinal plate expansion (Weisblat et al., 1984).] As the germinal plate expands during stages 9 and 10, the provisional integument retracts before, or is pushed back by, the leading edges of the germinal plate. During this period, definitive epidermis is being produced by the germinal plate. Thus, much or all of this micromere-derived squamous epithelium has been lost at the end of body closure.

Micromere fate maps in the late stage 7 embryo

Contributions of individual micromeres to the normal late stage 7 embryo were mapped by injecting individual micromeres in *H. robusta* embryos with lineage tracer at the time of their birth and then determining the distribution of their progeny in embryos fixed 44 hours after N teloblast formation (see Materials and Methods). This experimental endpoint was chosen for two reasons. First, it marks the developmental transition from the period of cleavage and germinal band formation to the period of germinal plate formation; thus, the locations of micromere progeny at this point can be used to draw infer-

ences as to their involvement in these processes. Second, at late stage 7, the germinal bands and the micromere cap are confined to a compact territory covering the animal pole, so it is possible to observe and count the cells without sectioning or rotating the embryo.

The contribution of each micromere to the late 7 embryo is relatively stereotyped with respect to the number of cells contributed (Table 2) and their position within the micromere cap (Fig. 2). We find that individual micromeres generate clones of squamous epithelial cells covering the germinal bands and the area in between them and/or deep cells which are confined to the distal ends of the germinal bands and to the area between their distal ends, where germinal band coalescence will begin. Our findings with respect to individual micromeres are described below.

The primary quartet of micromeres

The first four micromeres arise during the spiral third cleavage from blastomeres A, B, C and D and are designated as a', b', c' and d', respectively (Fig. 1). In the late stage 7 embryo, the progeny from each of these four micromeres form a ragged stripe, consisting of 8-10 superficial and 11-20 deep cells, in the prospective anterior region of the micromere cap, between the germinal bands (Figs 2, 3; Table 2). The deep cells derived from primary quartet micromeres interdigitate extensively among themselves and with deep cells derived from other clones (see following sections). We have not examined the details of this cell mixing. The superficial cells from each primary quartet micromere generally form coherent clusters, but not necessarily directly over the deep cells in the same clone. In keeping with the initial positioning of the primary quartet relative to the embryonic midline, the d' and a' clones exhibit rough mirror symmetry across the midline to the c' and b' clones, respectively. Moreover, the fact that the four clones lie roughly side by side from left to right at late stage 7 is consistent with observations by Nardelli-Haeffliger and Shankland (1993) that the a' and b' clones are moving dorsoposteriorly between the c' and d' clones at this stage.

The secondary and tertiary trios of micromeres

The secondary and tertiary trios of micromeres arise during the levo- and dextrorotatory fourth and fifth cleavage divisions, respectively, from the A, B and C quadrants and are designated as a''-c'' and a'''-c''' (Fig. 1). In the late stage 7 embryo, the progeny from all six of these micromeres consist entirely of deep cells clustered between the distal ends of the germinal bands, underneath or between the quartet micromere deep progeny (Figs 2, 4A-C,E-G; Table 2). The clones of the secondary and tertiary micromere trios, like those of the primary quartet, are in more than one deep cell layer and interdigitate extensively with each other and with other deep quartet cells. In some cases, individual clones have divided into separate clusters of cells by this point in development. Among these six micromere clones, a'' and a''' generally lie to the left of the embryonic midline while b'', b''', c'', and c''' lie to the right, but it is not possible from these data to define certain clones as contralateral homologs of others (see next section).

The dm' and dm'' micromeres

The mesodermal precursor, blastomere DM, arises as the vegetal daughter of macromere D' at fourth cleavage. As a

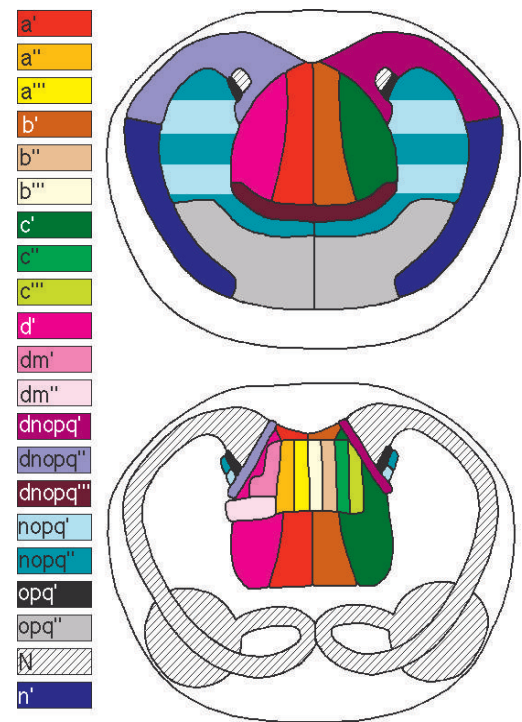


Fig. 2. Disposition of micromere progeny in superficial (upper map) and deep layers (lower map) within the micromere cap. These and all subsequent illustrations depict embryos at stage 6a + 44 hours (i.e. late stage 7), viewed from the animal pole and prospective dorsal aspect. The outer contour in each map represents the edge of the embryo; N teloblasts and their bandlets are denoted by hatching in the lower map. Otherwise, schematic representation of contributions by individual micromeres (and N teloblast-derived blast cells) are depicted by colored domains with dark contours according to the color coded boxes at left. The intermingling superficial progeny of ipsilateral nopq' and nopq'' cells (upper map) are depicted as alternating horizontal stripes within a single dark contour. The contours shown indicate approximate, averaged boundaries; individual clones have irregular boundaries that vary from embryo to embryo (see Figs 3-7). The positions of various deep cell groups are further stylized so that they can be projected to a single plane. In fact, progeny of primary quartet, DM-derived and trio micromeres interdigitate.

