A distinct patterning mechanism of O and P cell fates in the development of the rostral segments of the leech *Helobdella robusta*: implications for the evolutionary dissociation of developmental pathway and morphological outcome

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Summary

Despite a high degree of homonomy in the segmental organization of the ectoderm, the body plan of the leech is divided into two zones based on the distinct cell lineage patterns that give rise to the O/P portion of the segmental ectoderm. In the midbody and caudal segments, each segmental repeat of ectoderm arises in part from one 'o' blast cell and one 'p' blast cell. These two blast cells are positionally specified to distinct O and P fates, and give rise to differentiated descendant cells called O and P pattern elements, respectively. In the rostral segments, each segmental repeat of O and P pattern elements arises from a single 'op' blast cell. Based on their developmental fates and their responses to the ablation of neighboring cells, the granddaughters of the primary op blast cell are categorized into two O-type cells and two P-type cells. The O-type cells

do not require the presence of the rest of the op blast cell clone for their normal development. By contrast, normal development of the P-type cells depends upon interactions with the other OP sublineages. Additional experiments showed that the O-type cells are the source of a repressive signal involved in the normal fate specification of the P-type cells. Our data suggest that the cell interactions involved in fate specification differ substantially in the rostral and midbody segments, even though the set of differentiated descendants produced by the rostral OP pathway and the midbody O and P pathways are very similar.

Key words: Cell lineage, Cell interaction, Rostral segment, Leech, *Helobdella robusta*

Introduction

In segmented animals, segments are characterized as repeating morphological units aligned along the anteroposterior axis that share the same 'ground plan'. Serial homology refers to the existence of comparable pattern elements in different segmental units of the same individual. It is generally the case that serial homology is a result of the iterated actions of the same set of patterning mechanisms in different morphological compartments. For example, the segmental pattern of insects, and possibly other arthropods, is established by the iterated deployment of a developmental mechanism called the segmental polarity pathway (Martinez-Arias, 1993; Patel, 1994).

As discussed by Wray and Abouheif, developmental pathways and morphological homology can dissociate in different evolutionary lineages (Wray and Abouheif, 1998). In the vulval development of different nematode species, distinct modes of cell-cell interaction are used to construct the same conserved cell lineage pattern and morphology (Sommer, 1997). This implies that a developmental process can undergo evolutionary change (i.e. interspecific diversification) without an obvious alteration of its morphological outcome. Evolutionary change in the developmental underpinnings of a

conserved morphological outcome is usually assumed to be under little or no selective pressure compared with developmental changes that directly impact the final morphology (True and Haag, 2001), and hence represent a type of evolutionary flexibility similar in principle to the accumulation of silent mutations in the genetic code. As serial homology is likewise defined by a similarity in morphological pattern, serially homologous patterning mechanisms might also be amenable to this sort of evolutionary dissociation.

There is some evidence that serially homologous morphologies can arise from segmentally differentiated developmental mechanisms. In the segmental development of the crustacean *Diastylis*, identical cell arrangements can be generated in different segments by distinct cell lineage histories (Dohle and Scholtz, 1988). However, the role of cell lineage in the segmental patterning of the crustacean is unknown, and it has been argued that the segment polarity pathway plays a central role by a mechanism that is not dependent on cell lineage (Scholtz et al., 1994). Another example involves the development of *Drosophila* head segments, in which the segment polarity pathway is modified substantially relative to trunk segments (Gallitano-Mendel and Finkelstein, 1997). Unfortunately, this latter case is weakened

by the fact that homology between head segments and trunk segments of insects is highly controversial (reviewed by Rempel, 1975). The difference between head and trunk segments could have arisen by an imperfect cooptation of the ancestral trunk patterning mechanism into an originally unsegmented head region rather than by the diversification of homologous segments.

In this study, we take advantage of the high degree of segmental homonomy in an annelid, the leech *Helobdella robusta*, to further investigate the dissociation of developmental pathways and morphological outcome in serial homology. The segmented ectoderm and mesoderm of the leech originate from five bilaterally symmetric pairs of teloblastic stem cells (reviewed by Shankand and Savage, 1997). Each of the four ectodermal teloblast lineages, and the one mesodermal teloblast lineage, produces a distinct set of highly stereotyped differentiated descendants. Each teloblast undergoes repeated rounds of asymmetric cell division and gives rise to a string of primary blast cells that is termed a 'bandlet'. Two ectodermal teloblasts, O and P, and the mesodermal teloblast M, each give rise to a single class of primary blast cell that is named by the same letter as the progenitor teloblast but in lower case (i.e. o, p and m). In the O, P and M lineages, each blast cell derived from the same teloblast generates the same set of pattern elements, and as a result, each blast cell clone corresponds to one segmental repeat in these lineages. By contrast, the N and Q teloblasts generate two classes of primary blast cells in alternation, and thus create a periodically repeated segmental unit consisting of two blast cell clones.

The five bandlets on each side of the embryo merge to form right and left germinal bands. In each germinal band, the transverse arrangement of the four ectodermal bandlets represents the future dorsoventral axis, in which the n, o, p and q bandlets are arranged ventral to dorsal, respectively. The left and right germinal bands progressively move toward the ventral side of the embryo, where they fuse into a germinal plate that develops into the segmented trunk of the adult animal.

The mechanisms of cell fate specification in the teloblast lineages have been studied by extensive ablation experiments in glossiphoniid leeches of the genus *Helobdella* (Weisblat and Blair, 1984; Zackson, 1984). It was found that cell interactions play a major role in the specification of the O and P lineages (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Zackson, 1984; Ho and Weisblat, 1987; Huang and Weisblat, 1996). The o and p blast cells, which are distinguished by their respective dorsolateral and ventrolateral positions within the germinal band, represent an 'equivalence group' (i.e. equipotent cells that choose distinct developmental fates according to external instructions and/or interactions within the group), and are often referred to collectively as 'o/p' blast cells. Experimental studies reveal that the specification of O and P fates in the O/P equivalence group involves a P fate-inducing signal from the q bandlet (Huang and Weisblat, 1996), an O fate-inducing signal from a provisional epithelium derived from micromeres (Ho and Weisblat, 1987), and inhibitory interactions between the adjacent o and p bandlets (Shankland and Weisblat, 1984). The molecular basis of these interactions is unknown.

Studies of O/P fate specification have, to date, been restricted to the midbody and caudal segments that form the bulk of the leech body plan. The rostral body segments arise by a distinct pattern of cell divisions. The O/P teloblasts are generated by the symmetric division of their precursor, the OP proteloblast. The OP proteloblast undergoes several rounds of asymmetric cell division to produce 'op' primary blast cells prior to this symmetric division (Fig. 1A). Cell lineage analysis in the closely related species *H. triserialis* (Shankland, 1987c) revealed that the op blast cells contribute to the formation of the rostral segments, and that each of these blast cells gives rise to a set of descendant pattern elements that is serially homologous to the sum of pattern elements derived from one o primary blast cell and one p primary blast cell in the midbody or caudal segments. As pointed out by Shankland, different genealogical patterns are used by the OP lineage in the rostral segments, and by the O and P lineages in the midbody segments, to generate the same set of pattern elements (Shankland, 1987c); in other words, there is a dissociation of developmental process and morphological pattern, the final outcome of development.

