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Establishment of segment polarity in the ectoderm of the leech *Helobdella*

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SUMMARY

The segmented ectoderm and mesoderm of the leech arise via a stereotyped cell lineage from embryonic stem cells called teloblasts. Each teloblast gives rise to a column of primary blast cell daughters, and the blast cells generate descendant clones that serve as the segmental repeats of their particular teloblast lineage. We have examined the mechanism by which the leech primary blast cell clones acquire segment polarity – i.e. a fixed sequence of positional values ordered along the anteroposterior axis of the segmental repeat. In the O and P teloblast lineages, the earliest divisions of the primary blast cell segregate anterior and posterior cell fates along the anteroposterior axis. Using a laser microbeam, we ablated single cells from both o and p blast cell clones at stages when the clone was two to four cells in length. The developmental fate of the remaining cells was characterized with rhodamine-dextran lineage tracer. Twelve different progeny cells were ablated, and in every case the ablation eliminated the normal descendants of the ablated cell while having little or no detectable effect on the developmental fate of the remaining cells. This included experiments in which we specifically ablated those blast cell progeny that are known to express the *engrailed* gene, or their lineal precursors. These findings confirm and extend a previous study by showing that the establishment of segment polarity in the leech ectoderm is largely independent of cell interactions conveyed along the anteroposterior axis. Both intercellular signaling and *engrailed* expression play an important role in the segment polarity specification of the *Drosophila* embryo, and our findings suggest that there may be little or no conservation of this developmental mechanism between those two organisms.

Key words: Segmentation, Cell lineage, Leech

INTRODUCTION

Segmentation of the anteroposterior (AP) body axis is a structural motif shared by a number of animal phyla (Brusca and Brusca, 1990). Some authors argue that the last common ancestor of bilaterian animals was segmented, and hence that the developmental mechanism underlying segmentation is homologous between different segmented phyla (De Robertis, 1997; Kimmel, 1996). But others propose that segmentation originated multiple times within specific bilaterian clades (Patel et al., 1989a; Brusca and Brusca, 1990), a scenario that more readily explains the disparate location of the segmented taxa within the metazoan tree (Aguinaldo et al., 1997; de Rosa et al., 1999). One reason for this uncertainty is that the developmental mechanisms that generate body axis segmentation are poorly understood outside of a few model systems, most notably the fruitfly *Drosophila*. Much less is known about the mechanistic basis of segmentation in other arthropods or vertebrates, and even less in the annelids. To address the latter deficiency, we have investigated the sequence of cellular events that give rise to segment polarity in an annelid, the leech *Helobdella robusta*.

The *Helobdella* embryo generates its segmented ectoderm and mesoderm from a bilaterally symmetric set of embryonic stem cells called teloblasts (for an overview, see Shankland and Savage, 1997). Each of the five teloblasts undergoes an iterated sequence of highly asymmetric cell divisions, and produces a linear column of much smaller primary blast cell daughters. The five ipsilateral columns of blast cells come together in parallel to form a germinal band, and the right and left bands then fuse to produce a bilaterally symmetric germinal plate that differentiates into the 32 body segments of the adult leech.

Primary blast cells function as segmental founder cells for their respective teloblast lineages. The mesodermal teloblast M and two of the four ectodermal teloblasts, O and P, each generate a single class of primary blast cell that is designated by the same letter in lower case, i.e. m, o and p. The O and P teloblasts are equivalent in developmental potential (Weisblat and Blair, 1984), but their blast cell daughters become committed to distinct O or P developmental pathways in accordance with dorsoventral positioning in the germinal band (Shankland and Weisblat, 1984; Huang and Weisblat, 1996). Within each of these three lineages, all of the primary blast cells undergo a very similar and teloblast-specific sequence of development (Zackson, 1984) to produce descendant clones that serve as segmental repeats (Shankland, 1987a; Shankland, 1987b). The two remaining ectodermal teloblasts, N and Q, develop in much the same fashion, except that they produce

alternating pairs of primary blast cells that give rise to distinct anterior and posterior halves of their segmental repeats (Zackson, 1984; Weisblat and Shankland, 1985).

We have investigated the process of segment polarity specification in primary blast cell clones of the O and P teloblast lineages. By analogy with *Drosophila*, we use the term 'segment polarity' to refer to the establishment of subsegmental positional values along the AP axis of the repeat (Nüsslein-Volhard and Wieschaus, 1980). In the leech embryo, segment polarity is first evident when the primary blast cells initiate their subsidiary divisions. For example, a primary p blast cell undergoes two rounds of division parallel to the AP axis to generate grand-daughter cells p.aa, p.ap, p.pa and p.pp (Fig. 1C), thereafter switching to transverse divisions (Shankland, 1987b). A single p blast cell clone will eventually give rise to about 70 differentiated descendants (Shankland and Weisblat, 1984), and the relative AP positions of the four grand-daughter cells in the germinal band predicts the overall AP disposition of their descendants within the differentiated blast cell clone (Fig. 1D). It is not currently known how the anterior and posterior blast cell progeny acquire their differing fates, but the specification of those differences is a crucial step in establishing the segment polarity of each individual primary blast cell clone.

The primary o blast cell clone develops in much the same way as the p blast cell clone, although it differs with respect to the details of cleavage pattern (Shankland, 1987a) and the exact set of descendants produced (Shankland and Weisblat, 1984). The earliest o blast cell divisions are also parallel to the AP axis (Fig. 1A), and its progeny cells o.aa, o.apa, and o.app are arrayed within the germinal band in an order that predicts the AP disposition of their differentiated descendants (Fig. 1B). Progeny cell o.p has proven too small for lineage tracer injection (Shankland, 1987a), and its relationship to segment polarity remains unclear.

There is some evidence that the leech *Helobdella* employs a genetic mechanism of segment polarity specification similar to that found in the fruitfly *Drosophila*. In the fly embryo, zygotic expression of the *engrailed* (*en*) gene occurs at the cellular blastoderm stage in transverse stripes that precisely demarcate the future segments (DiNardo et al., 1988). Blastoderm cells that express the en transcription factor are specified to take on the most posterior positional value within each segment, and they also initiate a sequence of intercellular signals that – both directly (Heemskerk and DiNardo, 1994) and indirectly (Lawrence et al., 1996) – specify the positional value of cells throughout the remainder of the segment's length.

An ortholog of the *en* gene has been cloned and characterized in the closely related leech species *H. triserialis* (Wedeen and Weisblat, 1991). The leech *en* gene is expressed in all five teloblast lineages (Lans et al., 1993), and its expression is restricted to specific blast cell progeny. In the P lineage, expression of *en* is restricted to cell p.ap and its immediate descendants during the time of segment formation (Fig. 1C). In the O lineage, expression of *en* is restricted to cell o.aap and its immediate descendants (Fig. 1A). The cells that express *en* are arrayed in a transverse stripe that spans the DV axis of the germinal band (Lans et al., 1993), a finding that has led several authors to suggest that the segmental function of the *en* gene – and by extension, the entire process of segmentation – is homologous between arthropods such as the fly and annelids such as the leech (Wedeen and Weisblat, 1991; Shankland, 1994; Ramirez et al., 1995). However, at least some aspects of leech segmentation are not under the direct control of *en* expression, as *en*-expressing cells appear to play little or no role in the formation of intersegmental fissures in the nervous system of the leech *Theromyzon rude* (Shain et al., 2000).

In this paper we more closely examine the developmental mechanism that specifies segment polarity in embryos of the leech *H. robusta*. In contrast to many other segmented animals, the leech embryo displays a lineal stereotypy that allows us to uniquely identify the individual founder cells that comprise a nascent segment, and to precisely characterize the descendant fate of those cells in both normal (Shankland, 1987a; Shankland, 1987b) and experimentally manipulated embryos. We here use a laser microbeam to ablate various blast cell progeny from either the O or the P teloblast lineage, and find no evidence that the primary blast cell clone requires cell interactions along its AP axis in order to specify anterior and posterior cell fates. In particular, our results show that the en-expressing cells do not serve as a source of required intercellular signals for the normal patterning of the remainder of the segmental repeat. Thus, the developmental mechanism that establishes and maintains segment polarity in the leech embryo appears to be quite different from that characterized in fruitflies, a conclusion that raises doubt as to whether the annelids and arthropods could have inherited their segmentation mechanisms from a segmented common ancestor.