result of the levorotatory spiral fourth cleavage, cell DM and the two micromeres that it produces (Fig. 1) are situated to the left of the embryonic midline (as defined by the position of the ectodermal precursor cell, DNOPQ, the animal daughter of macromere D'). At late stage 7, the clone of the dm' micromere consists entirely of deep cells distal to the left germinal band, underneath the d', and sometimes the a', micromere progeny (Figs 2, 4D; Table 2). These cells lie to the left, or sometimes partially above, the a'' clone. The dm' clone varies in shape but always contains a set of cells nearer the margin of the micromere cap that lies deeper than those more centrally located. The cells previously identified as dm' micromeres (Fig. 3B in Ho and Weisblat, 1987) were in fact dnopq'' micromeres (see below). The dm'' micromere also generates exclusively deep cells which, at late stage 7, occupy a roughly oval domain posterior to and abutting the progeny of dm' and a'' (Figs 2, 4H; Table 2).

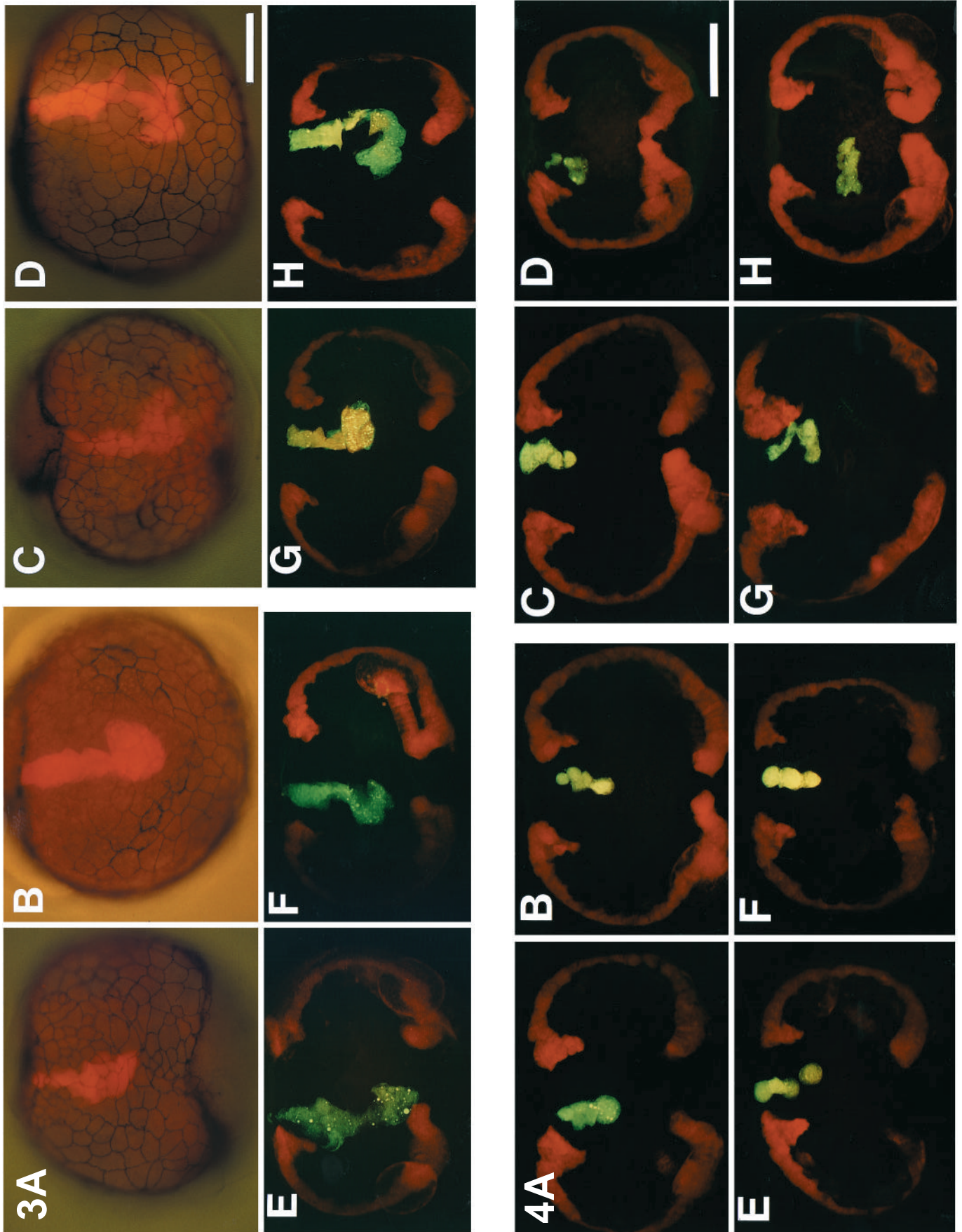


Fig. 3. Progeny of the primary quartet micromeres. (A-D) Double exposure (fluorescence and bright-field) photomicrographs of silver-stained embryos in which primary quartet micromeres had been injected with TRDA at stage 4a. Each clone contains both superficial and deep cells. (E,F) Confocal micrographs of embryos showing the position of the micromere progeny (in green) relative to the n bandlets (red). Progeny of each micromere occupy a different area in the central cap region. (A,E) Progeny of the d' micromere. (B,F) Progeny of the a' micromere. (C,G) Progeny of the b' micromere. (D,H) Progeny of the c' micromere. The shape of each micromere clone varies in each embryo. Scale bar, 100 μm in A-D; 90 μm in E-H.