To further explore how the mechanism that generates the O and P descendant cell fates in the rostral segments differs from that in the midbody segments, we here investigate the importance of interactions between the four op tertiary blast cells (i.e. the granddaughter cells of the op primary blast cell). First, we describe the pattern and timing of early blast cell divisions in the OP lineage. Second, we confirm that the previously described fate map of the OP lineage in *H. triserialis* (Shankland, 1987c) is conserved in *H. robusta*. Third, by cell ablation experiments, we characterize the cell interactions involved in fate specification of the O-type and P-type sublineages within an op blast cell clone. Our results indicate that the differences between the developmental mechanisms employed by the OP lineage and in the O and P lineages are significant. In both the developmental potential of individual blast cells and the pattern of cell-cell interactions, these two alternative pathways display a number of distinct characteristics.

Materials and methods

Animals

Embryos of *H. robusta* were taken from a breeding colony established with animals collected from Shoal Creek in Austin, Texas. The laboratory leech colony was maintained as described by Seaver and Shankland (Seaver and Shankland, 2000). The embryos were cultured at 23°C in a buffered saline medium supplemented with antibiotics (0.05 mg/ml tetracycline, 100 units/ml penicillin, 100 units/ml streptomycin). Staging and nomenclature of the leech embryo in this study follow that described by Stent et al. (Stent et al., 1992).

Nomenclature of nervous system pattern elements

The nomenclature for many of the neurons in the nervous system of the leech has been traditionally based on their clonal origin (Kramer and Weisblat, 1985). But neurons in the OP lineage have a different clonal origin from their serially homologous counterparts in the O and P lineages. For convenience of comparison, we have here adopted the nomenclature that is already in use for the O and P lineages (Shankland, 1987a; Shankland, 1987b), when referring to homologous cells in the OP lineage.

Fate map analysis

In stage 7 embryos, the junction of the op$_4$ clone, the most posterior op blast cell clone, and the anterior ends of the o and p bandlets

resembles a 'Y', and serves as a reliable landmark for visual identification of blast cells in the germinal band of mid-stage 7 embryos (Fig. 1A,C). We chose the op_4 clone as the subject of experimental analyses in order to optimize cell identification.

To label the op_4 clone distinctly, we used a double-labeling strategy. The op_4 primary blast cell was labeled by pressure injecting the OP proteloblast with a 1:2 mixture of 100 mg/ml tetramethylrhodamine dextran, lysine fixable (Molecular Probes), and 4% Fast Green (Sigma), in 0.2 M KCl shortly before the birth of the op_4 blast cell. Within one hour of the formation of the O/P teloblasts, both O/P teloblasts were pressure injected with a 1:1 mixture of fluorescein dextran, lysine fixable (Molecular Probes) (100 mg/ml in 0.2 M KCl), and 4% Fast Green, in 0.2 M KCl. Therefore, only the descendants of the op_4 primary blast cell were exclusively labeled with tetramethylrhodamine dextran; the rest of the cells in the embryo were either unlabeled, or double labeled with both tetramethylrhodamine dextran and fluorescein dextran.

To label individual progeny of the op_4 blast cell, the cell in question was iontophoretically injected with tetramethylrhodamine dextran (100 mg/ml in 0.2 M KCl) as described previously (Shankland, 1987a). We did not perform iontophoretic injection of the op_4 blast cell itself because of the difficulty of visualizing this cell prior to its first division owing to its deep location.

After dextran injections, embryos were cultured in buffered saline to allow further development. At stage 9, the embryos were paralyzed in a solution containing 4.8 mM NaCl, 1.2 mM KCl, 10 mM $MgCl_2$ and 8% ethanol, and then fixed in a 1:1 mixture of 8% formaldehyde (Pella) and HEPES-buffered saline (50 mM HEPES, 150 mM NaCl, pH 7.4). Fixed embryos were counterstained with 2.5 µg/ml Hoechst 33258.

Laser ablations

After the cell-labeling procedure, the embryos were allowed to develop until a desired stage for laser ablation. Laser ablation was performed in the manner described by Seaver and Shankland (Seaver and Shankland, 2000). In short, living embryos were positioned under a 40× water immersion objective on a compound microscope, and the target cell was visualized and identified with transmitted illumination prior to irradiation with an incident laser microbeam. The operated embryos were fixed and prepared for microscopic analysis at stage 9 (as described above).

Results

Normal development of the OP lineage
Formation of the tertiary blast cells

The number of op blast cells produced by the OP does not appear to be evolutionarily conserved. Whereas four op primary blast cells are produced in the leech *Helobdella triserialis* (Bissen and Weisblat, 1989) and the oligochaete *Tubifex hattai* (Goto et al., 1999), five op primary blast cells are produced in the leech *Theromyzon tessulatum* (Sandig and Dohle, 1988). We characterized the number of the op primary blast cells, and the timing of their birth, in *H. robusta* by injecting OP proteloblasts with rhodamine dextran at various time points, and then injecting the two O/P teloblasts with fluorescein dextran immediately after their formation (*n*=37). The number of segmental repeats, which represents the number of primary blast cells (each primary op blast cell gives rise to one segmental repeat, see below), labeled with only rhodamine was counted in older embryos. Our data suggest that each OP proteloblast invariably gives rise to four primary op blast cells (Fig. 1A). The first op blast cell (op_1) is produced approximately 2 hours after the birth of the OP proteloblast.
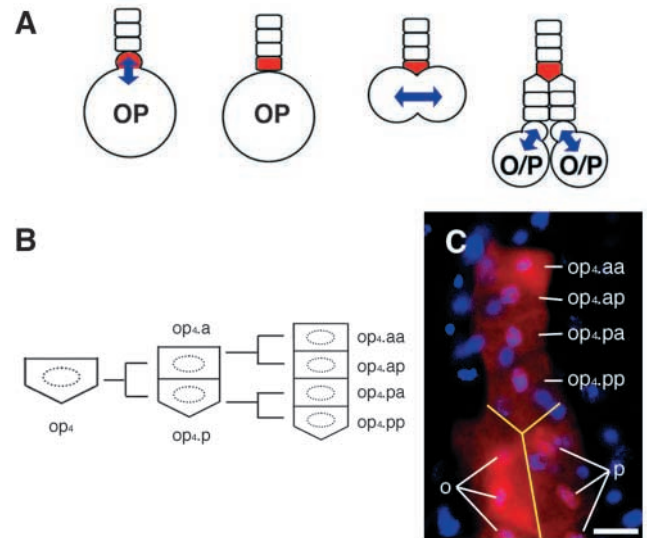
Fig. 1. Early development of the OP lineage. (A) The OP proteloblast undergoes four asymmetric cell divisions to form the four primary op blast cells. The op_4 blast cell is labeled red. The OP proteloblast undergoes a symmetric cell division to form the two O/P teloblasts. Each O/P teloblast then resumes asymmetric cell divisions to form the o and p bandlets. Blue arrows indicate the axis of cell division. (B) Divisions of the op_4 blast cell. (C) Fluoromicrograph showing the four tertiary blast cells of the op_4 clone (clonal age is 44 hours). The OP proteloblast was injected with rhodamine dextran immediately before the birth of the op_4 blast cell. The specimen was counterstained with Hoechst 33258, and is shown with anterior toward the top. The boundary between the op_4 clone and the o and p bandlets is marked by a yellow line. Note that the posterior edge of the $op_4.pp$ cell retains a unique chevron shape which borders the o bandlet to the future ventral side (left) and the p bandlet to the future dorsal side (right). Scale bar: 10 µm.