MATERIALS AND METHODS

Animals

H. robusta embryos were taken from a breeding colony founded with individuals collected from the mouth of Shoal Creek in Austin, Texas. *H. robusta* is closely related to *H. triserialis*, and displays essentially identical patterns of cell lineage and morphological development (Shankland et al., 1992; Huang and Weisblat, 1996; Seaver and Shankland, 2000). Leech care and embryonic staging are described by Stent et al. (Stent et al., 1992).

The *H. triserialis* used for ablations of cell o.aa were kindly provided by Matt Kourakis and Mark Martindale (University of Chicago).

Laser cell ablation

Single cells were ablated from the germinal band using a focused 440 nm laser microbeam as outlined in Fig. 2. A single O/P teloblast was pressure injected with a solution containing 50 mg/ml tetramethylrhodamine-dextran-amine (Molecular Probes) and 20 mg/ml Fast Green FCF (Sigma) in 0.2 M KCl. The embryo was then raised to a stage at which the oldest of the rhodamine-labeled primary blast cells had initiated its subsidiary divisions. Primary blast cell progeny were identified under the microscope by size, shape and position, and a single cell ablated with a single laser pulse (Seaver and Shankland, 2000). The beam was aligned to ocular crosshairs prior to ablation, and to ensure against loss of alignment we used the crosshairs to target a visible granule of Fast Green in the cell selected for ablation. We only scored experimental embryos in which laser irradiation caused the targeted granule to disappear. In most cases the laser pulse also resulted in sudden cytoplasmic displacements, an immediate loss of membrane integrity, and/or a rapid condensation of chromatin.

To minimize the potential for cell interaction, we routinely ablated target cells 0-60 minutes after they completed the preceding cell division. In a substantial fraction of experiments we followed the preceding cell division under the microscope, and then irradiated one of the two daughter cells as it began to lose the rounded shape of telophase and take on the rectangular profile seen at interphase. Laser pulses prior to this shape change destroyed both the target cell and its sibling.

At the time of ablation we noted the position of the operated blast cell clone relative to the anterior boundary of rhodamine-labeling, and used this information to locate the operated clone at later stages. It should be noted that successive primary blast cell clones overlap to some degree at their anterior/posterior borders (Weisblat and Shankland, 1985), and to minimize any ambiguities resulting from this overlap, we restricted our ablation analysis to the anteriormost labeled clone for cells o.aa, p.a, p.aa and p.ap. All experimental embryos were raised to stage 9, fixed with formaldehyde, counterstained with the nuclear dye Hoechst 33258, and dissected before being mounted for fluorescence microscopy (Seaver and Shankland, 2000).

Identification and statistical analysis of descendant pattern elements

Despite the stereotypy of leech development, we could not reliably identify all of the known pattern elements in every rhodamine-labeled blast cell clone. A pattern element was only scored as present if it could be unambiguously distinguished from other labeled cells by morphology and location. The frequency of unambiguous identification varied for different pattern elements, and was affected by the intensity of labeling and quality of dissection. To control for these variables we scored the cellular composition of a second labeled clone – situated two segments posterior to the site of ablation – in each experimental embryo.

To determine whether any of our ablations altered cell fates in the remainder of the blast cell clone, we used the χ^2 test to compare the set of rhodamine-labeled descendants in the operated clone with the predictions of a null hypothesis in which (1) the ablated sublineage gives rise to no descendants and (2) the unablated sublineages develop normally. The expectations of this null hypothesis were corrected to reflect the likelihood of identifying different pattern elements in the control data set from that same experiment. Ambiguous pattern elements (see Results) were not included in statistical analyses.

Blast cell injection

To ascertain the developmental fate of cell o.ap in *H. robusta*, we iontophoretically injected this cell with tetramethylrhodamine-dextran-amine as previously described (Shankland, 1987a).

RESULTS

To ascertain whether cell interactions are required to establish the segment polarity of primary blast cell clones, we used a laser microbeam to eliminate single cells and followed the developmental fate of the remainder of the clone with rhodamine-dextran lineage tracer. The relative timing and geometry of both o and p blast cell divisions were identical to previous descriptions from the closely related species *H. triserialis* (Fig. 1A,C). In most cases the ablation resulted in a clean gap in the pattern of labeled tissues observed at stage 9, but in a minority of cases the gap included fluorescent debris believed to be residue of the ablated cell. Both our present results and a previous study (Seaver and Shankland, 2000) indicate that the laser ablation procedure causes little or no damage of adjacent cells as judged by the cellular composition of their descendant clones.

As a framework to discuss the experimental results, we will first overview the normal blast cell fates. These overviews are consistent with previous descriptions (Shankland and Weisblat, 1984; Shankland, 1987a; Shankland, 1987b), but here we focus on the segment-by-segment reliability of identifying each individual pattern element. Pattern elements were only scored as present in these experiments if they could be unambiguously distinguished from other surrounding labeled cells on the basis of position and morphology.

Normal fate of the O lineage

Fig. 1B depicts a two-dimensional projection of an o blast cell clone from a stage 9 embryo with the various sublineages shown in color. The primary blast cell clone contains approximately 70 cells at this stage (Shankland and Weisblat, 1984), and straddles the boundary between two consecutive segments. In the present paper we scored a total of nine discrete pattern elements within each o blast cell clone. All but one of these elements could be unambiguously identified in >90% of the 56 rhodamine-labeled control clones.

Central nervous system

We scored four discrete O pattern elements within the segmental ganglia of the central nervous system (Fig. 1B). A large anterodorsal (AD) neuron cluster and a much smaller posteroventral (PV) neuron cluster are easily identified by distinctive positions within their respective ganglia. The crescent (CR) cluster of central neurons is also found in a distinct and reliable location, but is typically composed of an irregular grouping of smaller clusters that interdigitate with unlabeled cells. Finally, the o blast cell gives rise to two ‘packet’ glia/ganglion (Kramer and Weisblat, 1985), and one of these – the medial packet glia (mpg) – was visible in 89% of control clones as a large, faintly labeled stellate cell separated from the O-derived neurons.

Peripheral nervous system

The o blast cell clone generates three subepidermal neurons situated at distinctive locations within the body wall (Fig. 1B). The oz2 and LD2 neurons are located respectively at medial and lateral positions within the most posterior or ‘PP’ peripheral nerve arising from the segmental ganglion. The oz1 neuron from that same blast cell clone is located at a medial position in the most anterior or ‘AA’ peripheral nerve arising from the next posterior ganglion.

Squamous epidermis

An o blast cell clone contributes two discrete patches to the squamous epidermis that covers most of the body surface (Figs 1B, 3A). The larger patch of O-derived epidermis forms a mediolateral swath aligned with the posterior half of the ganglion. The o.aa sublineage gives rise to a single squamous epidermal cell located just medial of the LD2 neuron, but we did not routinely score this cell as it was often difficult to identify at the boundary between two labeled blast cell clones.