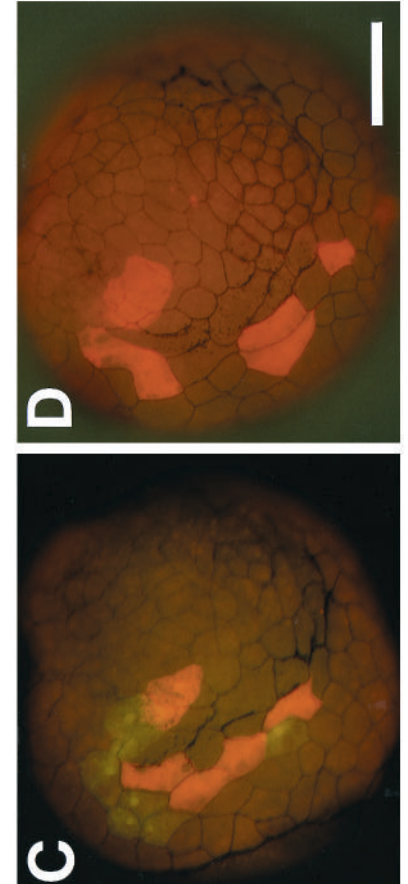
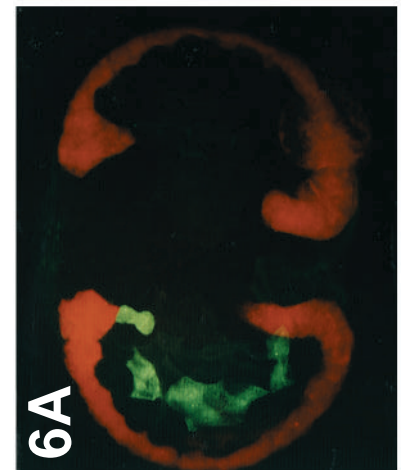
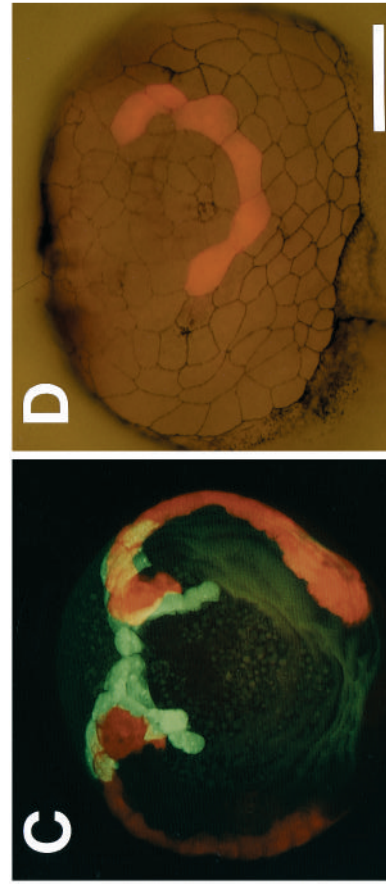
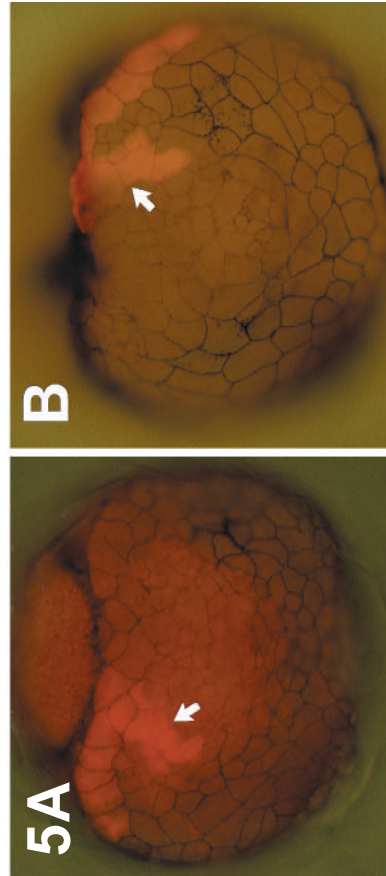
Fig. 4. Progeny of the secondary trio, tertiary trio and DM-derived micromeres. All these cells produce exclusively deep progeny. Confocal micrographs of embryos showing the position of the micromere progeny (yellow-green) relative to the n bandlets (red). (A) Progeny of the a'' micromere. (B) Progeny of the b'' micromere. (C) Progeny of the c'' micromere. (D) Progeny of the dm' micromere. (E) Progeny of the a''' micromere. (F) Progeny of the b''' micromere. (G) Progeny of the c''' micromere. (H) Progeny of the dm'' micromere. Scale bar, 90 μm .

Fig. 5. Progeny of the DNOPQ-derived micromeres. (A,B,D) Double exposure (fluorescence and bright-field) photomicrographs of silver-stained embryos in which

individual DNOPQ-derived micromeres had been injected with TRDA at stage 4b, showing the progeny of dnoq', dnoq'' and dnoq''', respectively. The medial lobes of dnoq' and dnoq'' progeny (arrows) contain both deep and superficial progeny. The lateral lobes of their progeny consist of superficial cells only, as does the entire dnoq''' clone. (C) Confocal micrograph showing the progeny derived from micromeres dnoq' and dnoq'' (green) at the ends of the right and left n bandlets, respectively (red); epithelial progeny lying over the n bandlets appear yellow. Scale bar, 100 μm in A,B,D; 70 μm in C.

Fig. 6. Progeny of the NOPQ-derived micromeres. (A) Confocal micrograph showing the position of the nopq'L progeny (green) relative to the n bandlets (red). Deep cells are at the distal end of the n bandlet and are non-contiguous with the superficial progeny.