Following the birth of the op_1 blast cell, subsequent cell divisions give rise to the op_2, op_3 and op_4 blast cells at intervals of approximately 1.5 hours. The symmetric division of the OP proteloblast into the two O/P teloblasts takes place 1-1.5 hours after the formation of the op_4 blast cell.

We next followed the cell divisions within the op_4 blast cell clone by examining the morphology of the clone at one-hour intervals (*n*=27). The averaged timing of early divisions of the op_4 blast cell clone is given below. The primary op_4 blast cell divides along its anterioposterior axis approximately 30 ± 1 hours after its birth, and gives rise to two equal-sized daughter cells: an anterior secondary blast cell, $op_4.a$, and a posterior secondary blast cell, $op_4.p$. The next division occurs more or less simultaneously for both the $op_4.a$ cell and the $op_4.p$ cell, when the clonal age is approximately 41 ± 1 hours. Both the op.a cell and the op.p cell divide symmetrically along the anteroposterior axis. The anterior daughter of the op.a cell is named op.aa and the posterior daughter is named op.ap. In parallel, the anterior daughter of the op.p cell is named op.pa and the posterior daughter is named op.pp. The cell lineage leading to the formation of these four tertiary blast cells is depicted in Fig. 1B.

The sequence of cell divisions among the four tertiary blast cells is in an invariant order of op.pa, op.ap, op.aa and op.pp, with an approximate one-hour interval between each division.

The first of these divisions occurs when the clone is approximately 48±1 hours old. The divisions of the tertiary blast cells take place with a similar geometry, in that each of these cells gives rise to two mediolaterally arranged, and roughly equal-sized, daughter cells.

Although the division pattern of the blast cells op$_1$-op$_3$ was not studied in detail, our anecdotal observations suggest that they divide in a manner similar to op$_4$.

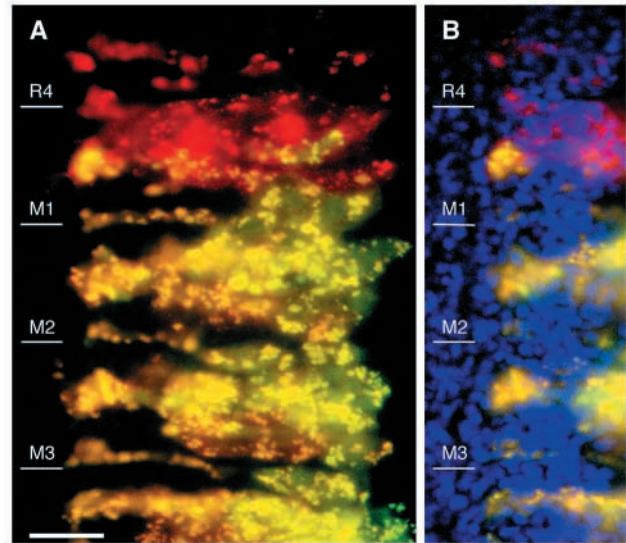## An op clone is serially homologous to the sum of an o clone and a p clone

To determine the descendant fate map of the OP lineage in *H. robusta*, we examined the morphological components of labeled op$_4$ clones in older embryos. In stage 9 embryos, the op$_4$ clone spans the boundary of the fourth rostral segment (R4) and the first midbody segment (M1), and consists in large part of neuron clusters in the ventral nerve cord ganglia, peripheral neurons and epidermal tissues (Fig. 2). These OP-derived pattern elements appear to be segmentally homologous to the O and P pattern elements in the midbody segments (see below; Fig. 4). Like the o and p blast cell clones of the midbody (Shankland, 1987a; Shankland, 1987b), the op$_4$ clone straddles the boundary of two anatomically defined segments, but is in fact only one segmental repeat in length. Although the cellular composition of the op$_1$-op$_3$ clones was not characterized in as great detail, the overall composition of these more rostral op clones is overtly similar to that of the op$_4$ clone.

Fate maps of individual tertiary op blast cells

To ascertain the fates of the tertiary op blast cells during normal development, we iontophoretically injected each individual tertiary blast cell in the op$_4$ clone with a fluorescent lineage tracer and scored its fluorescently labeled descendants in the stage 9 embryo, at which time the majority of tissues have undergone terminal differentiation. As detailed below, each tertiary op blast cell gives rise to a clone of differentiated descendants that is morphologically indistinguishable, except for its segmental location, from either one cell or a pair of cells in the O and P lineages of the midbody. For reference, the early o and p blast cell divisions are depicted in Fig. 4A. The developmental fates of the tertiary blast cells determined here for *H. robusta* are consistent with the previous brief description from *H. triserialis* (Shankland, 1987c).

We observed that the developmental fate of an op$_4$.aa cell is equivalent to the sum of the fates of an o.aa cell and a p.aa cell in the midbody segments. At stage 9, a labeled op$_4$.aa cell consistently gives rise to the CR neuron cluster in the R4 neuromere of the ventral nerve cord, and to the LD2 neuron, the oz2 neuron, the pz7 neuron, and a lateral patch of squamous epidermis in the periphery of the R4 segment (n=4) (Fig. 3A). The CR neuron cluster, the oz2 neuron and the LD2 neuron are the descendants of the o.aa cell in midbody segments (Fig. 3B), and the corresponding region of the epidermis and the pz7 neuron are derived from the p.aa cell in a midbody segment (Fig. 3C). In the midbody segments, the p.aa cell also gives rise to the pz1-3 central neurons (Shankland, 1987b). We could not determine whether homologous neurons are present in the op.aa clone as the presence of the fluorescently labeled CR neuron cluster would obscure any counterparts of the pz1-3 neurons owing to their overlapping localization.