Cell floret 2/nephridial tubule

The o blast cell clone contributes two cuboidal cells to a ventrolateral epidermal specialization called cell floret 2 (cf2),

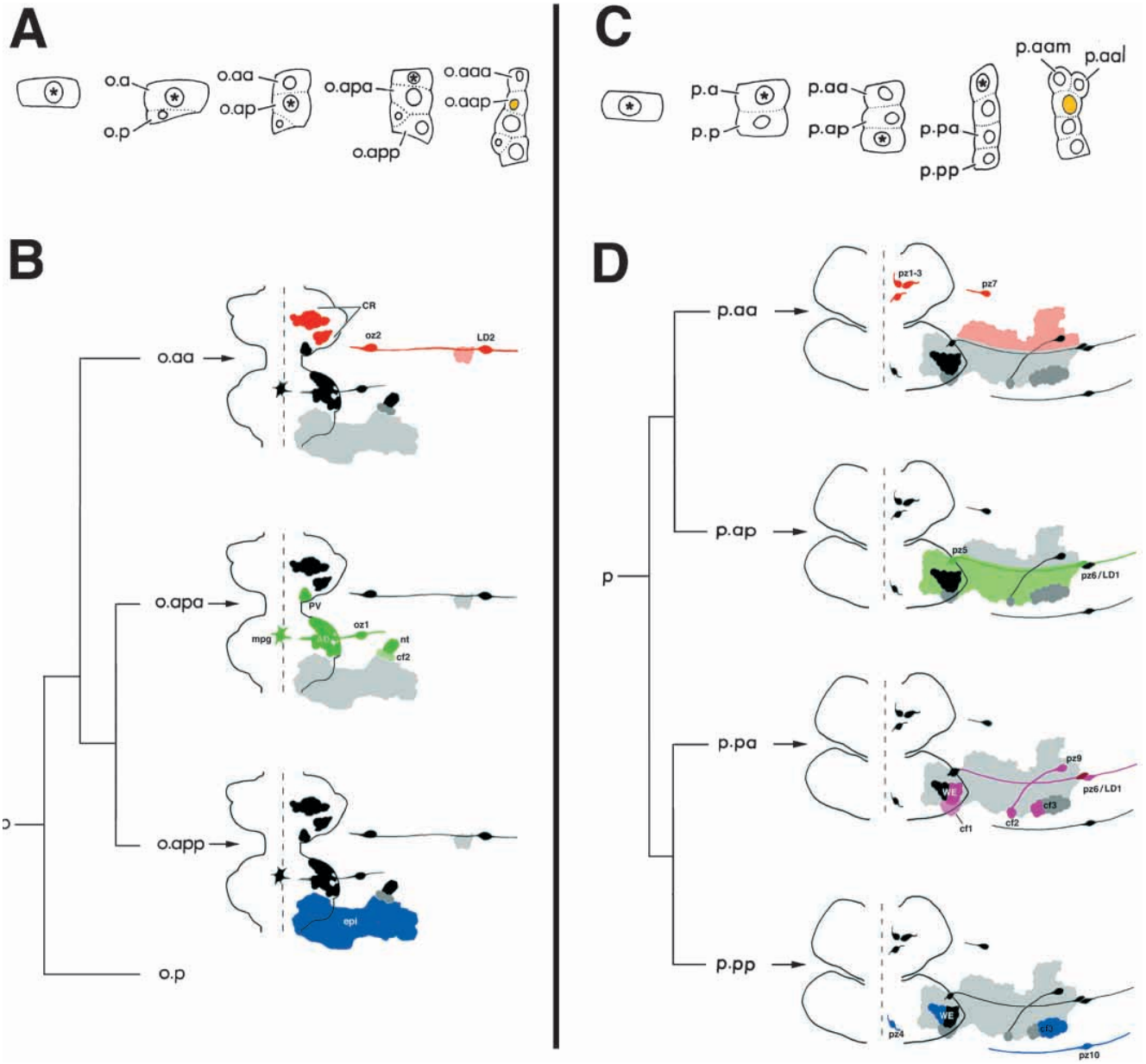

Fig. 1. Primary blast cell clones serve as segmental repeats in the O and P lineages of the leech embryo. Anterior is towards the top. (A) The first four divisions of the primary o blast cell (shown in temporal sequence from left to right) yield an elongate clone five cells in length. Asterisks mark the nucleus of the next cell to undergo division; daughters of each division are designated by adding the letter 'a' (anterior) or 'p' (posterior) to the parent cell's name. The pattern of *en* expression is shown in yellow (Lans et al., 1993). (B) By embryonic stage 9, a primary o blast cell clone (right side of embryo) gives rise to a set of differentiated descendants straddling the boundary between two successive segments. Segmental ganglia are shown in outline, and the ventral midline marked by a broken line. The same blast cell clone is depicted in triplicate with each of three different sublineages in color. Note that the AP position of these sublineages in the blast cell division pattern predicts the relative positioning of their descendants in the differentiated clone. Uniquely identifiable O pattern elements include three clusters of central neurons (AD, PV, CR), three peripheral neurons (oz1, oz2, LD2), the medial packet glial cell (mpg), cell floret 2 (cf2), the nephridial tubule (nt) and a large patch of squamous epidermis (epi). Axons are only shown for some of the neurons. Cell o.p does not give rise to any uniquely identifiable pattern elements, but its exact fate is unknown (Shankland, 1987a; see text). (C) The first three divisions of a primary p blast cell clone yield an elongate clone four cells in length; the fourth division is transverse. The pattern of *en* expression is shown in yellow (Lans et al., 1993). (D) By embryonic stage 9, a primary p blast cell clone gives rise to a set of differentiated descendants straddling the boundary between two successive segments. The same blast cell clone is depicted in quadruplicate with each of the four different sublineages in color. Note that the AP position of these sublineages in the blast cell division pattern predicts the relative positioning of their descendants in the differentiated clone. Uniquely identifiable P pattern elements include two clusters of central neurons (WE, pz1-3), another isolated central neuron (pz4), six peripheral neurons (pz5-10, LD1), cell florets 1-3 (cf1-cf3), and a large patch of squamous epidermis (epi). Axons are shown for only some neurons. The pz6 and LD1 neurons are morphologically indistinguishable. The ancestry of the pz4 neuron is drawn from the findings of this paper. Drawings are adapted from Shankland (Shankland, 1987a; Shankland, 1987b) and Seaver and Shankland (Seaver and Shankland, 2000).

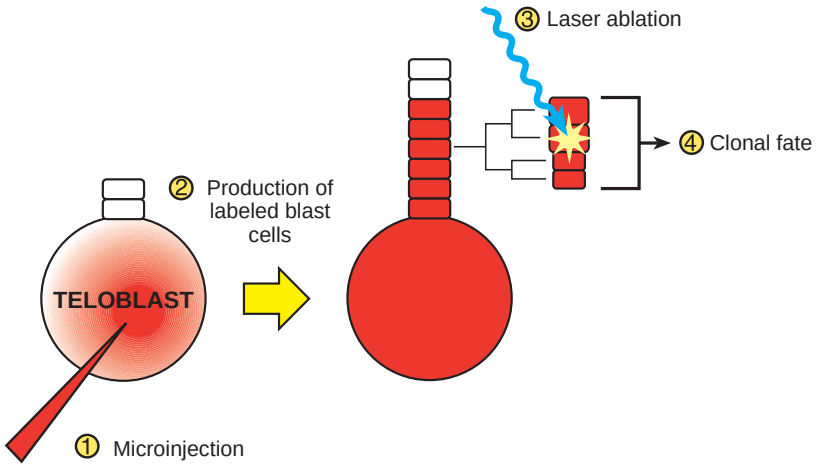

Fig. 2. Experimental paradigm. An O/P teloblast was injected with rhodamine-dextran lineage tracer (1) at embryonic stage 7. The injected teloblast generated a chain of rhodamine-labeled primary blast cell daughters (2). The primary blast cells underwent a stereotyped sequence of cell divisions, and one of the labeled progeny cells subjected to laser ablation (3). The embryo was raised to embryonic stage 9, and the lesioned blast cell clone examined to ascertain the developmental fate of the unablated cells (4).

plus a single cell immediately beneath the floret that forms the distalmost element of the nephridial tubule (nt). The cf2 cells are contiguous with the squamous epidermis, but can usually be distinguished by their rounded shape and smaller nuclei. The nt cell undergoes a programmed cell death in some body segments (Martindale and Shankland, 1988), so we scored these two structures as a single pattern element.