(B,C) Double exposure (fluorescence and bright-field) photomicrographs of silver-stained embryos in which nopq'L progeny are labeled with TRDA (red) and nopq'L progeny with FDA (green); although the exact shape of each clone varies, the domain that they occupy together is relatively constant. (D) Double exposure (fluorescence and bright-field) photomicrograph of silver-stained embryo in which the nopq'L progeny are labeled with TRDA (red) and the nopq'L micromere had been ablated; the nopq' clone still contains discontinuous superficial cells. Scale bar, (A) 70 μm ; (B-D) 100 μm .



The dnopq'-dnopq'' micromeres

The ectodermal precursor blastomere (DNOPQ) produces three micromeres before dividing to form the NOPQ proteloblasts (Fig. 1). Even though the dnopq' and dnopq'' clones are symmetrically disposed with respect to one another across the embryonic midline, we do not regard them as bilaterally homologous because the dnopq' and dnopq'' micromeres are born sequentially. These clones each consist of 10-13 superficial cells and about 5 deep cells (Figs 2, 5A,B; Table 2). The deep cells are located distal to the germinal bands (Fig. 5C). The superficial cells are located at the margin of the micromere cap, covering their sibling deep cells and the distal ends of the germinal bands, primarily the n bandlets. The unpaired dnopq'' micromere contributes exclusively superficial progeny, in a clone that straddles the embryonic midline posterior to those contributed by the quartet micromeres (Figs 2, 5D; Table 2).

The nopq' and nopq'' micromeres

The first cleavage division that generates explicit bilateral symmetry is that of cell DNOPQ'' to form the left and right NOPQ blastomeres. These cells generate the left and right ectoteloblasts but, first, each NOPQ produces two micromeres, nopq' and nopq'' (Fig. 1). In the late stage 7 embryos, the clone derived from each of these micromeres consists of approximately two deep cells and six epithelial cells (Figs 2, 6A-C; Table 2). The two pairs of deep cells derived from the nopq' and nopq'' micromeres form a cluster adjacent to the most distal n blast cells (Fig. 6A). Superficial cells derived from nopq' and nopq'' cover parts of the o, p and q bandlets in the

middle portion of the germinal band and some of the area between the germinal band and the embryonic midline. The combined contributions of the ipsilateral nopq' and nopq'' micromeres to the superficial epithelium form a stereotyped and mainly coherent domain but, within this domain, the contributions of individual nopq' and nopq'' micromeres vary from embryo to embryo relative to those of other micromeres (Fig. 6B,C). Superficial progeny of individual nopq' and nopq'' micromeres intermingle with each other and often occur as discontinuous clusters within the larger domain. The cells previously identified as nopq' (Fig. 2D in Ho and Weisblat, 1987) were probably dm'' micromeres.

The opq' and opq'' micromeres

After the NOPQ'' blastomeres have divided to form the N teloblasts and the OPQ proteloblasts, the latter cells each produce two micromeres, opq' and opq'' (Fig. 1). In the late stage 7 embryo, the opq' progeny form a small patch of roughly four superficial and seven deep cells at the anterior ends of the op and q bandlets (Figs 2, 7A,B; Table 2). The superficial progeny of the opq' micromere occupy the lateral portions of two domains of cells that exhibit smaller than average apical contours (Fig. 7B). The deep progeny of opq' lie medial to those derived from nopq' and nopq''. The cells previously identified as opq' (Figs 2F, 3D in Ho and Weisblat, 1987) were in fact opq'' micromeres (see below).

In contrast to opq', the progeny of opq'' are exclusively epithelial cells, which form fan-shaped patches over the proximal portion of the ipsilateral germinal band (Figs 2, 7D; Table 2). Sometimes (4 of 32 cases), the cells in the labeled

