

Developmental origin of segmental identity in the leech mesoderm

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SUMMARY

Segmentation in the leech embryo is established by a stereotyped cell lineage. Each of the 32 segments arises from homologous, bilaterally symmetrical complements of mesodermal and ectodermal blast cell clones. Although segments are homologous, they are regionally differentiated along the longitudinal body axis. Various segments display idiosyncratic ensembles of features, which constitute discrete segmental identities. The differentiation of segment-specific features, such as the mesoderm-derived nephridia, genital primordia and identified Small Cardioactive Peptide immunoreactive neurons, reflects a diversification of the developmental fates of homologous blast cell clones. We have investigated whether segment-specific differentiation of homologous mesodermal blast cell clones depends on cell-intrinsic mechanisms (based on the cells' lineage history) or on cell-extrinsic mechanisms (based on the cells' interactions with their environment) in embryos of *Theromyzon rude*. For this purpose, we first mapped the segment-specific fates of individual mesodermal blast cell clones, and then induced mesodermal clones

to take part in the formation of segments for which they are not normally destined. Two types of ectopic segmental position were produced: one in which a mesodermal blast cell clone was out of register with all other conssegmental cells and one in which a mesodermal blast cell clone was out of register with its overlying ectoderm, but was in normal register with the mesoderm and ectoderm on the other side of the embryo. Mesodermal blast cell clones that developed in either type of ectopic segmental position gave rise to segment-specific features characteristic of their original segmental fates rather than their ectopic positions. Thus, the development of segmental identity in the leech mesoderm is attributable to a cell-intrinsic mechanism and, either before or soon after their birth, mesodermal blast cells are autonomously committed to segment-specific fates.

Key words: cell lineage, cell commitment, cell ablation, transplantation

INTRODUCTION

Early in their development, the embryos of annelids, arthropods and chordates form a series of iterated metameres grouped as segments. Later, distinct morphological structures associated with particular segments develop and provide a specific identity to each.

The cellular mechanisms by which the segmented body plan is generated and its relation to cell lineage have been studied in insect development (Crick and Lawrence, 1975; Lawrence, 1981; Martinez-Arias and Lawrence, 1985). Recent genetic and molecular studies in *Drosophila* have deepened our understanding of not only metameric periodicity but also segmental identity (Akam, 1987). However, the developmental mechanisms responsible for the establishment of the segmentation pattern vary considerably in different insect species (Tear et al., 1988, 1990). These variations are even greater across the taxa, among such groups as earthworms (Dévries, 1974; Storey, 1989), leeches

(Shankland, 1991; Weisblat and Shankland, 1985; Weisblat et al., 1988), crustacea (Dohle and Scholtz, 1988), fish (Kimmel et al., 1988), and birds (Stern et al., 1988).

The differentiation of segment-specific features reflects a diversification of the developmental fates of the cell lines giving rise to each segment. Such differentiation might be attributable to mechanisms that are either intrinsic or extrinsic to the cell lines. For instance, a cell-intrinsic mechanism would be attributable to the commitment of each cell line to a particular fate if it were governed by its genealogical position in the cell lineage and to a cell-extrinsic mechanism if governed by segment-specific morphogenetic signals. The uniquely identifiable cells and highly determinate development of the leech embryo and the possibility to perform transplant-like experiments allowed us to examine whether its development of segmental identity is attributable to cell-intrinsic or cell-extrinsic mechanisms.

Early in leech development, a set of five bilaterally paired teloblasts is formed, of which the pair designated M is the

precursor of mesodermal tissues and the pairs designated as N, O, P, and Q are the precursors of ectodermal tissues (Figs 1, 2). Each teloblast undergoes a series of iterated, unequal divisions in the stem cell division mode, producing 5 paired bandlets of small primary blast cells designated m, n, o, p, and q. On either side, the five bandlets merge to form a germinal band. The two germinal bands eventually coalesce on the ventral aspect of the embryo to form the germinal plate, which is the rudiment of the 32 bilaterally symmetrical segments of the body wall and nervous system. This metameric pattern of segmental tissues arises by division, migration and differentiation of the primary blast cells and their descendants. Blast cells generated early (and their descendant clones) occupy more anterior positions along the germinal plate than blast cells (and their clones) generated later. Thus, the relative position of a blast cell clone along the longitudinal body axis reflects the birth rank of its primary blast cell progenitor (Weisblat and Shankland, 1985).

Each of the 32 hemisegmental metameres on either side arises from homologous complements of mesodermal and ectodermal blast cell clones. These clones are derived from one m, o, and p primary blast cell and from two n and q primary blast cells, designated n_s , n_f , q_s , and q_f . The clones are spread across the boundaries of contiguous morphological segments, so that metameric elements contributed by serially successive homologous primary blast cells interdigitate within a single morphological hemisegment (Weisblat and Shankland, 1985).

Although obviously homologous, the morphology of the segments is regionally differentiated along the longitudinal

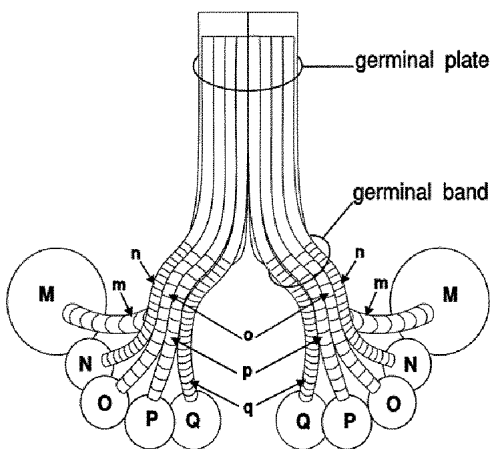


Fig. 1. Summary of leech development. Each of the five bilaterally paired teloblasts (M, N, O, P and Q) divides repeatedly to produce a bandlet of primary blast cells designated m, n, o, p and q, respectively. The bandlets merge, forming a germinal band composed of the ectodermal bandlets (n, o, p and q), superficially, and the mesodermal bandlet (m) underneath them. The two germinal bands coalesce along the ventral midline of the embryo to form the germinal plate, which will give rise to the body wall and nervous system of the leech. The first part of the germinal plate to be formed (top) produces the anterior end of the germinal plate.

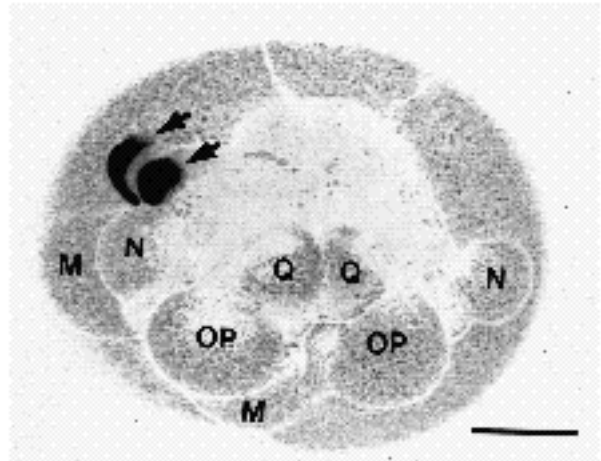


Fig. 2. Confocal micrograph of early stage 6b embryo viewed from its dorsal aspect. The fluorescence of the lineage tracer is rendered black against the light background. Blast cells m_3 and m_5 (arrows) of the left mesodermal bandlet were injected with RDA. The left and right mesodermal (M) and the ectodermal N, OP (precursors of O and P), and Q teloblasts are visible because of yolk autofluorescence. Scale bar, 100 μ m.

body axis, so that the segments can be grouped as 4 subesophageal segments (S1-S4), 21 midbody segments (M1-M21) and 7 caudal segments (C1-C7). Furthermore, most or all of the individual segments within a given group display morphological features that confer on each its own segmental identity.

To ascertain the extent to which the segment-specific morphological diversification of homologous mesodermal blast cell clones depends on mechanisms that are either intrinsic or extrinsic, we observed the developmental consequences of shifting a part of the m bandlet out of its normal segmental register in the leech, *Theromyzon rude*. Such shifting can be induced by ablating one or more nascent m primary blast cells shortly before their entrance into the germinal band (Shankland, 1984). This experimental procedure results in a transplantation-like operation, in that m blast cell clones are made to take part in the formation of segments that are either anterior or posterior to those for which they are normally destined by virtue of the birth rank of their founder blast cell.