The composition of labeled op$_4$.ap clones indicates that the


Fig. 2. The OP lineage of rostral segments is serially homologous to the sum of the O and P lineages in more posterior segments. This embryo was fixed and dissected at stage 9, by which time the primary blast cells have given rise to their segmental clones of differentiated descendants. The op$_4$ clone is labeled with rhodamine, and the more posterior O and P lineages are labeled with rhodamine and fluorescein (see Materials and methods). The preparation shown here is oriented with anterior to the top, ventral midline to the left and dorsal to the right. The segmental boundaries are marked on the left side of each panel. (A) The morphological pattern of differentiated descendants in the op$_4$ blast cell clone (red) is overtly similar to one segmental repeat of the O and P lineages combined (yellow). (B) The op$_4$-derived pattern elements in the ventral nerve cord are homologous to the segmental pattern elements derived from the O and P lineages. The ventral nerve cord is visualized with Hoechst 33258 counterstaining (blue). Scale bar: 20 μm.

developmental fate of the op$_4$.ap cell is equivalent to that of a p.ap cell in the midbody segments (Fig. 3D,E). The op$_4$.ap clone invariably gives rise to the pz5 peripheral neuron and a pz6/LD1 peripheral neuron in the M1 segment, and an elongated overlying stripe of epidermis (n=3).

Cell op$_4$.pa invariably gives rise to the PV neuron cluster in the R4 neuromere; the AD neuron cluster and medial packet glia in the M1 ganglion; and the oz1 peripheral neuron, a cell floret 2 (and associated tubule) and a patch of epidermis in the M1 segment (n=4) (Fig. 3F). These structures are serially homologous to an o.ap descendant clone in midbody segments (Fig. 3G). It should be noted that even though the rostral segments do not possess a definitive nephridium, there are mesodermal structures that appear to be serially homologous to the nephridium in the rostral segments (Martindale and Shankland, 1988). The op.pa-derived tubule cells associated with cell floret 2 in M1 and more rostral segments appear to be serially homologous to the o.ap-derived distal nephridial tubule cells seen in more posterior midbody segments.

The op$_4$.pp cell consistently gives rise to the WE neuron cluster and the pz4 neuron in the M1 ganglion, and to the pz6/LD1, pz9 and pz10 peripheral neurons, and cell florets 1 and 3, in the M1 segment (n=8). These pattern elements are comparable to the pattern elements derived from the p.p cell in a midbody segment (Fig. 3H,I).
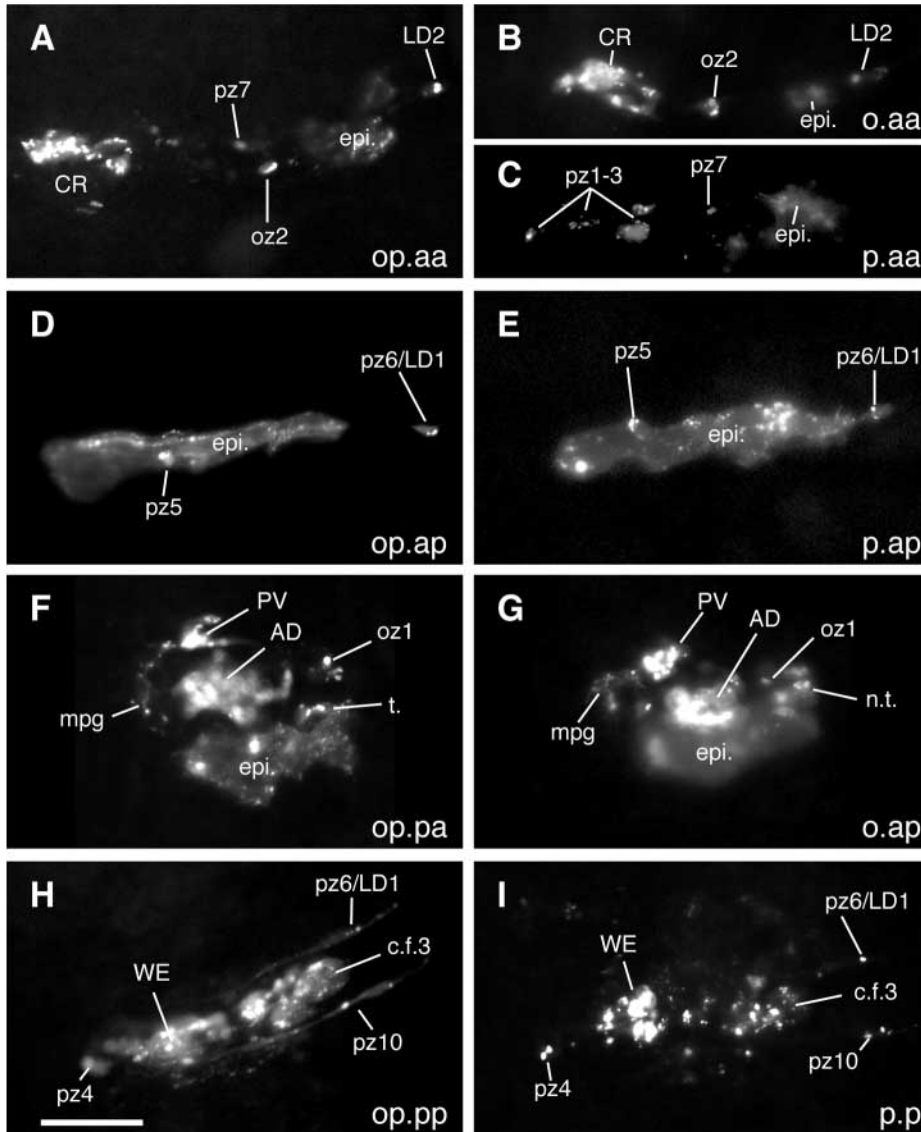
Fig. 3. Serial homology in cell identity between specific OP sublineages and O and P sublineages is shown by injection of rhodamine dextran lineage tracer. The labeled embryos were fixed and dissected at stage 9. The clonal origin of the labeled cells is shown at the lower right corner of each panel. The pattern elements derived from the op.aa cell (A) in the rostral segments are homologous to the pattern elements derived from the o.aa cell (B) and the p.aa cell (C) in more posterior segments. The pattern elements derived from the op.ap cell (D) in the rostral segments are homologous to the pattern elements derived from the p.ap cell (E) in more posterior segments. The pattern elements derived from the op.pa cell (F) in the rostral segments are homologous to the pattern elements derived from the o.ap cell (G) in more posterior segments. The pattern elements derived from the op.pp cell (H) in the rostral segments are homologous to the pattern elements derived from the p.p cell (I) in more posterior segments. See text for detail. CR, crescent neuron cluster; PV, posterior ventral neuron cluster; AD, anterior dorsal neuron cluster; WE, wedge-shaped neuron cluster; mpg, medial packet glia; c.f.3, cell floret 3; t., tubular structure homologous to the nephridial tubule (n.t.) in midbody segments (see text for detail); epi., epidermis. Orientation of the specimen is the same as in Fig. 2. Scale bar: 20 μm.