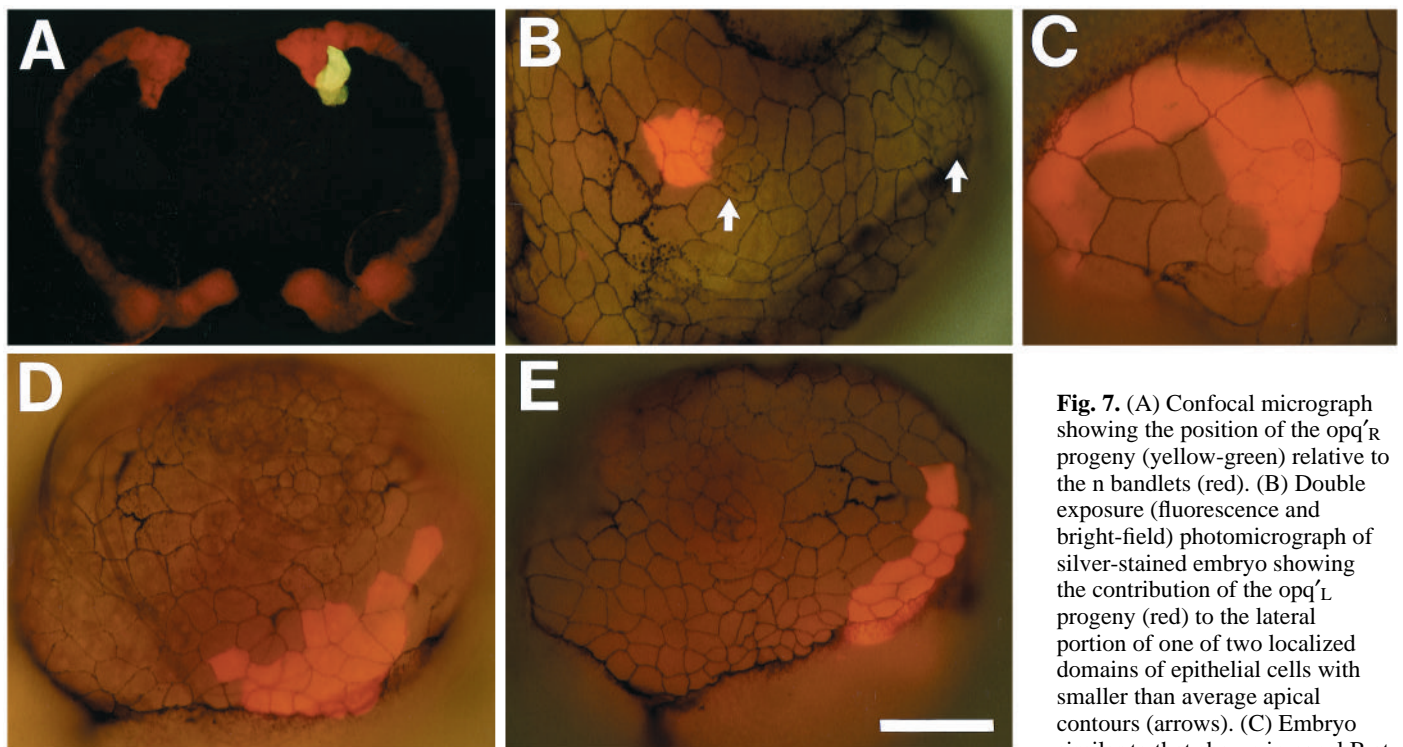


Fig. 7. (A) Confocal micrograph showing the position of the opq'_R progeny (yellow-green) relative to the n bandlets (red). (B) Double exposure (fluorescence and bright-field) photomicrograph of silver-stained embryo showing the contribution of the opq'_L progeny (red) to the lateral portion of one of two localized domains of epithelial cells with smaller than average apical contours (arrows). (C) Embryo similar to that shown in panel B at higher magnification, showing

that the distal end of the n_L bandlet (red) contributes superficial cells to the medial portion of the localized domain of epithelial cells with smaller than average apical contours. (D,E) Double exposure (fluorescence and bright-field) photomicrographs of embryos in which the opq'_R (D) and n'_R (E) progeny are labeled with TRDA (red). Scale bar (A) 90 μm; (B,D,E) 100 μm; (C) 55 μm.

opq'' clone lie on both sides of the midline, as if intermingling with the contralateral opq'' clone.

The n' micromeres

Each N teloblast forms three blast cells in a coherent column, then undergoes a shift in the orientation of its cleavage, so that its fourth daughter cell, micromere n', lies outside the nascent n bandlet, in the furrow separating the N teloblast from the OP proteloblast (Fig. 1; Sandig and Dohle, 1988; Bissen and Weisblat, 1989). With its fifth mitosis, the N teloblast resumes its original cleavage orientation and continues adding blast cells to the n bandlet. At late stage 7, the progeny of the n' micromere comprise a clone of approximately seven epithelial cells in a strip one or two cells wide at the margin of the micromere cap, covering the proximal portion of the germinal band (Figs 2, 7E; Table 2).

Teloblast contribution to the micromere cap

The fate map generated by the experiments described above indicates that 17 of the 25 micromeres contribute progeny to the superficial epithelium of the micromere cap (cells a'-d' and all those derived from the D' quadrant). To test this map for completeness by labeling all 17 of the identified progenitors in a single embryo is not technically feasible. However, two indirect observations indicate that the 17 micromeres do not, in fact, account for all of the superficial cells. First, the sum of the average number of cells contributed by each of the 17 micromeres (146) is slightly less than the average total number of superficial cells (157) in the micromere cap at the endpoint chosen for these experiments. Second, qualitatively superimposing the spatial domains of superficial cells arising from the 17 micromeres leaves two domains of cells partially unaccounted for. These two domains are the bilaterally paired sets of cells lying over the distal ends of the germinal bands which exhibit smaller than average apical profiles; as described earlier (Fig. 7B), the lateral cells of these domains arise from opq'.

Careful examination of silver-stained embryos in which N teloblasts had been injected with lineage tracer at birth revealed 7-8 tracer labeled superficial cells in the micromere cap of the

late stage 7 embryo in addition to those contributed by the n' micromeres (Table 2). These superficial cells occupy the medial portion of the domains of cells with small apical profiles (Fig. 7C). Moreover, these cells are located at the distal end of the n bandlet and are not labeled when an N teloblast is injected with lineage tracer after the birth of the n' micromere, indicating that they arise from one or more of the three n blast cells that are born prior to the n' micromere. No other teloblast was found to contribute progeny to the micromere cap.

Lineage-specific properties of micromere clones: clone size

The micromere fate maps obtained in the above experiments reveal reproducible differences during normal development with respect to clone location, composition (deep versus superficial cells) and rates of proliferation. For example, micromeres dnopq''' and opq'' generate clones of exclusively superficial progeny at the endpoint used in these experiments. At the time of fixation (44 hours after the birth of the N teloblasts), the clone derived from micromere dnopq''' is about 49 hours old and comprises typically 8 cells, while each opq'' clone is only about 42 hours old and yet already comprises typically 17 cells. Micromeres that generate exclusively deep progeny show similar differences; the dm' and a'' clones are both about 51 hours old at the time of fixation, yet the dm' clone comprises about 11 cells, while the a'' clone comprises only about 5 cells (Table 2).