By examining the distribution of mesoderm-derived, segment-specific features, we found that m primary blast cells shifted to ectopic segmental positions give rise to cell clones whose differential phenotype is characteristic of the blast cells' original segmental fate rather than of their ectopic position. It would appear, therefore, that development of segmental identity in the leech mesoderm is attributable to a cell-intrinsic mechanism, i.e., that m primary blast cells by the time of their shifting are autonomously committed to take on segment-specific fates in accord with their birth ranks, as has been proposed for the earthworm *Eisenia foetida* (Dévries, 1983, 1988). Similar conclusions have been drawn about the developmental autonomy of segment identity in the leech ectoderm (Martindale and Shankland, 1990b).

MATERIALS AND METHODS

The methods employed for culturing embryos of *Theromyzon rude* (at 18°C), for pressure-injecting the red-fluorescing lineage tracer tetramethylrhodamine-dextran-amine (RDA) or the green-fluorescing lineage tracer fluorescein-dextran-amine (FDA) into embryonic cells, for ablating identified embryonic cells by injecting them with the lethal enzyme DNAase I, and for fixing and dissecting embryos were those described by Stuart et al. (1989, 1991) and Torrence (1991). The m primary blast cells were visible on the surface of the embryo (Fig. 2) as they were being produced by the parent teloblast. They were distinguished from the surrounding micromeres by their larger size and position relative to the M teloblast. However, only the first 6 primary blast cells produced could be consistently injected by this procedure. The subsequent primary blast cells divide before they reach the embryonic surface, and the direct progeny of later ones do not reach the surface before being covered by the ectodermal bandlets. The developmental staging system referred to is that of Fernandez (1980), as modified by Weisblat et al. (1980) and Stent et al. (1992).

The distribution of CAS neurons over the segmental ganglia of experimental and control embryos was ascertained immunohistochemically, using a mouse monoclonal antibody against the molluscan Small Cardioactive Peptide B (SCP) (purchased from the University of Washington Monoclonal Facility). Embryos were grown to stage 11 (5 to 8 days after the appearance of eye pigment), fixed, dissected and incubated for four days at 4°C in Hepes-buffered saline (HBS: 50 mM sodium Hepes pH 7.4., 145 mM NaCl, 15 mM NaN₃), supplemented with 20 mg/ml Triton X-100, 30 mg/ml normal goat serum, 10 mg/ml BSA and a 1:10 dilution of the anti-SCP mouse antibody stock solution supplied by the manufacturer. The specimens were then rinsed for several hours in supplemented HBS, from which the mouse antiserum had been omitted (rinse solution). They were then incubated for 4 days at 4°C in rinse solution containing a 1:400 dilution of the fluorescein-conjugated goat anti-mouse gamma globulin (Antibodies Inc., lot no. 9F39W). Finally, they were rinsed, in rinse solution followed by HBS, and then cleared and whole mounted between coverslips in a solution of 80% glycerol and 20% 0.1 M Tris-HCl, pH 9.0 containing 40 mg/ml *n*-propyl gallate (Giloh and Sedat, 1982).

Live embryos were examined by epifluorescence microscopy, using Zeiss filter sets 487715 and 487717 to visualize tetramethylrhodamine and fluorescein, respectively. Fixed and dissected embryos were viewed by confocal microscopy, using a Bio-Rad MRC 600 system attached to a Zeiss Axioplan epifluorescence microscope. A series of digitized optical sections was collected from each specimen and subsequently reassembled to generate an image of the entire thickness of the specimen. For doubly labeled specimens, paired series of optical sections were collected, one for either label, which were subsequently merged and assigned computer-generated colors to represent the original labels. Monochrome confocal micrographs were electronically converted to negative images before being photographed from a high-resolution, flat-screen monitor (Lucius and Baer VM 1710) with Kodak Technical Pan film 2415. Colored confocal micrographs were photographed from the computer screen with Kodak Ektar 125 film.

RESULTS

The m primary blast cell clone

Since the interpretation of the present experiments depends on the characterization of the morphological features of hemisegmental mesodermal metameres under abnormal

developmental conditions, we ascertained the normal segmental distribution of the progeny of single m primary blast cells. Previous indirect surveys of that distribution in *Helobdella triserialis* had shown that metameric elements contributed by serially successive, homologous m primary blast cells interdigitate within single hemisegments (Weisblat and Shankland, 1985). These surveys had been carried out by scoring labeled cell types across lineage tracer boundaries generated by tracer injection of teloblasts. To confirm and extend this inference more directly for *T. rude*, the first five m blast cells produced by the M teloblast (m₁-m₅) were individually injected with lineage tracer and the distribution of the tracer was subsequently examined in the tissues of 40 late stage 10 embryos (Fig. 3).

The result of this experiment directly confirms the previous indirect inference of an interdigitation of serially successive m blast cell clones: it shows that in *T. rude* the descendant clone of a single m primary blast cell is distributed over a triplet of successive hemisegments. Specifically, each m blast cell clone contributes longitudinal and circular muscle fibers and connective tissue to the anterior and middle segments of the triplet and a set of central interneurons (mz neurons) to the posterior segment, as well as the nephridial tubule and most of the duct where a nephridium is present. In addition, each m blast cell gives rise to a set of provisional muscle fibers that course circumferentially over the part of the embryonic surface not occupied by the germinal plate (Fernandez, 1980; Weisblat et al., 1980; Torrence et al., 1989). In stage 10 embryos, the provisional muscle fibers descended from an m blast cell can be seen at the margin of the germinal plate next to the 4-8 segments posterior to the triplet of segments occupied by the major part of the descendant clone (Fig. 3).

Segment-specific contributions of m blast cells

In *T. rude*, among the features of mesodermal origin, nephridia are normally present only in segments M2-M4 and M8-M18 (Fernandez, 1980), genital primordia only in segments M5 and M6 (Bürger, 1902; Torrence, personal communication) (Fig. 5A). Furthermore, an interneuron, designated 'CAS,' is present only in segments M18-M21 and C1-C4 (Shankland and Martindale, 1989; Table 1).

The first two m primary blast cells (m₁ and m₂) produced by the M teloblast contribute some cells, including muscle fibers and connective tissue, to a poorly characterized part of the head that lies anterior to the first subesophageal segment. In addition, m₁ and m₂ contribute mz neurons to segments S1 and S2. The first m primary blast cell to contribute its progeny wholly to subesophageal segments is m₃, whose clone provides cells to the segment triplet S1-S2-S3. Blast cells m₁-m₅ contribute a structure that resembles a nephridial duct (Martindale and Shankland, 1988) to S1-M1, respectively, as well as cells and muscle fibers to the proboscis (Fig. 3A). Blast cells m₁₀ and m₁₁, whose clones contribute cells to segment triplets M4-M5-M6 and M5-M6-M7, each provides the genital primordia to the middle segment of their respective triplet. This result is based on tracer boundaries created by the injection of label into the M teloblasts, as described below (Fig. 4A). The m primary blast cells of origin for the segment-specific features surveyed in the present work are identified in Table 1.