When the 'isolated' cell changes its developmental fate in comparison with its normal fate, it signifies developmental regulation of the 'isolated' cell, and suggests that the fate of this cell is conditionally specified during normal development. Dextran injection of the OP proteloblast labels roughly half the ipsilateral ectoderm and, as a result, it is difficult to identify certain pattern elements in areas with densely overlapping labeled cells. To avoid such problems in the identification of pattern elements, only pattern elements that were routinely identifiable were scored. Among the pattern elements chosen for scoring, the CR neuron cluster and the LD2 neuron are op.aa descendants; one of the pz6/LD1 neuron pair is an op.ap descendant; the PV and the AD neuron clusters are op.pa descendants; and the WE neuron cluster, the other member of pz6/LD1 neuron pair, the pz10 neuron, cell floret 1 and cell floret 3 are op.pp descendants. Thus, each op tertiary blast cell lineage is normally represented by at least one pattern element that can be reliably scored.

For convenience of comparison, we will categorize the tertiary op blast cells into two different classes, O-type and P-type, based on that cell in the midbody segments to which they are serially homologous (Fig. 4). Clearly, the op.pa cell is an O-type cell, and the op.ap and op.pp cells are P-type cells. The classification of the op.aa cell is more ambiguous as it produces pattern elements homologous to both the o.aa and p.aa sublineages. However, we will herein refer to op.aa as an O-type cell as a result of: (1) the greater number of its o.aa pattern elements; and (2) the behavior of its descendant lineage in the experiments described below.

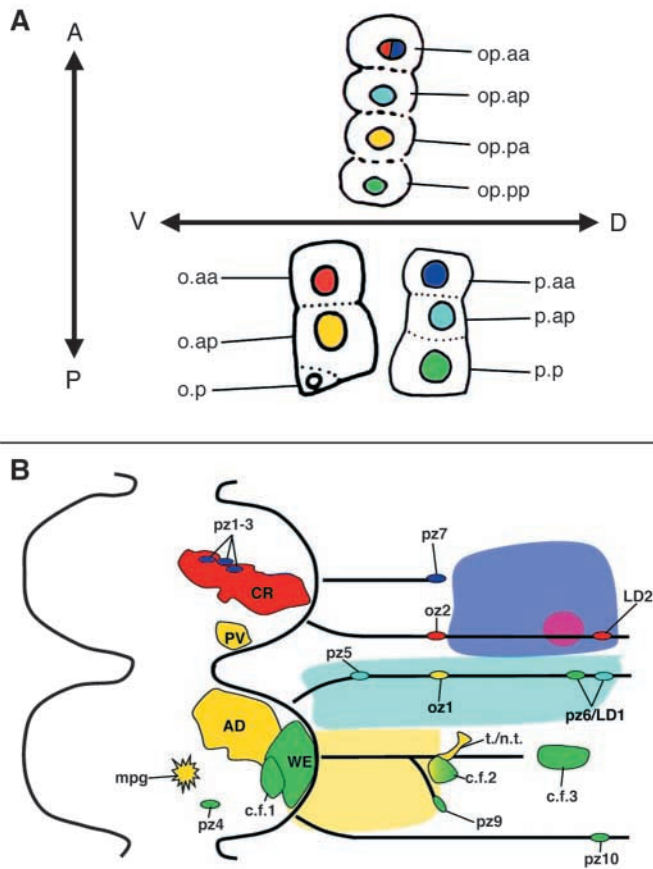### Development of 'isolated' tertiary blast cells

To determine whether the fate specification of each individual tertiary op blast cell requires interaction with the other members of its primary blast cell clone, we 'isolated' the cell by ablating its anterior and posterior neighbors with laser pulses at stage 7, and then scored the pattern of fluorescently labeled descendants produced by the 'isolated' cell at stage 9.

O-type cells develop normally in the absence of other cells in the op blast cell clone

As shown in Table 1, none of the 'isolated' op4.aa clones gave rise to any labeled descendants that were overtly inconsistent

Fig. 4. Schematic representation of the serial homology in cell identity between OP sublineages and O and P sublineages. (A) Comparison of an op blast cell clone (top) and the paired o and p blast cell clones (bottom) during embryonic stage 7. At the stage shown, the op blast cell has undergone two rounds of division (see Fig. 1B), and the o and p blast cells have undergone their second cell division in only the anterior half of the clone. Each homologous pair of cells gives rise to the same set of pattern elements, and their nuclei are labeled here with the same color. The positional relationship between the OP, O and P sublineages is indicated with anterior to the top, ventral to the left and dorsal to the right. (B) Descendant pattern elements arising from one op blast cell, or a consegmental pair of o and p blast cells, in the stage 9 embryo. The clonal origin of the pattern elements is represented by the same color scheme used in Fig. 4A. See Fig. 3 for labeling of pattern elements.

with the normal op.aa fate (Fig. 5A; Table 1). We obtained a similar result in the op$_4$.pa 'isolation' experiment (Fig. 5B; Table 1). Also, when either one or both of the P-type cells were ablated, no developmental regulation was detected among the surviving cells (Table 2). Thus, it appears that normal fate specification of the O-type tertiary blast cells does not require the continued presence of the other op-derived cells.

'Isolated' P-type cells display developmental regulation

As shown in Table 1, 'isolated' op$_4$.ap cells underwent regulation in a variable manner. Although the op.ap cell normally generates only one of the eight pattern elements scored in Table 1, when 'isolated' from the remainder of its clone, this cell can generate any of these pattern elements. Moreover, the frequency with which an 'isolated' op.ap cell generates a given pattern element ranged from 24-53%, and the exact subset of pattern elements varied from one experiment to the next. Because op$_4$.ap does not normally contribute any descendants to the ganglia of the ventral nerve cord, the frequent presence of op$_4$.ap descendants in the ganglion in these experiments (Fig. 6A) clearly indicates a significant transformation in the developmental fate of the 'isolated' op$_4$.ap cell.

An 'isolated' op$_4$.pp cell can likewise express a range of developmental fates in excess of its normal fate. In addition to certain of their normal fates, 'isolated' op$_4$.pp clones expressed a variable combination of O-fate elements and/or unidentifiable cell clusters that are not present in normal development (Fig. 6B; Table 1).

The regulative behavior of the 'isolated' P-type blast cells

Table 1. Developmental fates of 'isolated' tertiary blast cells in stage 9 embryos

Operation	CR*	LD2*	AD†	PV†	pz6/LD1‡	WE§	c.f.1§	c.f.3§	Unknown¶
(n=16)	**16** **100%**	**15** **92%**	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%	2 17%
(n=17)	6 35%	9 53%	9 53%	8 47%	**16** **94%**	4 24%	4 24%	8 47%	9 53%
(n=9)	0 0%	0 0%	**9** **100%**	**9** **100%**	0 0%	0 0%	0 0%	0 0%	0 0%
(n=16)	5 31%	7 44%	7 44%	7 44%	**16** **100%**	**16** **100%**	**15** **94%**	**15** **94%**	13 81%

*CR and LD2 are op.aa pattern elements in normal development.

†AD and PV are op.pa pattern elements in normal development.

‡pz6/LD1 are a pair of morphologically indistinguishable neurons produced by the op.ap blast cell and the op.pp blast cell, respectively, in normal development.

§WE, c.f.1 and c.f.3 are op.pp pattern elements in normal development.

¶'Unknown' refers to pattern elements that do not readily correlate to cell types seen in normal development.