Ablation of primary o blast cell daughters

In one series of experiments we used the laser microbeam to ablate either the anterior or posterior daughter of the primary o blast cell. As portrayed in Fig. 1A, the first o blast cell division produces (1) a large anterior daughter, cell o.a, that contributes all of the O pattern elements described above and (2) a much smaller posterior daughter, cell o.p, whose fate is unknown but which has no uniquely identifiable descendants (Shankland, 1987a). In the absence of cell fate regulation, this fate map suggests that ablation of cell o.a should give a result similar to the ablation of its primary blast cell parent.

We ablated cell o.a in a total of 12 embryos, and for the most part our results were in keeping with this prediction. In six embryos we ablated a single o.a cell from a chain of labeled blast cell clones, producing a gap in the pattern of labeled

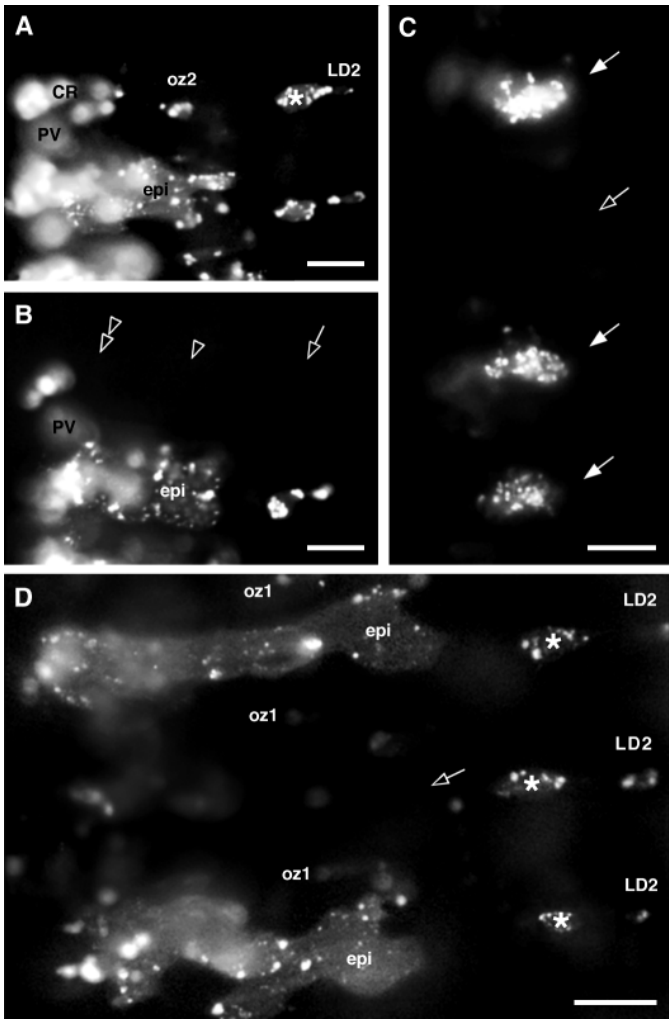

Fig. 3. Fluorescence micrographs showing the absence of O pattern elements in stage 9 embryos that were subjected to laser ablation of identified o blast cell progeny. Anterior is towards the top. (A) Anterior boundary of fluorescently labeled tissues resulting from rhodamine-dextran injection of the right O teloblast in an otherwise normal embryo. A number of O pattern elements are marked in the most anterior labeled blast cell clone according to Fig. 1; the small lateral patch of epidermis is marked with an asterisk. At this epidermal focal plane the CR and PV neuron clusters are out of focus and the AD neuron cluster is completely obscured. (B) Anterior boundary of fluorescently labeled tissues in an embryo in which cell o.aa was ablated from the most anterior labeled clone. Note that the anterior clone is lacking neuron LD2 and the small epidermal patch (arrow), neuron oz2 (single arrowhead), and most of the CR neuron cluster (double arrowhead). Cell o.ap gives rise to the PV neuron cluster and the large patch of epidermis (epi), both of which have formed normally. (C) Cell o.apa was ablated in one of four consecutive segments, resulting in the loss of neuron cluster AD (open arrow). Solid arrows mark labeled AD neuron clusters in unlesioned adjoining segments. Other labeled O pattern elements are outside the plane of focus. (D) Laser ablation of cell o.app results in a selective loss of its only descendant, the large medial patch of squamous epidermis (open arrow). Large patches of labeled epidermis (epi) are present in the next anterior and posterior clone. Peripheral neurons oz1 and LD2 as well as the smaller patch of lateral epidermis (asterisk) are present in all three segments. Note the lateral expansion of the epidermis and peripheral neurons in this stage 10 embryo compared to the stage 9 embryos shown in A,B. Scale bars: 20 μm.

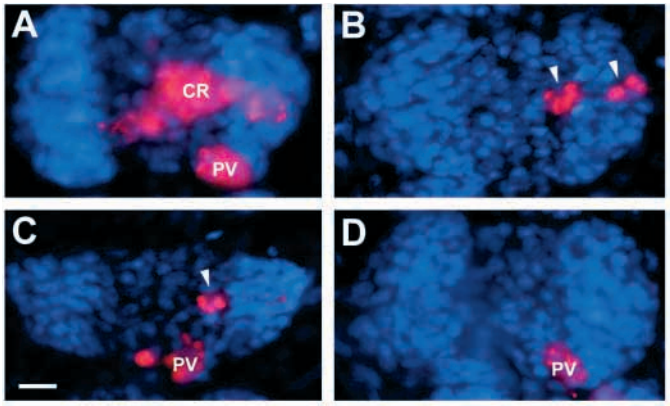

Fig. 4. Formation of the CR (crescent) neuron cluster in normal and partially ablated o blast cell clones. (A-C) The anteriormost labeled segmental ganglion from dissected stage 9-10 embryos in which the right O teloblast lineage had been labeled by injection of rhodamine-dextran. All preparations are counterstained with the blue-fluorescent DNA stain Hoechst 33258, and shown with anterior to the top. (A) In unoperated embryos, the labeled o blast cell clone gives rise to a large, irregular CR neuron cluster across the middle of the ganglion and a smaller PV (posteroventral) neuron cluster at the posterior edge of the ganglion. The AD cluster from this blast cell is located in the next posterior ganglion (not shown). (B) Laser ablation of the anterior daughter of the primary blast cell (cell o.a) eliminates the PV cluster and most of the CR cluster. But in three out of six cases we observed one or two labeled neurons (arrowheads) persisting in the region of the CR cluster. (C) Laser ablation of the anteriormost grand-daughter of the o blast cell (cell o.aa) eliminates most of the CR cluster, but in 13 out of 16 such ablations we observed one or two labeled neurons (arrowhead) persisting in the location of the CR cluster. Ablation of cell o.aa has no effect on the formation of the PV neuron cluster. (D) Rhodamine-dextran injection of cell o.ap – sibling of cell o.aa – labels the PV cluster and AD cluster (not shown), but in all cases failed to label neurons of the CR cluster. Scale bar: 10 μm.