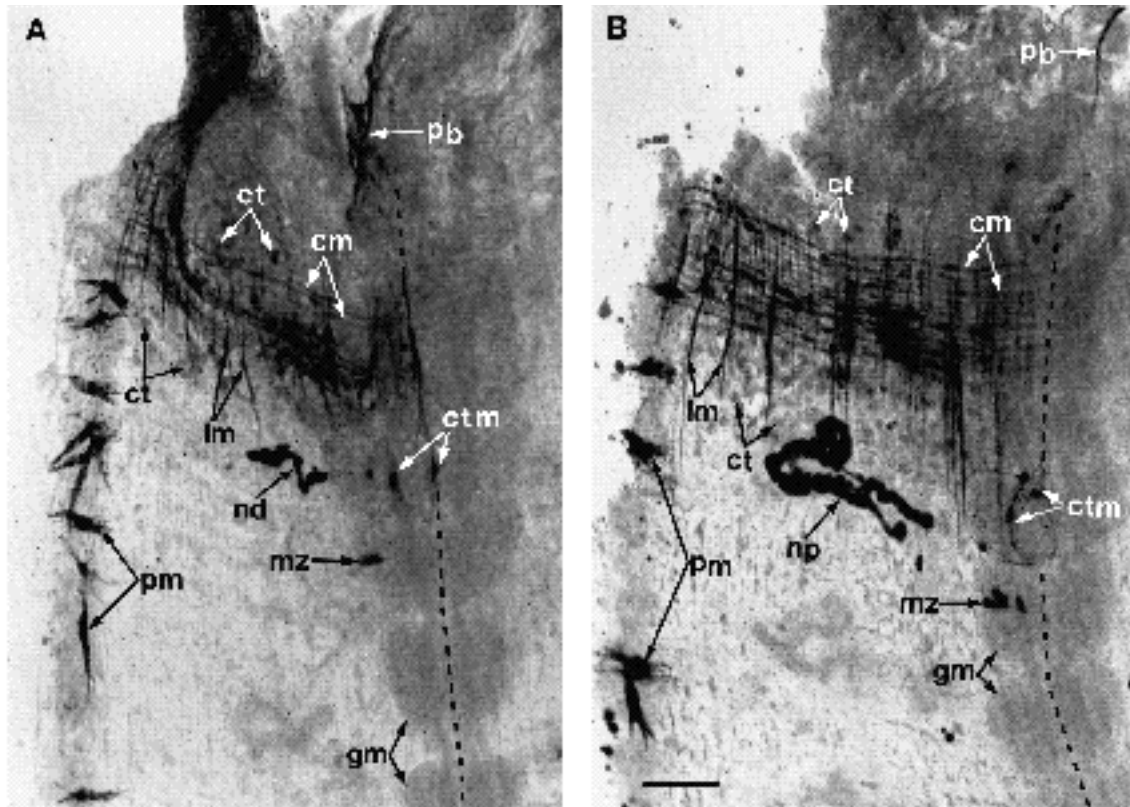


Fig. 3. Diversity of mesodermal blast cell clones. Confocal micrograph of dissected stage 10 embryos in which a single left blast cell was labeled with RDA in stage 6. In this and all subsequent figures, anterior is to the top. (A) The descendant clone of mesodermal blast cell m_4 comprises longitudinal (lm), circular (cm) and provisional (pm) muscle fibers; connective tissue (ct); a structure resembling a nephridial duct (nd); a group of five neurons (mz) in subesophageal neuromere 4; and cells in the proboscis (pb). (B) The descendant clone of mesodermal blast cell m_6 comprises longitudinal (lm), circular (cm), and provisional (pm) muscle fibers; a nephridium (np); connective tissue (ct), and a group of five neurons (mz) in ganglion M2. The dashed line indicates the ventral midline; gm, ganglionic margin. Scale bar, 50 μm .

Primary blast cell ablation and bandlet shifts

To induce parts of the m bandlet to shift into ectopic positions along the longitudinal embryonic axis, a gap in the bandlet was created by ablating one or more m primary blast cells by injecting them with DNAase I shortly before their entrance into the germinal band. Shankland (1984) has shown that the creation of such a gap in an ectodermal bandlet causes the part of the bandlet posterior to the gap to shift posteriad into ectopic positions. The creation of a gap in the m bandlet can cause either anterior or posteriad shifts. The direction and extent of this shift in the present experiments were ascertained by means of the following double-labeling protocol (Fig. 4). Both M teloblasts of stage 6 embryos were injected with RDA lineage tracer at a time when they had given rise to two or three m primary blast cells (m_1 - m_3). This tracer injection gave rise to an 'RDA tracer boundary', separating the anteriormost unlabeled mesodermal cells from the posterior cells that were labeled with RDA. Approximately 6 hours later, when each teloblast had produced about three more m primary blast cells (m_3 - m_6) and the anteriormost cells of the m bandlets had reached the embryo surface, one or two of the RDA-labeled m primary blast cells in the left m bandlet (e.g. m_5

and/or m_6) were ablated by intracellular injection of DNAase. In some specimens, which served as controls, the DNAase injection was omitted. Both M teloblasts were then reinjected with FDA lineage tracer. This tracer reinjection gave rise to an 'FDA tracer boundary', separating the anterior mesodermal cells that were labeled only with RDA from the more posterior mesodermal cells that were labeled with both RDA and FDA. All specimens were cultured to late stage 10, at which time the segmental positions of the RDA and FDA lineage tracer boundaries in the embryonic tissues were compared on left and right sides (Fig. 5).

The rationale of this double-labeling protocol was that if creation of a blast cell gap caused the part of the operated bandlet posterior to the gap to shift anterior, the FDA boundary on the operated left side should lie anterior to the FDA boundary on the unoperated right side. Conversely, if the part of the operated bandlet posterior to the gap shifted posteriad, the FDA tracer boundary on the left side should lie posterior to the FDA boundary on the right side. If the operated bandlet did not shift at all, the FDA tracer boundaries should be in segmental register on left and right sides. The RDA boundary should be in segmental register on left

Table 1. m primary blast cell of origin of segment-specific features

Blast cell of origin	Feature	Segment
m ₆	Nephridium	M2
m ₇	Nephridium	M3
m ₈	Nephridium	M4
m ₁₀	Genital primordium	M5
m ₁₁	Genital primordium	M6
m ₁₂ -m ₂₂	Nephridium	M8-M18
m ₂₂ -m ₂₅	CAS neuron	M18-M21
m ₂₆ -m ₂₉	CAS neuron	C1-C4

and right sides, regardless of whether the part of the operated bandlet posterior to the gap had shifted.

The interpretation of the results of the double-labeling experiments takes for granted that the division cycles of the bilateral M teloblast pair are sufficiently well synchronized that both teloblasts produce a primary blast cell of a given rank order, say m_i , at very nearly the same time. To confirm that this is true, control experiments were carried out in which the tracer boundaries were examined in doubly-labeled specimens that had not been subject to any blast cell ablations.

The result of the experiments was that the RDA tracer boundaries were in segmental register in 15/15 and the FDA tracer boundaries in 63/64 of the unoperated control specimens examined (Figs 5A, 6A). Moreover, in 55/55 of the operated specimens, the RDA tracer boundary, which lies anterior to the ablated primary blast cell and hence should not be affected by any bandlet shift, was similarly in segmental register in left and right m bandlets. Thus, the divisions of the bilaterally paired M teloblasts are indeed synchronized to a precision of about $1/134=0.8\%$ of the division cycle.

The interpretation of the results of experiments in which anterior or posterior shifts of the mesodermal m bandlet were observed following ablation of one or more m primary blast cells is predicted on the assumption that these

shifts were not accompanied by any concomitant shifts of the overlying ectodermal n, o, p and q bandlets. This assumption will be shown to be justified in a later section of this report.

Anterior shift

In 22% (31/139) of specimens in which one m primary blast cell of the left bandlet had been ablated, the FDA tracer boundary on the operated left side fell into one segment *anterior* to the corresponding boundary on the unoperated right side (Figs 5B, 6C). Hence in these specimens the part of the left m bandlet posterior to the gap had shifted anterior by one segment to close the gap.

In these specimens, no (or at most minor) deficits were observed in mesoderm-derived elements in the segments bracketing the site of the shift. Furthermore, the mesoderm on the operated left side expressed the normal pattern of segment-specific structures (Table 1): a series of three nephridia (unless the ablated cell happened to be m_6 , in which case there were only two nephridia) followed by two genital primordia, a segment containing neither nephridia nor genital primordia, and finally more segments with nephridia. However, this entire pattern on the operated left side was shifted one segment anterior with respect to the unoperated side: nephridia were present in segments opposite to M1-M3 (instead of M2-M4) of the unoperated right side and reappeared in a segment opposite to M7 (instead of M8), while genital primordia were present in segments opposite to M4 and M5 (instead of M5 and M6) of the unoperated side (Figs 5B, 6C). In the few anterior shift specimens in which the primary blast cell m_6 had been ablated (which would normally have contributed a nephridium to segment M2; Table 1) only two (rather than three) nephridia developed anterior to the first genital primordium on the operated side. These results show that anteriorly shifted m blast cell clones take on a fate in accord with the characteristics of their original segmental destination (i.e., with the birth rank of their founder cell) rather than with their actual ectopic segmental location.