The data shown in bold indicate that the pattern element in question is normally derived from the unablated blast cell.

Table 2. Developmental fate of op$_4$ clone following P-type blast cell ablation

Operation	CR*	LD2*	AD†	PV†	pz6/LD1‡	WE§	c.f.1§	c.f.3§
(n=4)	4 100%	4 100%	4 100%	4 100%	0 0%	0 0%	0 0%	0 0%
(n=15)	15 100%	15 100%	15 100%	15 100%	15** 100%	15 100%	15 100%	15 100%
(n=21)	21 100%	21 100%	21 100%	21 100%	21** 100%	0 0%	0 0%	0 0%

*Only one of the pz6/LD1 neuron pair was observed.
See Table 1 for additional explanation.

indicates that these cells have the potential to adopt a partial O fate in addition to their normal P fate. Given the mechanism of O/P fate specification in the midbody and caudal segments, these results were not expected. In those segments, the P lineage is largely unaffected by cell deletions in the O lineage, whereas cell deletions in the P lineage can produce partial or complete transfating of the O lineage to the P fate (Weisblat and Blair, 1984; Shankland and Weisblat, 1984). The degree of autonomy exhibited by O-type and P-type sublineages differs greatly in the OP lineage of the rostral segments compared with the O and P lineages of the midbody and caudal segments.
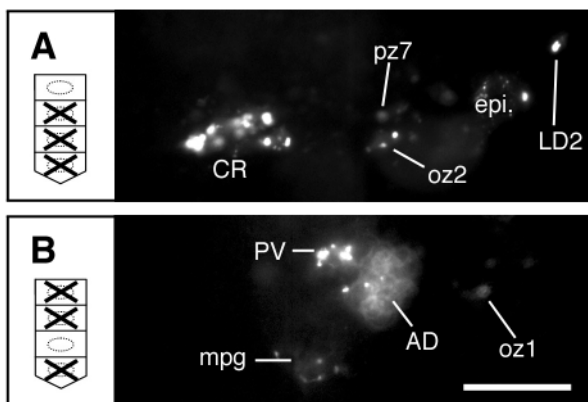
The role of cell-cell interactions between O-type and P-type cells in the fate specification of OP sublineages

The results from the 'isolation' experiments indicate that normal fate specification of a P-type blast cell in the OP lineage requires the presence of the other sublineages within the primary blast cell clone. This implies that interactions between the P-type cells and the other tertiary blast cells might be responsible for their fate restriction in normal development. Given the linear arrangement of alternating O-type and P-type cel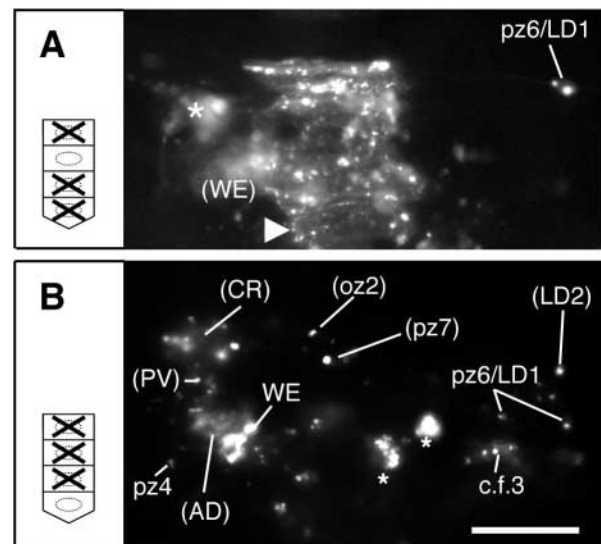ls in the op clone, it seems that such interactions might involve repressive signals originating from the adjacent O-type cells.

To investigate whether the O-type cells are responsible for restricting the developmental potential of P-type cells, the O-type cells op$_4$.aa and op$_4$.pa were deleted in unison, and the fate of the remaining two P-type cells was examined. We found that the two P-type cell clones together were capable of a nearly complete replacement of the missing O fate elements (Table 3).

We further examined the role of each of the two O-type cells in the development of the op clone by ablating them individually. In op$_4$.aa ablation experiments, we found that op.aa pattern elements were absent, whereas the other OP sublineage pattern elements were present, in all but one out of 19 cases (Table 3). The presence of a complete set of op.aa pattern elements in the one remaining embryo could have


Fig. 6. Developmental regulation in 'isolated' P-type cells. (A) An example of an 'isolated' op$_4$.ap cell clone. (B) An example of an 'isolated' op$_4$.pp blast cell clone. In both cases, the 'isolated' P-type cell gives rise to pattern elements that are normally derived from the ablated sublineages (marked by parentheses) and unidentifiable cell clusters (marked by asterisks). Note that 'isolated' P-type cells give rise to a variable combination of pattern elements (see Table 1), and differing combinations were seen in different embryos. Scale bar: 20 μm.

Fig. 5. 'Isolated' O-type cells show no sign of developmental regulation. (A) The 'isolated' op$_4$.aa cell consistently gives rise to its normal fate (Fig. 3A). (B) The 'isolated' op$_4$.pa cell consistently gives rise to its normal fate (Fig. 3F). Pattern element labels are given in Fig. 3. Scale bar: 20 μm.

Table 3. Developmental fate of op₄ clone following O-type blast cell ablation

| Operation	CR*	LD2*	AD†	PV†	pz6/LD1‡	WE§	c.f.1§	c.f.3§
(n=4)	4 100%	3 75%	4 100%	4 100%	**4** **100%**	**4** **100%**	**4** **100%**	**4** **100%**
(n=19)	1 5%	1 5%	**19** **100%**	**19** **100%**	**19** **100%**	**19** **100%**	**19** **100%**	**19** **100%**
(n=17)	**17** **100%**	**17** **100%**	7** 41%	8** 47%	**17** **100%**	**17** **100%**	**17** **100%**	**17** **100%**

*The outcome of these experiments varied with respect to bandlet slippage (see text).
See Table 1 for additional explanation.

resulted from a failure in cell ablation rather than from a regulation of cell identity. These results suggest that the op.pa cell is capable of restricting the op.ap cell to its normal fate in absence of the op.aa cell.

The role of the op.pa cell was investigated by its ablation. The outcome of this experiment depended upon a phenomenon known as 'bandlet slippage', a widening of the gap caused by the ablation of a blast such that the fragment of the bandlet posterior to the gap moves toward the posterior end of the embryo by an integral number of segments (Shankland, 1984). In embryos that displayed bandlet slippage following the ablation of op.pa, normal op.aa and op.ap pattern elements were located anterior to the gap, and seemingly normal op.pa and op.pp pattern elements were located posterior to the gap (Fig. 7A,B). Because the only uniquely labeled cell posterior to the gap was the op₄.pp cell, the op.pa pattern elements

observed in these experiments were almost certainly derived by regulation of the op₄.pp cell. They could not have arisen from the more posterior o or p blast cell lineages, as the latter cells were co-labeled with rhodamine and fluorescein (see Materials and methods). It should be noted that regulation by the op₄.pp cell never gave rise to either op.aa or op.ap pattern elements in any of these cases.