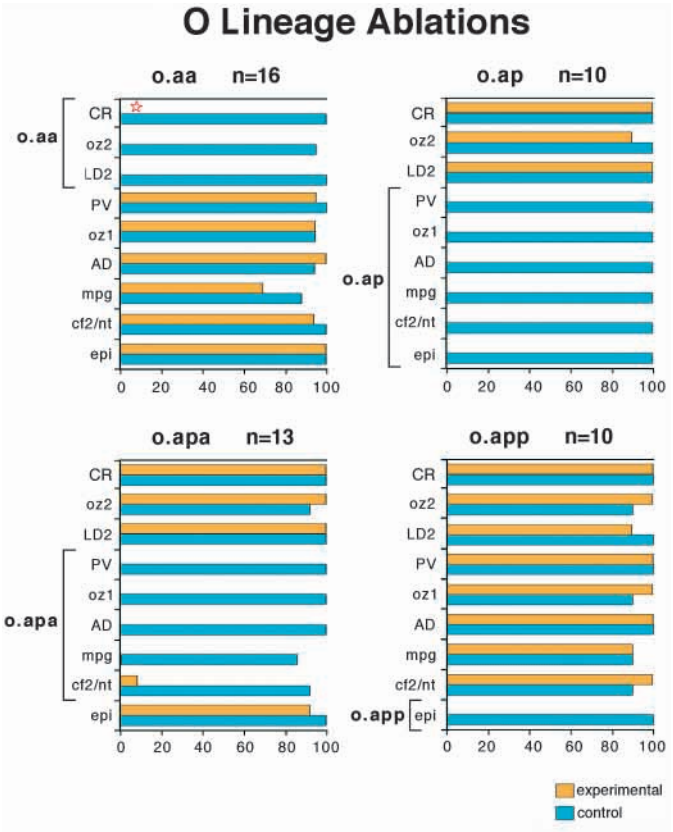

Fig. 5. Ablation of single o blast cell progeny has little or no effect on the developmental fate of the remainder of the blast cell clone. Histograms show the frequency at which differentiated pattern elements (see text) were identified as present in primary o blast cell clones from which one specific progeny cell had been ablated. In all four experiments, comparison of experimental clones (orange) and unoperated control clones (blue) reveals no significant change in the set of descendants produced by the unablated portion of the clone (χ^2 test, $P>0.9$). Brackets demarcate the subset of pattern elements that would normally arise from the ablated cell (Shankland, 1987a), and those elements were consistently missing from the experimental clones. The CR cluster of central neurons is usually derived from cell o.aa, but one or two isolated CR neurons were consistently produced by the remainder of the blast cell clone when cell o.aa was ablated (orange star). See text for details.

descendants. The unlesioned clones immediately anterior and posterior to that gap contained essentially normal sets of O pattern elements, as was seen following ablations of primary o blast cells (Seaver and Shankland, 2000). In the remaining six embryos, we ablated cell o.a from the anteriormost rhodamine-labeled clone (descended from the first labeled daughter of the injected teloblast), and thus could not assay the fate of more anterior clones nor see a visible gap. However, in three of these embryos we observed one (n=1) or two (n=2) rhodamine-labeled cell bodies in the ganglion expected to house the anterior half of the operated blast cell clone (Fig. 4B). The cell(s) in question appeared to be neuronal, and were located near the middle of the ganglion in the normal location of the CR cluster (Fig. 4A). These anomalous CR neurons could be descendants of the sibling of the ablated cell, o.p, whose normal fate is unknown. Otherwise, they must have arisen abnormally from one of the more posterior blast cell clones.

To ascertain whether the o.p cell plays a role in the specification of its sibling cell o.a, we ablated cell o.p in seven embryos, employing the same ablation criteria used for other cells. As the fate of cell o.p is uncertain, we were unable to determine whether its own normal descendants were missing. More importantly, ablation of the o.p cell had no detectable effect on the developmental fate of the sibling o.a cell, which gave rise to a seemingly normal set of O pattern elements (not shown).

Ablations within the o.a sublineage

To further investigate cell fate specification within the O lineage, we used the laser microbeam to individually ablate various progeny cells within the o.a sublineage, specifically progeny cells o.aa, o.ap, o.apa and o.app (Fig. 1A). Individual ablation results are shown in Fig. 3, and the results of all four ablations are summarized in Fig. 5. Each of these four experiments gave a conceptually similar result. First, the operated blast cell clones consistently lacked those pattern elements that would normally have arisen from the ablated precursor (Figs 3B-D, 5). Of 49 experimental clones examined, there was only one case in which the remainder of the clone appeared to have compensated for a pattern element whose normal progenitor had been ablated. In that one embryo,

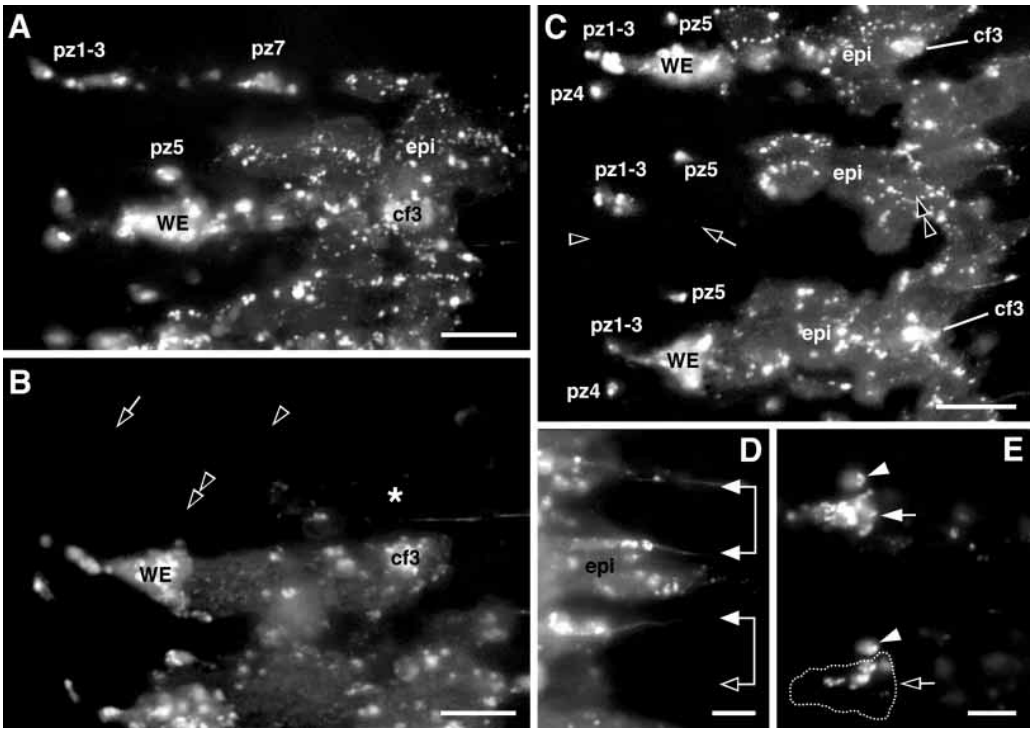

Fig. 6. Fluorescence micrographs showing the absence of P pattern elements in stage 9 embryos that were subjected to laser ablation of identified p blast cell progeny. Anterior is towards the top. (A) Anterior boundary of fluorescently labeled tissues resulting from rhodamine-dextran injection of the right P teloblast in an otherwise normal embryo. Identifiable P pattern elements are marked in the most anterior labeled blast cell clone. (B) Anterior boundary of fluorescently labeled tissues in an embryo in which cell p.a was ablated from the most anterior labeled clone. This ablation selectively eliminates central neurons pz1-3 (arrow), peripheral neuron pz7 (arrowhead), peripheral neuron pz5 (double arrowhead), and the labeled epidermis (asterisk) in that region. Descendants of cell p.p – including the WE neuron cluster and cf3 – are intact. (C) Cell p.p was ablated from the middle of three consecutive labeled segments. Loss of the p.p sublineage results in an absence of the WE neuron cluster (arrow), pz4 neuron (arrowhead), and cf3 (double arrowhead). Neurons pz1-3, neuron pz5, and the squamous epidermis (epi) derive from cell p.a, and are normal in all segments. (D) Laser ablation of cell p.pp prevents the formation of peripheral neuron pz10. In the lesioned blast cell clone (lower bracket), the AA nerve contains labeled axons (white arrowhead) extending beyond the lateral edge of the labeled epidermis (epi), but the PP nerve (black arrowhead) does not. The next anterior clone (upper bracket) was not lesioned, and the PP nerve contains a labeled axon extending from neuron pz10. (E) Ablation of cell p.pp also results in a partial loss of the WE neuron cluster. Arrowheads mark labeled pz5 neurons associated with two consecutive ganglia. The white arrow marks a normal WE neuron cluster in the unlesioned anterior clone, and the black arrow marks a partial WE cluster in the next posterior clone from which the p.pp sublineage has been ablated. The size and shape of a normal WE cluster is outlined for reference. Scale bars: 20 μm in A-C; 10 μm in D,E.

ablation of cell o.apa did not prevent the remainder of the clone from contributing to cf2 (Fig. 5). Given that cf2 is contiguous with the squamous epidermis (Fig. 1B) – whose precursor, cell o.app, was not ablated – this one anomalous result may be a consequence of aberrant cell mixing rather than a respecification of cell identity. In either case, it should be noted that there were no labeled cf2 cells in any of the 22 other experimental clones in which a lineal precursor (cell o.ap or o.apa) had been ablated.