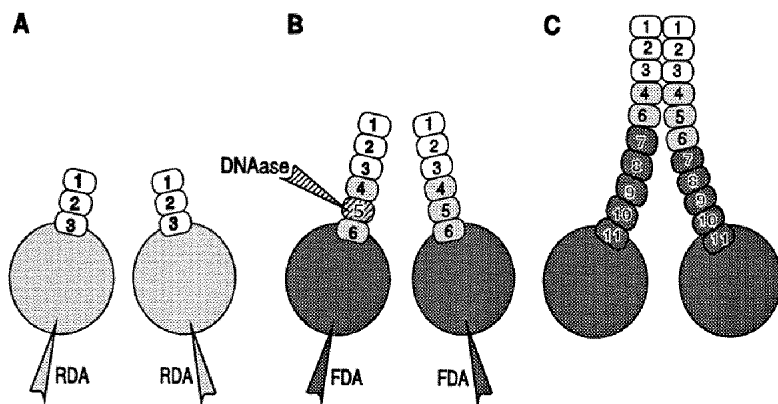


Fig. 4. Protocol used for inducing shifts of parts of the m bandlet. The large circles in each panel represent the left and right M teloblasts. A bandlet of blast cells is represented as growing upward from each teloblast. The number in each blast cell corresponds to its birth rank. (A) Both M teloblasts are injected with RDA lineage tracer (light gray) when they have each given rise to three m primary blast cells. Only the blast cells born after this time will be labeled with RDA. (B) When each teloblast has produced about 6 blast cells, one (or two) RDA-labeled blast cells, e.g. m_5 , are ablated by intracellular injection of DNAase, (hatching), and both teloblasts are reinjected with FDA, (dark gray). The first left and right blast cells labeled with RDA are in register. (C) In the case of a single

m blast cell ablation, the part of the left bandlet posterior to the ablation site either shifts anterior, closing the gap created by the absence of the ablated blast cell, here m_5 , or does not shift, preserving the gap (In the case of a dual m blast cell ablation the part of the left bandlet posterior to the ablation site shifts posterior, opening the gap). As a result of the anterior shift in this example, the first FDA-labeled blast cell in the left bandlet, m_7 , is out of register with its contralateral homologue.

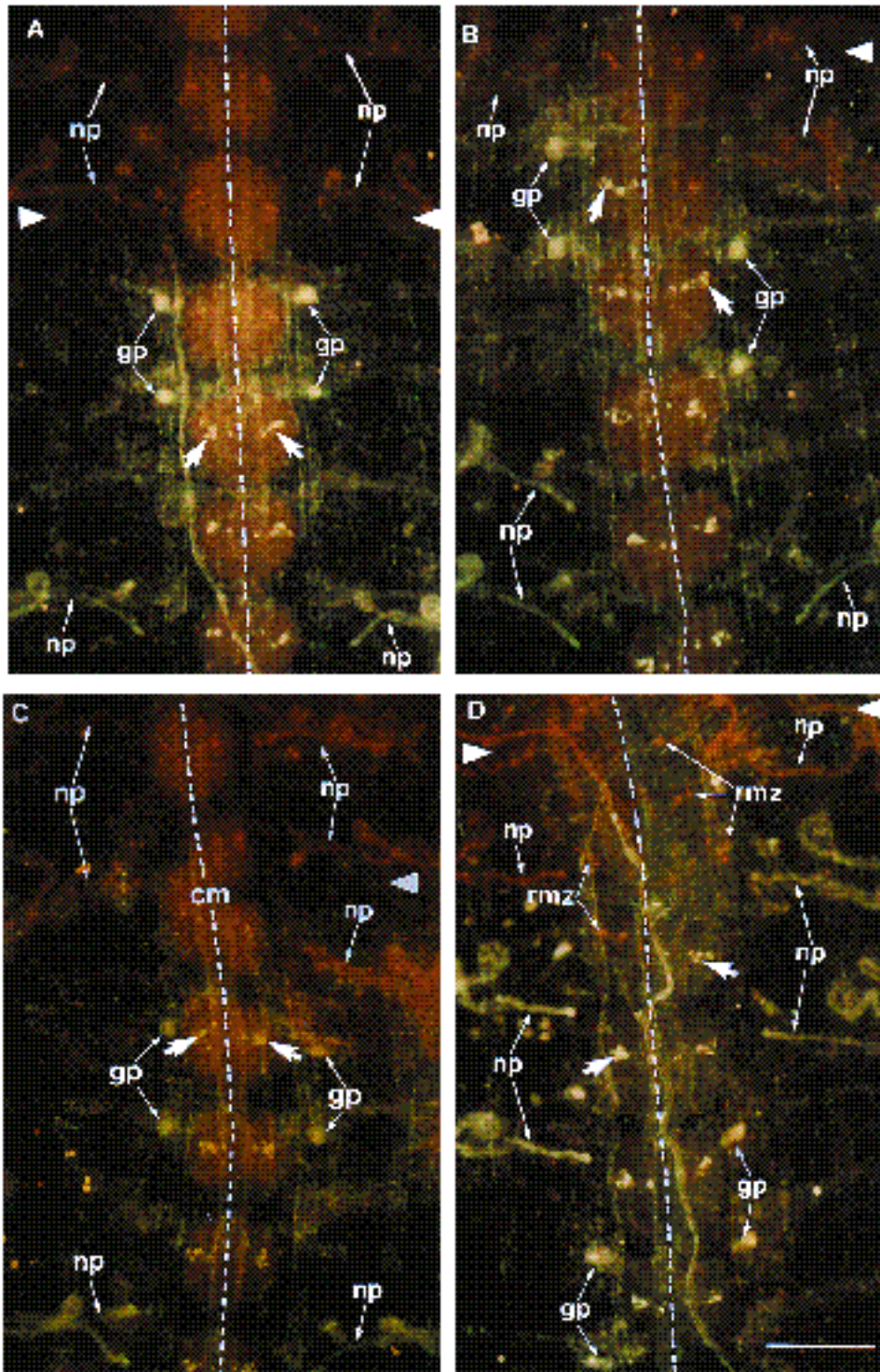


Fig. 5. FDA boundary positions and distribution of nephridia (np), genital primordia (gp), mz neurons (large arrows), and the anteriormost FDA-labeled muscles (arrowheads) in unoperated control and operated, as described in Fig. 4, stage 10 embryos. The dashed line indicates the ventral midline. (A) Segments M3-M8 of a control embryo. The left and right FDA boundaries are in register, as can be seen by the anteriormost FDA-labeled muscles and mz neurons. Nephridia and genital primordia are in register on both sides of the embryo. (B) Six segments of an embryo in which the left mesoderm shifted anterior in response to the ablation of one left m blast cell. A corrective mismatch occurred posterior to the segments shown here. The anteriormost extent of FDA-labeled muscles on the unoperated right side is marked with an arrowhead. FDA-labeled muscles extend one segment further anterior on the left. Similarly, the anteriormost FDA-labeled mz neurons are seen one segment more anterior on the left than on the right. Thus, the FDA boundary on the operated left side lies one segment anterior to the border on the unoperated right side. Nephridia and genital primordia are out of register between left and right sides, displaced by one segment anterior on the left side in relation to the right side. (C) Approximately 7 right and 6 left hemisegments of an embryo in which the left mesoderm shifted anterior in response to ablation of one left m blast cell, but in which the corrective mismatch involves the anteriormost FDA-labeled left and right hemisegments. The FDA boundaries are in register on both sides of the embryo, as revealed by the anteriormost FDA-labeled mz neurons on both sides. The corrective mismatch (cm) is seen between the hemisegment containing the third nephridium

(second visible from the anterior) on the left side and the second and third nephridia (second and third visible from the anterior) on the right side. The anteriormost FDA-labeled muscles on the right (arrowhead) are slightly posterior to the corresponding muscles on the left (not visible here but just anterior to the third nephridium). Body symmetry is reestablished posterior to the mismatch: the genital primordia and the first nephridia posterior to the hemisegments that do not contain nephridium nor genital primordium are once more in segmental register. (D) Approximately 6 segments of an embryo in which the left mesoderm shifted one segment posterior in response to ablation of two left m blast cells. The FDA boundary on the operated left side lies one segment posterior to the border on the unoperated right side as revealed by the anteriormost FDA-labeled mz neurons on both sides. The distribution of nephridia and genital primordia follow the same out-of-register pattern. All the singly RDA-labeled mz neurons present in the field of view are labeled (rmz). The anteriormost region of the operated side is deficient of mesoderm-derived elements as can be seen by the absence of singly RDA-labeled mz neurons in the hemiganglia opposite hemiganglia M1 and M2 (the latter containing the first nephridium) on the unoperated right side. Scale bar, 50 μ m.