By contrast, in most of the op.pa-ablated embryos that did not display bandlet slippage, the op.pa pattern elements were missing, whereas the op.aa, op.ap and op.pp pattern elements remained intact (Fig. 7C,D). It appears that regulation by the op₄.pp cell generated the op.pa and op.pp fates when bandlet slippage caused it to lie at some distance away from the op.aa and op.ap cells; this developmental regulation usually failed to happen when this cell remained in its original close proximity to the op.aa and op.ap sublineages.
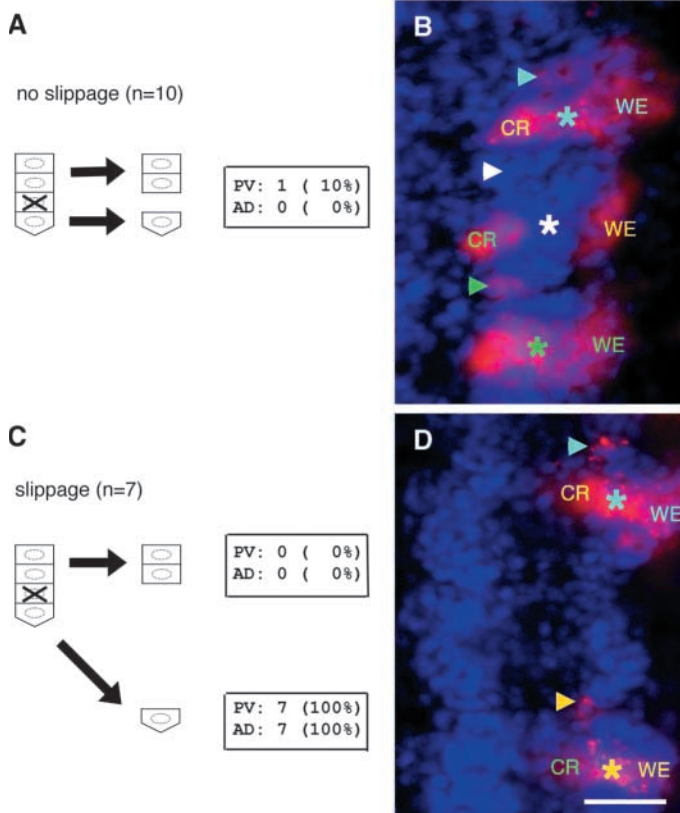
Discussion

To understand how serially homologous structures can arise from distinct developmental pathways, we investigated the role of cell-cell interactions between the O-type and P-type sublineages in the patterning of the rostral segments of the leech *H. robusta*, and compared those interactions with the O/P

Fig. 7. A correlation between bandlet slippage and developmental regulation in the op.pp lineage following ablation of the op.pa cell. The OP lineage was labeled with rhodamine lineage tracer and the op₄.pa cell ablated during stage 7. The operated embryos were fixed at stage 9 and counterstained with Hoechst 33258. (A,B) In those embryos in which bandlet slippage did not take place, the PV and AD clusters are usually missing from the op₄ clone. The normal sites of the missing op₄ pattern elements are marked by the white arrowhead and asterisk. (C,D) In embryos that experience slippage, a gap forms between the portion of the blast cell clone immediately anterior to the ablated cell and the portion immediately posterior to the ablated op₄.pa cell. Within the gap, no fluorescently labeled cells are observed, and the ganglia in the gap show a reduced size. A PV cluster (yellow arrowhead) and an AD cluster (yellow asterisk) are seen immediately posterior to the gap, and are therefore likely to be derived by regulation of the op₄.pp cell. Orientation of the specimen is the same as in Fig. 2. Yellow, op₄ descendants; light blue, op₃ descendants; green, the descendants of the anteriormost o and p blast cells. Arrowhead indicates PV cluster; asterisk indicates AD clusters. See Fig. 3 for labeling of additional pattern elements. Scale bar: 20 μm.

patterning mechanism seen in midbody segments of the leech. In the midbody, the presence of the P lineage promotes the O fate and represses the P fate in the adjacent O lineage (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Zackson, 1984; Huang and Weisblat, 1996). By contrast, the predominant interactions we observe in the OP lineage of more rostral segments seem to involve repressive signals that emanate from the O-type sublineages and restrict the fate of the P-type sublineages. This dissimilarity in the process of cell fate specification suggests an evolutionary diversification in the developmental pathways that generate the O/P cell fates in different segments of the leech, and hence a potential for dissociating developmental pathway and morphological outcome in the generation of those fates.

Distinct cell-cell interactions in the development of serially homologous cell lineages

In a midbody segment, the progenitors of the O and P pattern elements, i.e. a pair of o/p blast cells, originate from two separate O/P teloblasts. The evidence for interaction between the o/p blast cells comes from experiments in which one of the two O/P lineages is ablated: if the p bandlet is ablated, the neighboring o bandlet switches to the P fate, whereas if the o bandlet is ablated, the p bandlet retains its normal P fate. This is generally interpreted as meaning either: (1) that the p bandlet sends a signal that induces O fate in the o bandlet (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Zackson, 1984); or (2) that the p bandlet physically prevents a P-inducing signal produced by the q bandlet from reaching the o bandlet (Huang and Weisblat, 1996). Although these two models appear to be quite different from each other, they are not mutually exclusive. The p bandlet might play dual roles as an O-fate inducer, and also as a barrier to P-fate inducing signals from the q bandlet.

In a rostral segment, the O and P pattern elements homologous to those seen in the midbody segments arise from a single op blast cell, a daughter of the OP protoeloblast (Shankland, 1987c). Each of the four tertiary op blast cells is equivalent in fate to a set of one or two specific O and/or P sublineages. Based on their serial homology to the O and P sublineages in a midbody segment, and on their responses to ablation experiments, we have divided the op tertiary blast cells into two groups, namely O-type cells and P-type cells.

By ablating all but one of the four tertiary blast cells in an op blast cell clone, we found: (1) that fate specification of each of the two O-type cells appears to be independent of any other OP sublineage; and (2) that a P-type cell generally adopts both O and P fates when the rest of the OP sublineages are removed. Next, we deleted just the two O-type cells and found that the two surviving P-type cells compensated by producing O fates in addition to P fates, resulting in a nearly normal set of O and P pattern elements. Taken together, these data suggest that signals derived from the O-type cells repress O fate in the P-type cells of the rostral segments. By contrast, it is the P lineage that provides repressive signals to the O lineage in the midbody segments.

The rostral and midbody segments appear to differ not only in the directionality of the cell interactions but also in the kind of regulation. The O-to-P transformation reported in the O/P equivalence group of the midbody segments is replacement regulation; for example, a regulating cell abandons its normal

O fate and adopts a P fate instead (Shankland and Weisblat, 1984). By contrast, the developmental regulation seen in P-type cells of the op clone appears to be compensatory, i.e. the regulating cell actually expands upon its normal P fate to compensate, in part or in whole, for the loss of the O-type cells.