Second, the operated blast cell clones gave rise to the precise complement of O pattern elements that would normally derive from the cells that were not ablated. These pattern elements were observed at frequencies that did not differ significantly (P>0.9, χ^2 test) from the control clones (Fig. 5), and we did not see any apparent duplication of pattern elements or encounter other labeled cell types atypical of an o blast cell clone. In short, there was little or no detectable change in the developmental fate of the remainder of the o.a sublineage when any one of its progeny cells was eliminated by laser ablation.

One experiment gave unexpected results. Cell o.aa normally gives rise to the CR cluster of central neurons (Shankland, 1987a), but we observed one or two labeled neurons located in the normal vicinity of the CR cluster in 13 of 16 *H. robusta* embryos and 3 of 3 *H. triserialis* embryos in which cell o.aa had been ablated (Fig. 4C). We always ablated cell o.aa from the most anterior labeled clone, so these isolated CR neuron(s) must have derived from one of the two unablated sublineages within that clone (o.ap or o.p) or from some more posterior blast cell clone. It was previously reported that cell o.ap will occasionally give rise to a single, isolated CR neuron in otherwise unperturbed *H. triserialis* embryos (Shankland, 1987a), and to determine whether cell o.ap might be the source of the anomalous CR neuron(s) seen here, we labeled the o.ap sublineage with rhodamine-dextran lineage tracer in 13 *H. robusta* embryos (Fig. 4D). In no case did the injected o.ap cell give rise to a CR neuron, and in only one case did a labeled o.ap cell give rise to a descendant (which had the stellate morphology of a phagocyte or glial cell) in the relevant portion of the ganglion. From these data we conclude that the isolated CR neuron(s) seen following o.aa ablations are not normal descendants of cell o.ap. However, our present data are not sufficient to determine whether those neuron(s) are a normal but previously uncharacterized derivative of the o.p sublineage, or are being produced abnormally by cell o.ap in response to the o.aa ablation.

Normal fate of the P lineage

Fig. 1D depicts a two-dimensional projection of a p blast cell

clone from a stage 9 embryo with the various sublineages shown in color. The primary blast cell clone contains approximately 70 cells at this page (Shankland and Weisblat, 1984), and straddles the boundary between two consecutive segments. In the present paper we scored a total of eleven discrete P pattern elements. Six of these pattern elements could be unambiguously identified in >90% of the 67 rhodamine-labeled control clones; identification of the remaining pattern elements is discussed in more detail below.

Central nervous system

The p blast cell clone gives rise to three pattern elements that we could routinely score within the ganglia of the CNS (Fig. 1D). The first consists of three clustered neurons (pz1, pz2 and pz3) situated medially on the ventral surface of the ganglion. The second pattern element is a more laterally situated cluster of neurons called the wedge (WE). The third pattern element is the isolated neuron pz4, whose cell body can be distinguished from the remainder of the P-derived central neurons by its more posterior location.

It has previously been shown that neuron pz4 arises bilaterally (M. S., unpublished) and that either the right or the left homologue later disappears within each segmental ganglion (Kramer and Weisblat, 1985; Shankland and Weisblat, 1984). In the present experiments we identified neuron pz4 in 90% (60/67) of labeled control clones, a discrepancy that suggests the majority of our experimental embryos were fixed prior to the time of cell death. We therefore labeled the right and left P lineages by teloblast injection in otherwise normal embryos, and found that midbody ganglia still contained an average of 1.53 viable pz4 neurons (n=34 ganglia, five embryos) at mid-stage 9, our usual time of fixation. We found an average of 1.20 viable pz4 neurons at the end of stage 9 (n=40 ganglia, six embryos) and only 1.05 viable pz4 neurons at late stage 10 (n=22 ganglia, three embryos), suggesting that the programmed death of one pz4 neuron is usually completed by the latter stage.

Peripheral nervous system

Six of the peripheral neurons generated by the p blast cell clone were scored in this study (Fig. 1D). The pz10 neuron is the only P-derived cell in the PP nerve (Fig. 6D), and we could reliably visualize its dorsally projecting axon even in embryos in which the cell body was covered by labeled epidermis. The pz7 and pz9 neurons are associated with the middle or 'MA' segmental nerve, and were more difficult to identify. We were only able to identify pz9 reliably in 87% of control clones and pz7 in 69% of control clones.

There are three P-derived neurons in the AA nerve. Neurons pz6 and LD1 are situated in the lateral body wall, and have been distinguished from one another on the basis of neurotransmitter content but not morphology (Stuart et al., 1987). These two neurons are subepidermal, and when the overlying epidermis was also labeled with dextran – as during normal development (Fig. 1D) – it was difficult to visualize whether one or two labeled cell bodies were present. The pz5 neuron is also associated with the AA nerve, but its cell body is located medially in the nerve root. We were only able to identify pz5 in 72% of control clones.

Squamous epidermis

The p blast cell clone gives rise to the majority of ventral epidermis within a region of one segment's length. Previous work has shown that this patch of P-derived epidermis comprises two sublineages (Shankland, 1987b) – a part that is anterior to the segment boundary that derives from granddaughter cell p.aa, and a part that is posterior to the segment boundary that derives from granddaughter cell p.ap (Fig. 1D). During normal development, it is the latter patch of epidermis that overlies the pz6/LD1 neurons from the same blast cell clone.

Cell florets

The p blast cell clone also contributes cuboidal cells to the three most ventral cell florets (Fig. 1D). Cell floret 3 (cf3) is composed of roughly a dozen cells at this stage, and is relatively easy to distinguish from the surrounding squamous epidermis (Fig. 6A,C). In contrast, cf2 contains only one P-derived cell (Shankland and Weisblat, 1984), and we could unambiguously distinguish the labeled floret cell from the surrounding epidermis in only 87% of our control clones. Cell floret 1 was not identified in these experiments.

Ablations within the p blast cell clone

We used a laser microbeam to individually ablate different progeny cells from p blast cell clones that had undergone 1-3 cell divisions. In particular, we examined the effect of ablating either daughter of the primary p blast cell (cells p.a and p.p) or any one of its four granddaughters (cells p.aa, p.ap, p.pa and p.pp; see Fig. 1C). Individual ablation results are shown in Fig. 6, and the results of all four ablations are summarized in Fig. 7.

Ablations within the p blast cell clone gave results comparable with those obtained for the o blast cell ablation experiments described above. First, the operated clones lacked those pattern elements that would normally have arisen from the ablated precursor (Figs 6A-D, 7). Of 67 experimental clones examined, the only cases in which the remainder of the clone appeared to have compensated to produce a missing pattern element involved the formation of squamous epidermis (see below). In no case did an operated p blast cell clone give rise to neurons or cell florets that would normally derive from the cell that had been ablated.

Second, the operated blast cell clones gave rise to the precise complement of P pattern elements that would normally derive from the cells that were not ablated. These pattern elements were observed at frequencies that did not differ significantly (P>0.9, χ^2 test) from the control clones (Fig. 7), and we did not see any apparent duplication of pattern elements or encounter other labeled cell types atypical of a p blast cell clone. In short, there was little or no detectable change in the developmental fate of the remainder of the p blast cell clone when any one of its early progeny cells was eliminated by laser ablation. In some experiments the ablated progeny cell (e.g. p.p, p.pp) was situated at the posterior end of the operated blast cell clone, and in those cases we examined the cellular composition of the next posterior blast cell clone as well. Consistent with our previous findings (Seaver and Shankland, 2000), ablations within one blast cell clone did not produce any detectable alteration in the developmental fate of the adjoining posterior clone.