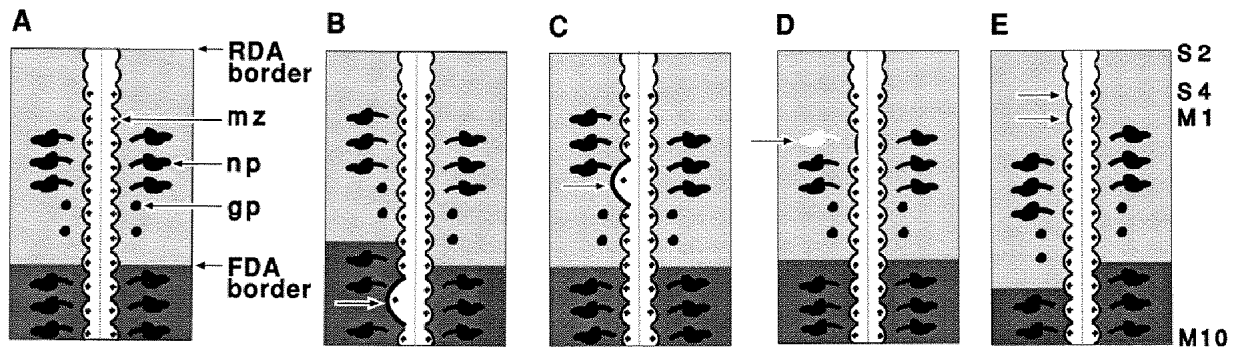


Fig. 6. Diagram of the results of the m bandlet shift experiment depicted in Fig. 4. Each panel represents 13 segments (S2-M10) of a stage 10 embryo. Each of the segmental ganglia contains a group of m-derived neurons (mz). The segment-specific distribution of mesodermal elements is represented by nephridia (np) and genital primordia (gp). The RDA tracer boundaries represented by the top of each panel, are in register in all panels. The region of the embryo containing only RDA is represented by light gray, the region containing RDA and FDA by dark gray. (A) Control embryo. Left and right FDA boundaries and segment-specific structures are in register. The labeling of segment numbers on the far right of the diagram applies to both sides of the panel. (B-D) Types of results produced by ablation of one left m primary blast cell. The left side of each panel corresponds to the embryo's operated side. The labeling of segment numbers on the far right of the diagram applies to the right unoperated side of each of the panels. (B) Anterior shift, corrective mismatch (arrow) represented by one left hemiganglion stretched, covering the distance of contralateral hemiganglia M8-M9, occurred posterior to FDA boundaries. FDA boundaries and segment-specific features are out of register. (C) Anterior shift, corrective mismatch (arrow) occurred anterior to FDA boundary. Like C, but the stretched hemiganglion covers the distance of contralateral hemiganglia M4 and M5, bringing more posterior segments back into register, and, as a result, FDA boundaries are aligned. (D) No shift - FDA boundaries are in register and there is a deficiency of mesodermal elements (arrow), as represented by misshapen segmental hemiganglion, the absence of mz neurons, and a hollow nephridium in the left hemisegment opposite the right hemisegment M2. (E) Result produced by ablation of two left m primary blast cells. The labeling of segment numbers on the right applies to the right unoperated side of the panel. Posterior shift - FDA boundaries and segmental features are out of register. Deficiencies of mesodermal elements (arrows) are represented by misshapen hemiganglia and the absence of mz neurons in the left hemisegments opposite the right hemisegments S4 and M1.

Corrective mismatch

Besides the anteroad shift of segment-specific structures, all specimens in which part of the left m bandlet shifted anteroad, showed a site of segmental mismatch somewhere posterior to the site of the shift. This mismatch was manifest in the opposition of one segmental hemiganglion on the operated side with two hemiganglia on the unoperated side. The single hemiganglion on the operated side was stretched and contained a single set of mz neurons. It was flanked by a single, stretched hemisegmental complement of muscle cells, and (when appropriate for the birth rank of the progenitor m blast cells at the site of the mismatch) by a single nephridium or genital primordium. In contrast, the two apposed hemiganglia on the unoperated side were compressed and each was accompanied by its own hemisegmental mesodermal complement. Thus, at the site of mismatch, the number of hemiganglia as well as of the elements of the hemisegmental mesodermal complement on the unoperated side was twice that on the operated side. In a few cases (3/139) the corrective mismatch involved two hemiganglia on the operated side apposed to three hemiganglia on the unoperated side. In another small group of experimental specimens (5/139) a misalignment of the left and right hemisegmental ganglionic margins and corresponding mesodermal complements that spanned over almost the whole length of the embryo was observed.

Posterior to the mismatch site, the number of hemiganglia and of hemisegmental mesodermal elements was the same on both sides of the embryo and the most posterior hemisegments containing a nephridium (normally formed by primary blast cell m22) were in register. In specimens

in which the corrective mismatch occurred anterior to the genital segments, the genital primordia of M5 and M6 and the hemisegmental mesodermal complements of M7 (which comprises neither a genital primordium nor a nephridium) were correctly matched on both sides. These observations show that body symmetry is reestablished posterior to the mismatched hemisegments and that the hemisegmental identities are again in register on operated and unoperated sides (Figs 5C, 6C). This mismatch thus seems to function as a corrective mechanism, which restores bilateral symmetry to the caudal segments with respect to the pattern of segment-specific mesodermal elements and birth rank of the m primary blast cells that founded the mesodermal cells clones on opposite sides.

In 27% (38/139) of operated specimens in which a single m primary blast cell had been ablated, the FDA tracer boundary was in segmental register on left and right sides, even though an anteroad bandlet shift had occurred. These specimens turned out to be cases in which the corrective mismatch occurred soon enough to have fallen into a segment *anterior* to the FDA tracer boundary. According to this interpretation, such anterior shift specimens showed discrepancies in segmental identities between left and right sides just anterior to the site of the corrective mismatch (Figs 5C, 6C).

No shift

In 50% (70/139) of the experimental specimens in which one m primary blast cell of the left m bandlet had been ablated, the FDA tracer boundary and the mesoderm-derived segment-specific elements were in segmental reg-

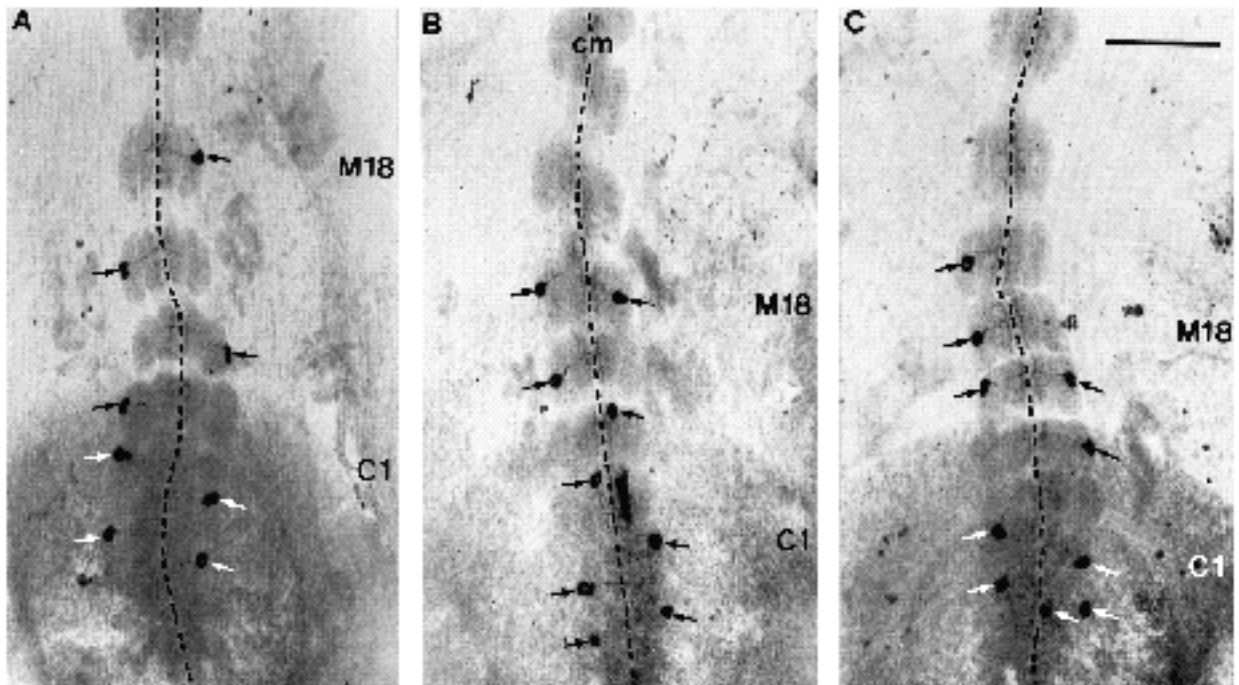


Fig. 7. Distribution of immunoreactive CAS neurons in a normal and operated stage 11 embryos. Segments were identified by the presence of nephridia and genital primordia (not shown here). Segments M18 and C1 are noted in each micrograph. The assignment of CAS neurons (arrows) to individual neuromeres in the tail ganglion was based on their axonal projection pattern. Two contralateral CAS neurons were judged to lie in same neuromeres if their main axons met at the ventral midline and to lie in different neuromeres, if not. (A) Segments M17-C7 of a normal embryo. CAS neurons are present in eight segments, from M18-M21 and C1-C4. (B) An operated embryo with anterior shift of the left m bandlet and corrective mismatch (cm) involving hemisegments M15-M16 on the unoperated side. CAS neurons distribution as in A. (C) An operated embryo with anterior shift of the left m bandlet and no corrective mismatch. The segments shown include hemisegments M15-C7 of the unoperated, right side. The anteriormost CAS neuron appears on the operated side, opposite hemiganglion M17 on the unoperated side. CAS neurons are present in nine, instead of the normal eight, segments. Scale bar, 100 μm .