To assess the extent to which these differences in rates of proliferation reflect intrinsic differences between micromere lineages, cell populations were counted in the superficial layer of the micromere caps of embryos in which development had been perturbed by cell ablation. Of the three micromeres that make exclusively superficial cells, the dnopq''' clone proliferates most slowly and might therefore be expected to have the greatest potential for regulative behavior. So in one set of experiments, both opq'' micromeres, which contribute the greatest number of superficial cells, were ablated and the dnopq''' micromere was injected with lineage tracer. In sibling

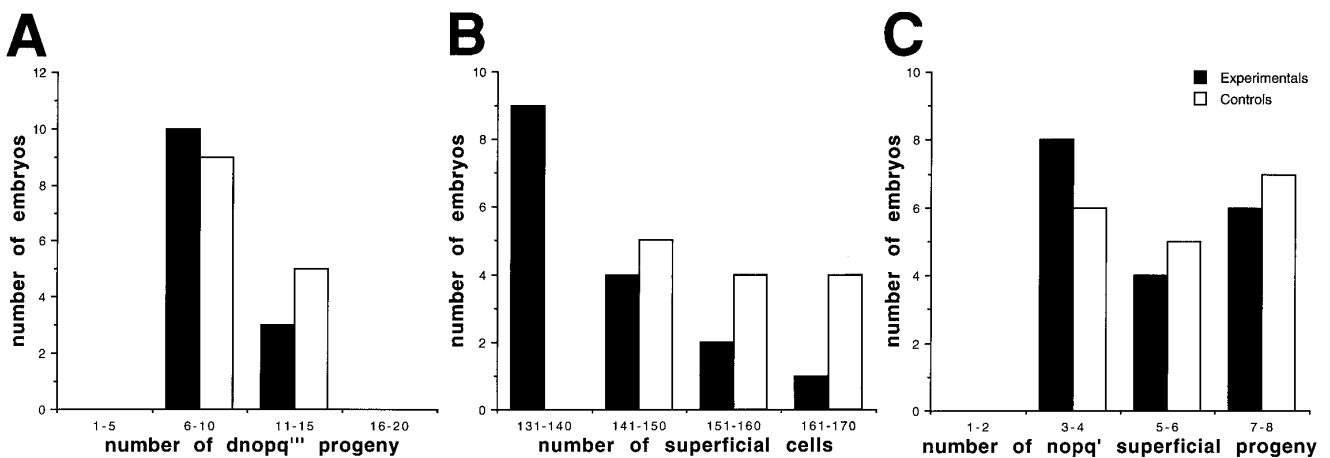


Fig. 8. Testing for regulation in size of micromere clones in response to experimental perturbation. (A) Size distribution of dnopq''' clones in embryos in which both opq'' micromeres were ablated (solid bars) and sibling controls (open bars). (B) Total numbers of superficial cells for embryos in which an opq'' micromere was ablated (solid bars) and for sibling controls (open bars). (C) Size distribution of superficial nopq'L progeny in embryos in which the nopq'L micromere was ablated (solid bars) and in sibling controls (open bars).

control embryos, $dnopq'''$ was injected with lineage tracer and no ablations were carried out (Fig. 8A). At the time of fixation, the $dnopq'''$ clone in the experimental embryos had, on average, 8.9 cells (s.d.=2.0; $n=13$), while the clones in unablated control embryos had, on average, 9.6 cells (s.d.=1.6; $n=14$), a difference which was not statistically significant [$\alpha > 0.1$; test of the difference between means; Alder and Roessler (1968)]. Thus, there was no evidence for numerical regulation from this experiment. These results parallel those of Ho (1988), who demonstrated that the clone now identified as opq'' exhibits no numerical regulation in response to ablation of the ipsilateral n' micromere. As expected (Ho and Weisblat, 1987; Ho, 1988), the micromere cap of embryos in which micromeres were ablated was intact.

In another set of experiments, total cell counts were obtained for the superficial layer in silver-stained late stage 7 embryos after one of the opq'' micromeres was ablated at birth. Sibling embryos in which an opq'' micromere was injected with lineage tracer served as controls (Fig. 8B). There were an average of only 141.3 epithelial cells (s.d.=9.1; $n=16$) in embryos in which an opq'' micromere was ablated, 12.7 less than the control average of 154.0 (s.d.=9.1; $n=13$). This difference is highly significant ($\alpha < 0.001$) and is in good agreement with the contribution of the opq'' clone in the control embryos (15.5 ± 2.3 ; $n=14$). These data also suggest that there was no regulation of cell number in any of the cell lines contributing superficial progeny to the micromere cap.

Lineage-specific properties of micromere clones: cell mingling

Differences between specific micromere clones in normal development were also observed with respect to the extent of cell 'mingling', as defined by the frequency with which progeny of a given micromere form discontinuous populations of cells within the superficial layer of the micromere cap at late stage 7. In particular, $nopq'$ and $nopq''$, which each contribute about 6 cells to the superficial layer, gave rise to discontinuous sets of superficial progeny in 9/17 and 7/14 cases, respectively, whereas n' and $dnopq'''$, which each contribute similar numbers of superficial cells (about 8 each) generated discontinuous sets of superficial progeny only rarely (0/21 and 1/23 cases, respectively). Moreover, the $nopq'$ and $nopq''$ progeny appeared to prefer to mingle with each other and not with members of other clones, as if the two clones together were defining a developmental compartment.

To test this possibility, experiments were carried out in which the one $nopq'$ micromere was injected with lineage tracer and the ipsilateral $nopq''$ micromere was ablated at stage 5; in sibling control embryos, both ipsilateral $nopq$ micromeres were labeled, using two different lineage tracers. Both sets of embryos were fixed and analyzed as usual at late stage 7 (Figs 6, 8C).