These conclusions are mitigated by certain ambiguities in cell type identification. One ambiguity involves the far lateral peripheral neurons in the AA nerve. During normal development, both cell p.a (and its daughter, p.ap) and cell p.p (and its daughter, p.pa) give rise to one of the two morphologically indistinguishable neurons (pz6 and LD1) at this location. Thus, all progeny cell ablations were expected to leave at least one viable neuron at this location. Other, less ambiguous descendants of cell p.a (pz1-3, pz5, pz7, squamous epidermis) and cell p.p (WE cluster, pz4, pz9, pz10, cf2, cf3) were always missing when their precursor was ablated (Figs 6A-C, 7), from which we conclude that there was little or no cell fate regulation following either p.a or p.p deletions.

Another potential ambiguity arises from the fact that certain ablations surpassed the limits of previous cell lineage analysis. Both WE and cf3 are clusters of cells that arise in part from the p.pa and p.pp sublineages (Fig. 1D). We usually detected a reduction in the size of the WE neuron cluster when either p.pa (11/11) or p.pp (5/7) was ablated (Fig. 6E), but could not be certain whether there was any respecification of cell fates between the two halves. Likewise, we usually detected a reduction in the size of cf3 when either p.pa (6/11) or p.pp (6/7) was ablated, but could not discern any respecification of cell fates between the two halves. It should be noted that reduction in size of WE and cf3 was only seen following ablations of p.pa or p.pp, and that other less ambiguous descendants (cell p.pa – cf1, cf2 and pz9; cell p.pp – pz10) were always missing when their normal precursor was ablated (Figs 6D, 7). Hence, there would appear to have been little or no cell fate regulation following either of those two ablations.

Finally, there was some ambiguity in distinguishing the squamous epidermis produced by cells p.aa and p.ap. These two epidermal lineages are normally distinguished by their respectively anterior and posterior positions with respect to the segment boundary (Fig. 1D). Ablation of either cell consistently produced an overall reduction in the amount of labeled epidermis, but the labeled epidermis generated by the remaining cell often extended into the region normally occupied by the ablated sublineage (Fig. 7). Following ablations of cell p.aa, we scored anterior epidermis as missing only if it did not extend anterior to the segment boundary (n=4/8). In the four remaining p.aa ablations, we observed a significant reduction in the amount of labeled epidermis, but its position had shifted such that a part of it was located anterior to the segment boundary. Likewise, in ablations of cell p.ap, we only scored the posterior epidermis as missing if it had not shifted posteriorly to cover the AA nerve (n=5/9). In the four remaining p.ap ablations we observed a significant reduction in the amount of labeled epidermis, but its position had shifted such that a portion overlay the AA nerve. It has been previously shown that ablation of epidermal lineages causes the remaining epidermis to spread into the resulting gap (Blair and Weisblat, 1984), and we suspect that the repositioning observed here reflects such spreading rather than a respecification of cell identity. The other less ambiguous descendants of cell p.ap (pz5) and p.aa (pz1-3, pz7) were always missing when their normal precursor was ablated (Fig. 7), from which we conclude that there was little or no regulation of cell identity following either p.aa or p.ap ablations.

Our present findings supercede the previous fate map of the p blast cell clone in one regard. A previous lineage tracer analysis (Shankland, 1987b) showed that the pz4 neuron is normally derived from the posterior daughter of the p blast cell, p.p, but failed to distinguish which p blast cell granddaughter gives rise to this neuron. We never observed a labeled pz4

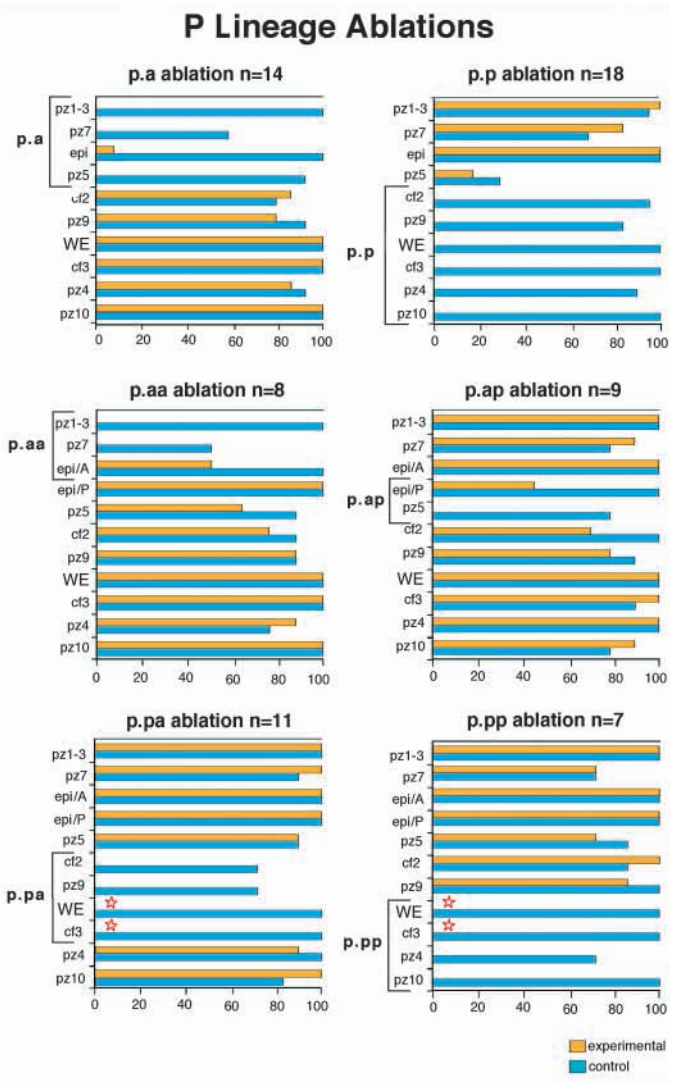

Fig. 7. Ablation of single p blast cell progeny has little or no effect on the developmental fate of the remainder of the blast cell clone. Histograms show the frequency at which differentiated pattern elements (see text) were identified as present in primary p blast cell clones from which one specific progeny cell had been ablated. In all six experiments, comparison of experimental clones (yellow) and unoperated control clones (blue) reveals no significant change in the set of descendants produced by the unablated portion of the clone (χ^2 test, P>0.9). Brackets demarcate the subset of pattern elements that would normally arise from the ablated cell (Shankland, 1987b), and those elements were consistently missing from the experimental clones. Note that cf3 and the WE neuron cluster are normally produced by the combined descendants of cells p.pa and p.pp; these two pattern elements were present but reduced in size (orange stars) when either of those two progeny cells was ablated. In the four grand-daughter cell ablations, squamous epidermis was distinguished as anterior (epi/A) or posterior (epi/P) by position. Ablation of cell p.aa or cell p.ap caused the expected reduction in the amount of squamous epidermis, but the remaining epidermis often spread into the region of deficit. See text for details.

neuron in p blast cell clones from which cells p.p (*n*=18) or p.pp (*n*=7) had been ablated (Fig. 7), but we frequently observed this neuron in p blast cell clones from which any one of the other progeny cells had been ablated (38/42, 90%). The simplest explanation of these data is that the pz4 neuron derives from granddaughter cell p.pp in both normal and operated embryos (Fig. 1D); however, we can not rule out the possibility that pz4 normally comes from some other cell lineage and that its derivation was altered by certain of our cell ablations.