ister on left and right sides (Fig. 6D). All of these specimens showed obvious deficits in the pattern of mesoderm-derived elements in the segments to which the aborted blast cell clone should have made its contribution: the muscles, mesoderm-derived interneurons and nephridium (if any) were all missing. Moreover, some ectoderm-derived features were abnormal in the mesoderm-deficient hemisegment - especially the structure of the segmental hemiganglion on the operated side. The disruptive effects of mesoderm deprivation on ectodermal morphogenesis in the leech have been described previously (Blair, 1982; Torrence et al, 1989; Torrence, 1991). All other segments, however, presented a normal morphology on both the operated and unoperated sides. Hence the mere absence of an m primary blast cell clone and the persistence of a one-clone-gap in the blast cell bandlet does not appear to cause any visible disturbance in the development of the identity of other segments.

Posterior shift

In all specimens in which *two* adjacent primary blast cells of the left m bandlet had been ablated, the FDA tracer boundary on the operated left side fell into a segment *posterior* to its position on the unoperated right side. Hence the part of the left m bandlet posterior to the gap had shifted posteriad, extending rather than closing the gap. In 24%

(9/37) of these specimens, the FDA boundary was displaced posteriad by just one segment on the operated side (Figs 5D, 6E), while in the remainder 76% (28/37) of the specimens, the FDA boundary was displaced posteriad for greater distances, ranging from 5 to 17 segments.

Specimens in which part of the left m bandlet posterior to the gap had shifted posteriad (and extended the gap) by one segment, showed severe deficits in the pattern in the hemisegments on the operated side that were deprived of their normal m blast cell clone contributions: the number of muscle fibers was reduced; the spacing between muscle fibers was larger than normal and they did not form the normal orthogonal grid; mz neurons and nephridia were absent. As happened with specimens in which the m bandlet did not shift, some abnormalities in the morphology of ectoderm-derived features, especially in the segmental hemiganglia, were manifest in these mesoderm-deprived regions: hemiganglia were severely misshapen and sometimes fused. Posterior to these deprived segments, the distribution of segment-specific mesodermal features on the operated side did not match that on the unoperated side (Figs 5D, 6E). On the operated side, nephridia were present in segments opposite M3-M5 (instead of M2-M4) and M9-M19 (instead of M8-M18) of the unoperated side, while genital primordia were present in segments opposite M6 and M7 (instead of M5 and M6) of the unoperated side.

Hence this result shows that posteriorly shifted m blast cell clones, like anteriorly shifted ones, take on a fate in accord with the birth rank of their founder cell rather than with their ectopic segmental location.

In specimens in which two m primary blast cells had been ablated and the part of the left m bandlet posterior to the gap had shifted posteriad for distances ranging from 5 to 17 segments, the operated side showed severe deficits in, or even a complete absence of, mesodermal tissues over this region. Posterior to this gap, the pattern of mesodermal features was too disorganized to allow scoring for the segmental distribution of nephridia and genital primordia. This mesodermal disorganization was accompanied by a pronounced disorganization of the consegmental ectodermal features.

CAS neuron distribution

The mesoderm-derived features that are present in some but not all segments include an interneuron designated 'Caudal Alternating SCP-like Immunoreactive (CAS) Neuron,' that contains an antigen that binds with a monoclonal antibody against the neuropeptide 'Small Cardioactive Peptide B' (SCP) (Shankland and Martindale, 1989). The distribution of the CAS neuron is limited to the four most posterior mid-body ganglia (M18-M21) and the four most anterior caudal ganglia (C1-C4) (Fig. 7A). Although a bilateral pair of CAS neurons is normally present in each of these ganglia at early stage 10, by stage 11 most ganglia contain only one immunoreactive CAS neuron. The position of this neuron tends to alternate between left and right hemiganglia in successive segments. This alternating, unilateral distribution appears to result from competitive interactions between the bilaterally paired and serially successive CAS neuron precursors (Shankland and Martindale 1989; Martindale and Shankland, 1990a). About 30% of unoperated stage 11 *T. rude* specimens contain a ganglion with two CAS neurons.

To determine whether the CAS neuron is another segment-specific feature that m blast cell clones express according to their original segmental destination rather than with their actual segmental location, the distribution of these neurons was examined in embryos with shifted m bandlets. The experimental procedure was the same as that used in the experiments described in the preceding section, except that no secondary injection of the M teloblasts with FDA lineage tracer was undertaken; only a single primary blast cell of the left m bandlet was ablated; and the specimens were allowed to develop well into stage 11, at which time they were fixed and stained with an antibody against SCP.

Of the 42 experimental specimens examined, 51% (22/42) showed the normal segmental distribution of nephridia and genital primordia on operated and unoperated sides of the embryo. Hence, these were specimens in which their operated left bandlets had not shifted and the gaps created by ablation of the m primary blast cell had been neither closed nor extended, or these were specimens in which the left m bandlet had shifted anteriorly but the corrective mismatch occurred anterior to M1. The segmental distribution of CAS neurons in these specimens was the same as that of unoperated embryos. This result reinforces the pre-

vious finding that the mere absence of an m primary blast cell and the persistence of a one-clone-gap in the blast cell bandlet does not appear to cause any disturbance in the development of the identity of other segments.

The remaining 49% (20/42) of the experimental specimens showed a one segment anterior shift in the distribution of nephridia and genital primordia on the left operated side. Hence these were specimens in which the posterior part of the operated left m bandlet had shifted anteriorly in response to the ablation. In 19/20 of these specimens, however, corrective mismatches had occurred in segments ranging from M2 to M16 of the right side, so that the m blast cell clones occupied their normal segmental positions in segments M18-C4 (Fig. 7B). In all of these specimens, the anteriormost CAS neuron appeared, as is normal, in segment M18: on the left side in 10/19, on the right side in 6/19 and bilaterally paired in 3/19 specimens. As will be shown in the discussion, this result shows that neither the ipsilateral nor the contralateral segmental ectoderm determines the segment-specific distribution of the CAS neurons.

In the one specimen (Fig. 7C) whose anterior shift of the left m bandlet was not corrected by a mismatch anterior to segment C4, the anteriormost CAS neuron appeared in a hemiganglion on the operated side opposite segment M17 of the control side. The m blast cell clone that gave rise to this CAS neuron is the clone that would normally give rise to the CAS neuron of M18. The next 8 ganglia each contained one CAS neuron (except M19, which contained a pair of CAS neurons). Thus in this one embryo CAS neurons were present in 9 ganglia (Fig. 7C), in contrast to all other operated and control specimens, in which they were present in only 8 ganglia.

The segmental distribution of CAS neurons found in this one uncorrected anterior shift specimen thus confirms the conclusion already reached from the distribution of nephridia and genital primordia in anterior shift specimens: anteriorly shifted m blast cell clones take on a fate in accord with the characteristics of their original segmental destination rather than with their actual ectopic segmental location.

Birth-rank discordance of consegmental mesoderm and ectoderm

To verify the assumption made at the outset of these experiments that ablation-induced shifts of the mesodermal m bandlet are not accompanied by a concomitant shift of the overlying ectodermal bandlets, m blast cell ablation-induced bandlet shift experiments were carried out in which an FDA-tracer boundary was examined in the ectodermal o and n bandlets. For this purpose, the same experiment depicted in Fig. 4 was performed, except that no secondary injection of the M teloblasts with FDA lineage tracer was undertaken. Instead, the right and left O or N ectodermal teloblasts were injected with FDA shortly after their birth and after the usual ablation of one left m primary blast cell. The timing of these injections was such that the ectodermal FDA borders were posterior to the site where the clone of the ablated m primary blast cell would have developed and almost always anterior to any corrective mismatch that occurred in embryos in which the m bandlet shifted anteriorly. The two O-teloblast labeled specimens with m ban-

dlet anterior shift where the ectodermal FDA borders were posterior to a corrective mismatch were excluded, although their borders supported the conclusions presented below for the region posterior to the corrective mismatch.