The ablations caused no significant change in the continuity of $nopq$ -derived epithelial progeny. In the control embryos, 11 of 18 $nopq'$ clones (and 12 of 18 $nopq''$ clones) contained discontinuous sets of superficial epithelial cells. In 10 of the 20 experimental embryos in which the ipsilateral $nopq''$ micromere had been ablated, the $nopq'$ clone had discontinuous epithelial cells, despite the lack of $nopq''$ progeny. Thus, the combined $nopq'$ and $nopq''$ clones do not constitute a compartment, as defined in the *Drosophila* embryo (Garcia-Bellido

et al., 1973; Crick and Lawrence, 1975). Furthermore, the $nopq'$ clone in the experimental embryos had, on average, 5.6 epithelial cells (s.d.=1.6; $n=18$), while $nopq'$ clones in unablated control embryos had, on average, 5.9 epithelial cells (s.d.=1.6; $n=18$). Thus, these experiments provide no evidence for regulation of cell number in the squamous epithelium either ($\alpha >> 0.1$).

DISCUSSION

Studies of leech development have often focused on the genesis of segmental tissues from the teloblasts and their progeny. In contrast, the experiments reported here provide a basis for elucidating the roles of the micromeres and their progeny in various aspects of leech development, including germinal band formation (Fernandez and Stent, 1980; Fernandez and Olea, 1982); development of the provisional integument, supraesophageal ganglion and other nonsegmental tissues (Weisblat et al., 1984; Nardelli-Haeffliger and Shankland, 1993); and epiboly (Smith et al., unpublished data).

The fate maps described here for all 25 micromeres reveal that these cells make stereotypical contributions to the late stage 7 embryo in terms of the number of progeny produced, their location and their distribution between the deep and superficial layers of the micromere cap. Moreover, ablation experiments indicate the differences between the fate maps of the various micromeres reflect lineage-specific differences. Significant differences in rates of cell proliferation were observed even between clones that generate otherwise similar sets of exclusively epithelial progeny. Whether these differences reflect different modes of cell division (i.e. geometric versus arithmetic progression in clone size over time) or different cell cycle times between clones remains to be determined.

Micromere derivatives in leech development

In normal development, micromeres contribute progeny to definitive, non-segmental structures, including the supraesophageal ganglion and proboscis of the prostomium. They also contribute to the epithelium of the provisional integument, a temporary embryonic structure that is shed at the completion of dorsal closure (stage 10-11). It is not yet possible in every case to equate the deep and superficial micromere progeny in the late stage 7 embryo with exclusively prostomial and epithelial fates, respectively. However, it is known that the micromeres that have exclusively superficial progeny at late stage 7 (e.g. n' , opq'' , $dnopq'''$) contribute exclusively to the squamous epithelium at stages 8-9 (Smith et al., unpublished data), and it seems likely that only the micromere progeny which are deep during stage 7 contribute to the prostomium.

The primary quartet progeny ($a'-d'$) make both superficial and deep cells. All the remaining epithelium, both provisional and definitive, derives from descendants of the DNOPQ lineage. While some of the micromeres descended from the DNOPQ lineage contribute deep cells, all the micromeres descended from the DNOPQ lineage make epithelial cells.

The remaining micromeres descended from the A-C macromere quadrants (the $a''-c''$ and $a'''-c'''$ trios of micromeres) make solely deep cells. In this regard, it is interesting that dm' and dm'' , the two micromeres derived from

blastomere DM (the D quadrant homolog of macromeres A''-C''), also make exclusively deep progeny. It is assumed that both the dm' and dm'' clones contribute definitive progeny to the bilaterally symmetric leech (since their progeny lie in the region of the prospective prostomium), yet these cells lack any obvious contralateral homologs. While the spatial distribution of their definitive progeny remains to be determined, our fate map suggests that the definitive progeny of dm' and dm'' may be homologous with those of micromeres c'' and c'''. Alternatively, dm' and dm'' may not contribute definitive progeny, or they may each contribute bilaterally symmetric progeny themselves as a result of extensive cell migrations, or they may contribute definitive progeny that lack bilateral homologs.

Commitment in micromere lineages

Differences between micromere clones in the rate of cell proliferation appear to reflect intrinsic differences between the clones, since there is no regulation of cell number within the epithelium in response to the ablation of individual micromeres. We conclude that the lineage-specific differences in cell proliferation are cell autonomous traits rather than the result of mechanical influences from cells in other clones. The formation of an intact epithelium in embryos with the reduced number of cells resulting from these ablations is evidence of functional regulation that may be accomplished by such means as increased cell size and/or shifting of cell positions. Functional regulation in response to ablation has also been observed within the definitive epidermis of the leech (Blair and Weisblat, 1984).

At late stage 7, the epithelial progeny of the two NOPQ-derived micromeres intermingle with greater frequency than do the epithelial progeny of any other micromeres. However, experiments in which one of those micromeres was ablated showed that there was no significant change in the continuity of the epithelial cells within the remaining ipsilateral clone. Thus, the nopq progeny do not seem to form a special compartment within which the cells are free to mingle and out of which they are not. The apparent propensities for mingling among the epithelial progeny of the nopq' and nopq'' micromeres may reflect regional differences in the forces to which cells in the micromere cap are subjected during this time in development. In this regard, extensive discontinuities in the clones of primary quartet micromeres have been observed by stage 9-10 (Weisblat et al., 1984) and in dnopq''' clones by the end of stage 8 (Smith, unpublished data).

Contributions of n blast cells to the micromere cap

The observation that one or more of the firstborn blast cells derived from the N teloblast contribute epithelial progeny to the micromere cap was unexpected. However, it had already been suggested that N progeny contribute to a nonsegmental structure, the adhesive organ, which the late embryo uses to attach to the ventral aspect of the parent before its posterior sucker becomes functional (Weisblat et al., 1984). Ho (1988) later demonstrated that it is the firstborn n blast cells that contribute to this structure. In light of these results, we suggest that the adhesive organ originates from the bilaterally paired domains of cells that exhibit smaller than average apical profiles. The medial cells in these domains arise from the firstborn n blast cells and the lateral cells originate from the

opq' micromeres; thus, it seems likely that opq' micromeres also contribute to the formation of the adhesive organ.