In addition to these functional differences in the cell-cell interactions involved in the specification of O and P fates, there is also a significant difference in the spatial orientation of such signals in rostral segments and midbody segments. In an op blast cell clone, the O-type and P-type cells are arranged linearly along the anteroposterior axis of the germinal band in an alternating pattern (Fig. 4A). As a result, the interactions between O-type cells and P-type cells would appear to take place along the anteroposterior axis in these segments. Our present results represent the first experimental demonstration of any fate-specifying interactions oriented along the anteroposterior axis between blast cells of the leech germinal band (see Seaver and Shankland, 2000; Seaver and Shankland, 2001; Shain et al., 2000). By contrast, the cell-cell interactions in the O/P equivalence group of the midbody segments take place along the dorsoventral axis. The repressive interaction between the O-type and P-type cells in the rostral segments is not only reversed with respect to the O and P fates, but also manifests a 90° rotation from the dorsoventral axis to the anteroposterior axis.

Mechanisms for OP sublineage patterning

We have shown that normal fate specification of P-type cells depends on an O-fate repressing signal from the O-type cells of that same clone. We further examined this phenomenon by deleting single O-type cells. It should be noted that a P-type cell is normally in contact with one anterior and one posterior O-type cell. In one experiment, it was shown that the ablation of the op.aa cell has no effect on normal fate specification of the P-type cells in that same op clone (including its immediate neighbor op.ap) when the more posterior O-type cell, op.pa, is still present (Table 3). Similarly, developmental regulation was never detected for the op.ap cell when its posterior neighbor, op.pa, was ablated and its anterior neighbor, op.aa, left intact. It appears that the interactions between op.ap and its two O-type neighbors are redundant, and that a signal from either O-type cell is sufficient to restrict op.ap to its normal fate.

Our data suggest that proper specification of the other P-type cell, op.pp, is somewhat more complicated. We have shown that an 'isolated' op.pp cell regulates when the other members of the op blast cell clone are ablated. But we do not know whether op.pp regulates when only the O-type cells op.aa and op.pa are ablated. In the latter experiment, it could be that both op.ap and op.pp regulate, or that one of them regulates while the other one does not. If both op.pp and op.ap undergo regulation in response to the ablation of the two O-type cells, one might conclude that interaction with an O-type cell is the only factor required for proper fate specification of op.pp. But if op.pp does not regulate when both O-type cells are ablated, it would suggest that an interaction with the other P-type cell, op.ap, is sufficient to specify the normal fate of the op.pp cell. Although the ablation of the P-type cell op.ap alone does not result in developmental regulation, it remains possible that op.ap acts together with the O-type cells to redundantly specify the normal fate of op.pp.

Regardless of the exact source of the signal(s) that specify

the normal fate of op.pp, the correlation between bandlet slippage and the developmental regulation of the op.pp progeny following the ablation of op.pa (Fig. 7) suggests a position-dependent mechanism of cell interaction. In these experiments, the op.pp cell rarely exhibited developmental regulation unless separated from the remaining OP sublineages by a distance of more than one segment. It appears that the remaining OP sublineages can direct the proper fate specification of the op.pp cell over a short distance, possibly by a diffusible signal or by cell contact through filopodial extensions, as seen in *Drosophila* imaginal discs (Ramírez-Weber and Kornberg, 1999), but not over longer distances. In any case, one important conclusion to be drawn from these slippage experiments is that contact of the op_4.pp cell with the anteriormost o and p blast cell clones (which persist in these experiments) is not sufficient for its normal specification. Hence, the o and p blast cells (and their progeny) do not seem to generate the same repressive signals that emanate from the O-type cells within the op_4 clone.

It should be noted that our ablation approach has certain limits in detecting cell interactions. For instance, some interactions may occur so soon after the cell is born that they can not be disrupted by its ablation (see Goldstein, 1992). Thus it remains possible that the O-type cells are initially subject to developmental regulation, and that some early signal specifies O-type cells before we can ablate their neighboring cells. The experiments presented here are also limited with respect to resolving the mechanisms that establish individual cell identity among the P-type cells. Although a regulating P-type cell produces a small and highly variable set of O pattern elements that it does not normally give rise to, it nonetheless always gives rise to a complete set of P pattern elements that represent its normal fate. Thus, a cell lineage-dependent mechanism may already have been involved in the partial specification of the P-type cell prior to its repressive interaction with the O-type cells. It remains possible that a set of earlier cell interactions or some cell-autonomous mechanisms are involved in the creation of this lineage-dependent tendency.

In addition to their limitation in detecting all of the cell interactions within the op clone, the experiments presented here do not directly examine the possible role of signals emanating from other teloblast lineages. In the O/P equivalence group of the midbody segment, interaction of the p bandlet with the q bandlet is required for normal fate specification (Huang and Weisblat, 1996). By contrast, the linear arrangement of tertiary op blast cells insures that both O-type cells and P-type cells are in contact with the q bandlet, and makes it rather unlikely that mere contact with the q bandlet directly dictates the choice of O versus P fates in the OP sublineages. However, the q bandlet could still be involved through some other mechanism, such as differential responsiveness of the O-type and P-type cells to signals from the q bandlet. These possibilities will be the subject of a separate study (D.-H.K. and M.S., unpublished).

Evolutionary dissociation of serial homology and developmental pathway

Oligochaetes and leeches share a similar pattern of early development (reviewed by Anderson, 1973). Intracellular lineage tracer injection analysis of an oligochaete, *Tubifex*, revealed a strikingly similar cell lineage pattern to that of the

leech *Helobdella* (Goto et al., 1999). But the O/P patterning mechanism in *Tubifex* is quite different from that of the leech (Arai et al., 2001). Despite an evolutionarily conserved morphology (e.g. cleavage pattern, germinal band formation and fate map), it appears that the developmental pathway underlying O/P patterning can be dissociated from its morphological end product, and has diverged significantly since the last common ancestor of the sludge worm *Tubifex* and the leech *Helobdella*.

In this study, we have shown that a serially homologous morphological pattern in the rostral and midbody segments of the leech is generated by distinct patterns of cell lineage and cell interaction. The evolution of distinct developmental pathways in the rostral and midbody segments requires a reorganization of developmental pathways conceptually similar to that seen in the divergence of O/P fate specification pathways among annelid species.

The evolutionary dissociation of developmental pathway and the outcome of development may be attributed in part to the modularly organized hierarchy of developmental pathways (Wagner and Altenberg, 1996; Kirschner and Gerhart, 1998; Eizinger et al., 1999; von Dassow and Munro, 1999; Mann and Carroll, 2002). Although our data are not molecular, the serial homology of differentiated descendant cells between the OP lineage, and the O and P lineages, suggests that homologous descendants may be specified by the same set of cell identity selector genes. It might be the case that the upstream pathways that regulate the expression of the selector gene are divergent in the rostral and midbody segments, whereas the downstream pathways of selector gene expression remain uniform in every segment.

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