As previous studies with the leech *Helobdella* have shown, the division cycles of bilaterally paired ectodermal teloblasts are not perfectly synchronized (Wordeman, 1983; Shankland, 1984; Martindale and Shankland, 1988, 1990b). To ascertain the extent to which such asynchrony of ectodermal teloblast divisions exists also in *T. rude*, control experiments were carried out in which no m blast cell was ablated. The results of these control experiments showed that in 90% (9/10) of O-teloblast-injected specimens and in 85% (17/20) of N-teloblast-injected specimens the FDA tracer boundaries were in register on both sides. In the four out-of-register specimens, the positions of the FDA boundaries differed by one segment on right and left sides. These results show that in *T. rude* the division cycles of paired ectodermal teloblasts are less closely synchronized than in mesodermal teloblast homologues. Nevertheless, the synchronization of the paired ectodermal teloblast homologues is sufficient for the FDA tracer boundaries to reveal whether an actual shift in the position of a bandlet occurs in a series of operated specimens.

Among the operated O-teloblast-labeled embryos, the m bandlet did not shift in 46% (19/41) of the specimens and the distribution of mesodermal segment-specific features on left and right sides was symmetrical. In the remaining 54% (22/41) of the specimens, the distribution of nephridia and genital primordia along part of the operated side was displaced by one segment anterior relative to the unoperated side. Hence, in these latter specimens, the gap created by ablation of the m primary blast cell has been filled by a one-segment anterior shift of the m bandlet. In 90% (37/41) of both types of O-teloblast labeled specimens - with or without m bandlet shift - the FDA tracer boundaries in the o bandlets were in register on left and right sides. In the two specimens of each type in which the tracer boundaries were not in register, the left boundary was displaced anteriorly in one specimen and posteriorly in the other.

Among the operated, N-teloblast-labeled embryos, the m bandlet did not shift in 35% (22/63) of the specimens, and the distribution of mesodermal segment-specific features on left and right sides was symmetric. The FDA tracer boundaries in right and left n bandlets were in register in 86% (19/22) of these specimens. In the three specimens in which the tracer boundaries were not in register, the left boundary was displaced anteriorly in two specimens and posteriorly in one specimen. In the other 65% (41/63) of the operated embryos, the m bandlet shifted anteriorly. The FDA tracer boundaries in right and left n bandlets were in register in 83% (34/41) of these specimens. In the seven specimens in which the tracer boundaries were not in register, the left boundary was displaced anteriorly in three specimens and posteriorly in four specimens.

In both m-bandlet shifted and non-shifted operated specimens, the frequency of out-of-register FDA tracer boundaries in o and n bandlets is no higher than in the non-operated controls. It can be concluded, therefore, that on the operated side the ectodermal o and n bandlets do not generally accompany the mesodermal m bandlet on its abla-

tion-induced shifts. Thus the shift of the m bandlet creates a disturbance of the normal birth-rank relations between consegmental mesodermal and ectodermal primary blast cells, causing mesodermal as well as ectodermal blast cell clones to divide and differentiate in abnormal segmental environments.

The disturbance of the normal birth-rank relations between consegmental mesodermal and ectodermal blast cell clones is not corrected by the corrective mismatches described above. By using two different ectodermal markers, we found that a single ectodermal segmental complement lies in the stretched hemisegment of the corrective mismatch on the operated side, facing two ectodermal segmental complements on the unoperated side. Out of 55 operated embryos displaying the corrective mismatch and labeled with an antibody against serotonin (Stuart et al., 1987), 53 (96%) showed a single complement of serotonergic cells (derived from the N teloblast) in the stretched hemiganglion. In all 24 operated embryos that displayed the corrective mismatch and in which the O teloblast had been labeled with FDA, a single complement of O-derived peripheral elements (e.g., nephridial distal tubule cell; Martindale and Shankland, 1988) was found in the stretched hemisegment. Hence, in the domain of the corrective mismatch, one ectodermal segmental complement and one mesodermal segmental complement on the operated side confronts two ectodermal and two mesodermal segmental complements on the unoperated side. Thus, posterior to the corrective mismatch the ectodermal clones on the operated side remain out of birth rank register with the ipsilateral mesodermal clones and come out of birth rank register with both mesodermal and ectodermal contralateral clones on the unoperated side.

Time of occurrence of bandlet shift

That shifted m blast cell clones take on a characteristic fate in accord with their original segmental destination rather than with their ectopic segmental location is not, in itself, decisive proof that their diversification is attributable to a mechanism that is bandlet-intrinsic rather than extrinsic. For a bandlet-extrinsic mechanism could impose on the clones their original segmental identity if the bandlet shift were to occur only after the primary blast cells, or their clonal descendants, have already reached definitive axial positions appropriate for their birth rank. To ascertain, therefore, the developmental stage at which bandlet shift actually occurs, the experiment described by Fig. 4 was repeated, and the living embryos were observed under the fluorescent microscope at regular intervals after m blast cell ablation. The following information about the exact size and number of clones at the 6 and 12 hour observation times is based upon analysis of separate fixed specimens in which the M teloblast had been labeled with lineage tracer. These findings confirmed earlier ones of Schimmerling (1986).

Observations began 6 hours after blast cell ablation, by which time the M teloblast had produced three or four additional m primary blast cells. The gap created in the RDA-labeled part of the left m bandlet by ablation of one or two blast cells could still be clearly seen, and it was possible to infer the number of ablated m primary blast cells from the size of the gap. The gap was located before the point

where the bandlets come together to form the germinal band and the ablated primary blast cells would have been around the time of their first division. From the specimens in which one blast cell had been ablated, 12 were chosen to be followed individually through subsequent development.

By 12 hours after ablation, about one-third of the final number of *m* primary blast cells had been produced. The anteriormost blast cell clones, including those on either side of the gap and containing approximately 4 cells, had joined the germinal band, which normally begins at the point where the *m* primary blast cell clones become 4 cells each. At this time, the gap had been closed in six specimens.

By 48 hours after ablation, coalescence of left and right germinal bands was well under way, with the anteriormost third of the germinal plate, including the ablation site, having been formed. Within the germinal plate, neighboring *m* blast cell clones had started to interdigitate, as each clone began to spread out into its definitive distribution over three segments. In another two specimens, the ablation-induced gap had closed. In the remaining four specimens a gap was still visible at the site of blast cell ablation, although it was much narrower because of the spreading of the individual clones.

By 64 hours after ablation, no gap could be discerned in any specimen. However, the ablation sites of those six embryos in which the gap was not closed at 12 hours, showed a very disrupted morphology of mesodermal tissues. In these sites, the metameric arrangement of cells characteristic of mesodermal tissues at this stage, was not observed, and the number of cells was apparently smaller than in more anterior and posterior regions.

The specimens were scored for the positions of the FDA lineage tracer boundaries on operated and control sides 88 hours after ablation, by which time formation of the germinal plate was complete (early stage 9). Of the six embryos in which the gap had closed by 12 hours after the ablation, four showed an anterior shift of the mesodermal hemisegments on the operated side and two had died. The two embryos in which the gap had closed between 12 and 48 hours also died after the 64 hour observation. Among the four embryos in which a gap was still detected at 48 hours, one showed a one-segment posterior shift, which extended the gap, and three showed no shift, preserving the gap.

Also, 12 specimens in which two blast cells had been ablated were followed individually through development. By 48 hours after ablation, the gap had closed in one specimen, was still visible in two specimens and had extended, leaving part of the germinal plate devoid of mesoderm in nine specimens. Among the former three specimens, the one in which the gap closed died and the other two showed a one-segment posterior shift of the operated *m* bandlet. Among the latter nine specimens, one died and eight showed a posterior shift of many segments and severe morphological disruption, or even absence, of mesoderm on the operated side.