Comparisons with other spiralian

The cell lineages leading to micromere production have been examined carefully in two genera of glossiphoniid leeches, annelids within the class *Hirudinea* (Sandig and Dohle, 1988; Bissen and Weisblat, 1989; this paper). The early lineages leading to micromere formation are closely conserved within this family. Although the cell lineage information for representatives of the polychaete and oligochaete annelid classes is generally less complete or less certain (Anderson, 1973; for a concise synopsis, see Sandig and Dohle, 1988), the early patterns of cell division are similar and at least some of the micromeres appear to have direct homologs with those described for the glossiphoniid leeches (e.g., see Schneider et al. 1992; Shimizu, 1982). However, polychaete and oligochaete annelids differ markedly from the leeches in details of body plan, regenerative capacity and/or the inclusion of trochophore larval stages. The results presented here, in addition to advancing our knowledge of leech development, should also provide a basis for comparative studies aimed at understanding how the dramatic differences between the annelid classes evolved.

We are grateful to Shirley Bissen for introducing *H. robusta* into laboratory culture, to Steve Torrence and Duncan Stuart for help with image processing, and to Karen Symes for her generous help with printing the map figure. This work was supported by NSF grant IBN-9105713.

REFERENCES

- Alder, H. L. and Roessler, E. B. (1968). *Introduction to Probability and Statistics*. Fourth Edition. San Francisco: W. H. Freeman.
- Anderson, D. T. (1973). *Embryology and Phylogeny in Annelids and Arthropods*. Oxford: Pergamon Press, Ltd.
- Arnolds, W. J. A. (1979). Silver staining methods for the demarcation of superficial cell boundaries in whole mounts of embryos. *Mikroskopie (Wein)* **35**, 202-206.
- Bissen, S. T. and Weisblat, D. A. (1989). The durations and compositions of cell cycles in embryos of the leech, *Helobdella triserialis*. *Development* **106**, 105-118.
- Blair, S. S. and Weisblat, D. A. (1984). Cell interactions in the developing epidermis of the leech *Helobdella triserialis*. *Dev. Biol.* **101**, 318-325.
- Crick, F. H. C. and Lawrence, P. A. (1975). Compartments and polyclones in insect development. *Science* **189**, 340-347.
- Fernandez, J. (1980). Embryonic development of the glossiphoniid leech *Theromyzon rude*: characterization of developmental stages. *Dev. Biol.* **76**, 245-262.
- Fernandez, J. and Olea, N. (1982). Embryonic development of glossiphoniid leeches. In *Developmental Biology of Freshwater Invertebrates* (eds. F. W. Harrison and R. R. Cowden), pp. 317-361. New York: A. R. Liss, Inc.
- Fernandez, J. and Stent, G.S. (1980). Embryonic development of the glossiphoniid leech *Theromyzon rude*: structure and development of the germinal bands. *Dev. Biol.* **78**, 407-434.
- Garcia-Bellido, A., Ripoll, P. and Morata, G. (1973). Developmental compartmentalisation of the wing disk of *Drosophila*. *Nat. New Biol.* **245**, 251-253.
- Ho, R. K. (1988). Factors that affect cell divisions and cell fates in the embryo of the leech *Helobdella triserialis*. Ph.D. thesis, Department of Zoology, University of California, Berkeley, California, USA.
- Ho, R. K. and Weisblat, D. A. (1987). A provisional epithelium in leech embryo: cellular origins and influence on a developmental equivalence group. *Dev. Biol.* **120**, 520-534.
- Nardelli-Haeffiger, D. and Shankland, M. (1993). *Lox 10*, a member of the

- NK-2* homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella*. *Development* **118**, 877-892.
- Nelson, B. H. and Weisblat, D. A.** (1992). Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo. *Development* **115**, 103-115.
- Sandig, M. and Dohle, W.** (1988). The cleavage pattern in the leech *Theromyzon tessulatum* (Hirudinea, Glossiphoniidae). *J. Morph.* **196**, 217-252.
- Schneider, S., Fischer, A. and Dorresteijn, A. W. C.** (1992). A morphometric comparison of dissimilar early development in sibling species of *Platynereis* (Annelida, Polychaeta). *Roux's Arch. Dev. Biol.* **201**, 243-256.
- Shankland, M.** (1991). Leech segmentation: cell lineage and the formation of complex body patterns. *Dev. Biol.* **144**, 221-231.
- Shimizu, T.** (1982). Development in the freshwater oligochaete *Tubifex*. In *Developmental Biology of Freshwater Invertebrates* (eds. F. W. Harrison and R. R. Cowden), pp. 283-316. New York: A. R. Liss, Inc.
- Stent, G. S., Kristan, W. B., Jr., Torrence, S. A., French, K. A. and Weisblat, D. A.** (1992). Development of the leech nervous system. *Int. Rev. of Neurobiology* **33**, 109-193.
- Weisblat, D. A., Kim, S. Y. and Stent, G. S.** (1984). Embryonic origins of cells in the leech *Helobdella triserialis*. *Dev. Biol.* **104**, 65-85.
- Whitman, C. O.** (1878). The embryology of *Clepsine*. *Quart. J. Microsc. Sci.* **18**, 215-315.
- Wilson, E. B.** (1892). The cell-lineage of *Nereis*: a contribution to the cytogeny of the annelid body. *J. Morphol.* **6**, 361-480.
- Zackson, S.** (1982). Cell clones and segmentation in leech development. *Cell* **31**, 761-770.

(Accepted 1 September 1994)