These observations show that the anteriad shift and the closing of the gap in the operated *m* bandlet of anterior shift specimens occur in at least the majority of cases long before the final number of *m* primary blast cells has been produced and before the clone has reached its final posi-

tion relative to all the other ectodermal bandlets, macromeres and micromeres. The *m* primary blast cell clones can shift anteriad when the most anterior and developed clone that shifts contains as few as about 4 cells. Hence, it seems unlikely that a bandlet-extrinsic mechanism imposes on the clones their original segmental identity. Rather, it would appear that a bandlet-intrinsic mechanism is responsible for the segment-specific diversification of mesodermal blast cell fates.

DISCUSSION

The observed shifts in segmental position of nephridia and genital primordia on the operated sides of experimental embryos always correlated precisely with the observed position shifts of the FDA tracer boundaries of *m* bandlets (when corrective mismatches are taken into account). Moreover, in the one experimental specimen in which the bandlet shift was not corrected anterior to the relevant segments, the shift in the distribution of the mesoderm-derived CAS neurons matches the shift in the distribution of nephridia and genital primordia.

These results indicate that *m* blast cells give rise to these segment-specific structures according to their original birth rank rather than to their actual segmental position. The origin of segment-specific differentiation of the mesodermal features studied here is therefore intrinsic to the *m* bandlet, and does not depend upon extrinsic signals from ectodermal bandlets or from other embryonic tissues.

The shift of mesodermal blast cell clones and the resulting bilateral asymmetry created by the ablation of a single *m* blast cell was, in almost all cases, corrected by the pairing of one mesodermal hemisegmental complement on the operated side with two complements on the unoperated side. Such corrective mismatch cannot arise before the bandlets of the left and right sides first come into contact, as they coalesce to form the germinal plate. Since muscle fibers arising from the left and right *m* bandlets begin to interdigitate with one another very soon after bandlet coalescence (Torrence et al., 1989), it is most likely that the correction takes place *during* the process of coalescence. Corrective mismatches between left and right sides in almost all cases involve integral numbers of hemisegments; mismatches of less than a full segment's length are rarely observed. During coalescence, therefore, blast cell clones on the left and right sides must align themselves longitudinally, so that left and right hemisegments come into metameric register.

By the time of coalescence, the anteriad shift of posterior blast cells has closed the gap created by the ablation of an *m* blast cell and restored a continuous bandlet now containing one fewer blast cell than before the operation. As left and right blast cell clones align during coalescence, the bandlets are still attached to their parental teloblasts and, consequently, blast cell clones in the shorter, operated bandlet could be extended more than those in the contralateral bandlet. A correction may occur when the posterior margin of the more extended *m* blast cell clone fails to align with the homologous posterior margin of the contralateral clone, and instead aligns with the posterior margin of the next more posterior contralateral clone. The mechanism respon-

sible for the alignment of margins under corrective mismatch is likely to be the same as that which assures normal alignment of left and right clonal margins during coalescence.

It should be noted that while the corrective mismatch does restore bilateral symmetry with respect to the birth rank of the m primary blast cell founders of the hemisegmental *mesodermal* cell clones, it results in a bilateral *asymmetry* with respect to the birth rank of the primary blast cell founders of the hemisegmental *ectodermal* cell clones. Although the anteriad shift of the m bandlet on the operated side creates there a discordance in the normal birth-rank relations between consegmental mesodermal and ectodermal primary blast cells, the hemisegmental ectodermal cell clones on operated and unoperated sides initially remain in birth-rank register. Since posterior to the site of mismatch, however, the contralateral mesodermal cell clones come *into* birth-rank register, it follows that the contralateral ectodermal cell clones come *out* of it.

Although the segments posterior to the corrective mismatch provide no information regarding the general question of the control of segment-specific fate of the m blast cell, they provide additional evidence that at least the *ipsilateral ectoderm* cannot be the determinant of the segment-specific distribution of the nephridia, genital primordia and CAS neurons. This follows from the observation that posterior to the corrective mismatch, the distribution of these segment-specific features is normal, despite the fact that in these segments the birth ranks of the founder blast cells of ipsilateral mesodermal and ectodermal clones are out of register. It follows, furthermore, that the *contralateral ectoderm* cannot be the determinant of the segment-specific distribution of the CAS neurons. Otherwise, the anteriormost CAS neuron on the unoperated side would have to appear posterior to M18, since posterior to the correction each ectodermal clone on the operated side lies one segment posterior to the segment in which it would normally lie.

Anteriad shifts of the m bandlet were induced by laser ablation of a single m blast cell by S. S. Blair in the leech *Helobdella robusta* (personal communication). In these experiments the occurrence of an anteriad bandlet shift was inferred from finding corrective mismatches in more posterior regions of the embryo. Just as in the anterior shift embryos presented here, so also in these embryos was the distribution of CAS neurons posterior to the corrective mismatch similar to that of the control embryos.

Bandlet-intrinsic marking of the birth rank of blast cell clones is not confined to the mesodermal m blast cells: Induction of posteriad shifts of the ectodermal n bandlet in *Helobdella triserialis* has shown that the segment-specific distribution of the Rostral Alternating SCP-like Immunoreactive (RAS) neuron is in accord with the original segmental destination of the shifted n blast cell from which the RAS neuron is derived, rather than with its ectopic location (Martindale and Shankland, 1990b). Moreover, it has been suggested that the differences in intrasegmental developmental fate of the successively born n_f and n_s primary ectodermal blast cells are established by the time of birth (Bissen and Weisblat, 1987).

It is possible that the birth-rank marking of m primary blast cells arises from the differential expression of genes

encoding morphogenetic factors that play a role in the development of their segment-specific phenotype, in a manner similar to the expression of homeotic genes that have been implicated in the establishment of the segmentation pattern of insects and vertebrate CNS structures, such as the hindbrain (Akam, 1987; 1989; Wilkinson and Krumlauf, 1990). This possibility is suggested by the identification of leech homologues of homeotic genes (Shankland et al., 1991; Wysocka-Diller et al., 1989), at least one of which is expressed in a spatially localized pattern (Wysocka-Diller et al., 1989).

Several hypotheses could account for a bandlet-intrinsic mechanism by which m primary blast cells or their clones acquire segmental identities in accord with their birth rank. One hypothesis invokes an intrabandlet cell-to-cell count-off. However, the fact that the gap created in the m bandlet blast cell ablation is closed long before the final number of m primary blast cells has been produced places severe limitations on this hypothesis. Another hypothesis is that birth-rank identity is conferred on m primary blast cells before or around the time when they are produced by their parent teloblast (Martindale and Shankland, 1990b). This ranking could reflect relative times of birth, as indicated by a clock within the parent teloblast that reflects a change in concentration of some morphogenetic factors in the cytoplasm. The clock could also be outside the parent teloblast and reflect progressive changes in the local environment, such as the clock that has been proposed to explain the commitment according to birth rank (Rakić, 1974) of neurons generated in the ventricular zone by progenitor cells to a particular laminar fate in the formation of the vertebrate neocortex (McConnell, 1991; McConnell and Kaznowski, 1991).

Alternatively, the birth ranking of primary blast cells could reflect a spatial differentiation of the teloblast cytoplasm or cortex with respect to morphogenetic factors. Contiguous patches of these factors are parceled out one-by-one to successive daughter blast cells as they are born by an orderly progression of cytokinetic sites on the teloblast surface. Thus, the teloblast might resemble the syncytial fly embryo, in which the products of homeotic genes responsible for the segment-specific distribution of morphogenetic factors are present in a spatially differentiated pattern (Akam, 1987).

Some features of segmental identity are *not* intrinsically specified in the primary blast cells and have been shown instead to be specified extrinsically, by interactions with the local segmental environment. For instance, the cell derived from the ectodermal o bandlet that forms the distal end of the nephridial tubule arises in every midbody segment, but dies in early stage 10 in segments that lack nephridia. Induced posteriad shifts of the o bandlet have shown that the survival or death of the presumptive distal tubule cell is determined by the local segmental environment (Martindale and Shankland, 1988). Similarly, in the leech *Hirudo medicinalis*, the distinct patterns of cell morphology and synaptic contacts of the serotonergic Retzius neurons in the genital segments devolve from interactions of the immature Retzius cell with the genital primordia (Loer et al., 1987; Loer and Kristan, 1989a,b).

In these examples of a bandlet-extrinsic specification of

segment-specific ectodermal features by interactions with the local segmental environment, the relevant segment-specific environmental factors - genital and nephridial primordia - are intrinsically specified mesodermal features. This suggests that at least some ectodermal features owe their segmental identity indirectly to an early segmental differentiation of the consegmental mesodermal blast cell clones.

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