Slippage

Some of our experimental embryos displayed a phenomenon known as 'bandlet slippage' in which there is an artifactual widening of the gap produced by the ablation (Shankland, 1984). Such widening affects only the lesioned teloblast lineage, and occurs because blast cell clones behind the site of ablation undergo a posterior frameshift (of an integral number of segments) relative to the other teloblast lineages. We had no direct means of assessing whether or not slippage had occurred in experiments in which we ablated the most anterior labeled cell. For the remaining experimental embryos we observed bandlet slippage in 44% (22/50) of our O lineage ablations and 60% of our P lineage ablations. Slippage was observed at a similar frequency following the laser ablation of primary o or p blast cells (Seaver and Shankland, 2000).

As seen previously (Martindale and Shankland, 1988; Martindale and Shankland, 1990; Nardelli-Haefliger et al., 1994), slippage has little or no effect on the developmental identity of the displaced cells. For instance, we ablated cell p.pa in 11 embryos, and in five cases the trailing fragment of the lesioned blast cell chain experienced ≥1 segment of posterior slippage. Despite this slippage, in every case the p.pp cell immediately posterior to the ablation gave rise to its normal descendants, including peripheral neuron pz10, a portion of the WE cluster of central neurons, a portion of cf3, and – in four out of five cases – the central neuron pz4. The p.aa and p.ap cells immediately anterior to the ablation also gave rise to their normal descendants in all five cases, including central neurons pz1-3, peripheral neurons pz5 and pz7, another peripheral neuron in the pz6/LD1 location, and a large ventral patch of squamous epidermis. Hence, ablation of a single p blast cell granddaughter does not appreciably alter the developmental fate of the three remaining granddaughter cells, even when one of those granddaughter cells slips posteriorly so as to differentiate in a segment that is inappropriate to its lineage history.

DISCUSSION

We have examined the establishment of segment polarity in an annelid, the leech *Helobdella*. Segment polarity refers to the orderly specification of positional values along the AP axis of each segmental repeat (Nüsslein-Volhard and Weischaus, 1980), and is distinct from the positional specification of entire segments or parasegments with respect to the body as a whole (McGinnis and Krumlauf, 1992). In leech embryos, the segmental ectoderm arises from a stereotyped cell lineage in which primary blast cell clones function as segmental repeats. The earliest blast cell divisions are parallel to the AP axis, and produce progeny cells with discrete anterior and posterior cell fates (Shankland, 1987a; Shankland, 1987b). We here present experimental results suggesting that the segment polarity of these clones is established by a developmental mechanism that has little or no requirement for cell interactions among the clonal progeny of the primary blast cell.

Specification of segment polarity

To ascertain whether cells of the clone must interact to specify their anterior and posterior cell fates, we used a laser microbeam to ablate single identified progeny cells at stages when the primary blast cell clone was two to four cells in length. We examined the effect of 12 different progeny cell ablations, in either the O or the P cell lineages. If the specification of segment polarity depended upon cell interactions within the blast cell clone, we would expect that some ablations would change the AP fate of one or more of the remaining cells. However, in every case we found little or no detectable change in the set of descendant pattern elements produced by the other, unablated cells. We have previously shown that both the O and the P lineages can generate normal segment polarity in the absence of interactions between adjacent clones (Seaver and Shankland, 2000), and together these two studies suggest that the segment polarity of the leech ectoderm does not depend upon inductive signals being transmitted exclusively along the AP axis (see below). Several of the blast cell divisions studied here display a predictable morphological asymmetry (Shankland, 1987a; Shankland, 1987b), and it may be that these cells have autonomous mechanisms for ensuring the asymmetric segregation of developmental potential as well.

Our ablations did result in a few, relatively minor changes of cell fate. For instance, several ablations led to a spreading of surviving epidermis into gaps left by the ablation of other epidermal lineages. Epidermal spreading has been observed in *Helobdella* embryos following a variety of ectodermal cell ablations (Blair and Weisblat, 1984), and appears to be more akin to wound-healing than any sort of respecification of cell identities. A second possible fate change involves the formation of one to two central neurons of the CR cluster when cell o.aa was ablated (Fig. 4B,C). Cell o.aa normally generates the CR neurons (Shankland, 1987a), and it is possible that the ablation of cell o.aa caused its sibling, cell o.ap, to regulate and produce the anomalous CR neuron(s) in addition to its roughly 40 normal descendants. However, one alternative possibility is that these anomalous CR neuron(s) are normal descendants of the o.p sublineage, whose fate is uncharacterized (Shankland, 1987a). Indeed, this latter scenario seems more likely as we also observed one to two anomalous CR neurons in three embryos in which o.a cell ablations had eliminated both the o.aa and the o.ap sublineages.

It should be noted that certain cell interactions can not be revealed by cell ablation methodologies. In *C. elegans*, induction of the EMS blastomere by the sibling P2 blastomere has been clearly demonstrated by cell isolation and recombination experiments (Goldstein, 1992), but was previously undetected in cell ablation studies. This cell interaction is complete within minutes after the cytokinesis that gives rise to the signaling cell, and with this limitation in mind we irradiated blast cell progeny within minutes of the preceding division. Nonetheless, we can not rule out the

possibility that o and p clones experience cell interactions that were not disrupted by our laser ablation technique.

However, our findings would seem to rule out signaling between non-sibling cell pairs. Consider, for example, the hypothesis that cell p.ap induces the normal fate of the non-sibling cells p.pa and/or p.pp (see Fig. 1C). Not only do the latter cells develop normally when p.ap is ablated, they also develop normally when its parent cell p.a is ablated several hours earlier (Fig. 7). It is conceivable that the ablated p.a cell retains some signaling properties or leaves behind debris that can influence cell fates, but one would not expect such debris to contain gene products whose onset of expression normally occurs after p.a completes its next division. Thus, ablation of cell p.a should effectively prevent the expression of *en* gene products within that one p blast cell clone (see Fig. 1C), and the fact that p.pa and p.pp develop normally under those conditions argues that they do not require expression of *en* – or other de novo gene products – by cell p.ap to do so.

Although we find little or no evidence of inductive cell interactions being conveyed along the AP axis of the O and P teloblast lineages, our experiments do not address the possibility that segment polarity is patterned by signals conveyed – in whole or in part – along the dorsoventral or superficial-deep axes of the germinal band. Such cues could originate from other teloblast lineages, or from the overlying micromere derivatives. But while we can not exclude this formal possibility, it should be noted that *Helobdella* embryos have been subjected to a wide variety of blastomere ablations, and that none of the ablations reported to date has any clear-cut effect on the segment polarity of the surviving lineages. For example, the dorsoventral specification of an o/p blast cell clone depends upon cues from three other cell lineages (Shankland and Weisblat, 1984; Ho and Weisblat, 1987; Huang and Weisblat, 1996), including the other O/P lineage on that side. But removing those cues does not alter the segregation of anterior and posterior fates within the descendant clone of the blast cell (Shankland, 1987b). Indeed, one might expect the P lineage ablations described in this paper to result in a partial O-to-P (ventral-to-dorsal) respecification of the nearest o blast cell clone(s) (Shankland and Weisblat, 1984), but even if such changes took place, they did not alter the AP specification of blast cell progeny within the labeled P lineage.

Comparison of segment polarity specification in annelids and arthropods

Before comparing segmentation between annelids and arthropods, it is instructive to consider the degree to which the homologous segmentation pathways have diversified within the arthropods. Different arthropod taxa have widely divergent modes of development with regards to important traits such as holoblastic versus meroblastic cleavage, determinate versus indeterminate cell lineage, and embryonic versus larval segmentation (Brusca and Brusca, 1990). But despite these differences, certain genetic mechanisms seem to be widely conserved within this phylum, particularly the step of segment polarity specification.

Segment polarity is first established in the *Drosophila* embryo by the formation of adjoining stripes of *en* and *wg* (wingless) expression in the posterior half of each segment primordium (DiNardo et al., 1988), and is then elaborated throughout the remainder of the segment by intercellular signaling primarily mediated by the Wg and Hedgehog (Hh) proteins (Heemskerk and DiNardo, 1994; Lawrence et al., 1996). The detailed relationship of *en* expression and segment formation is conserved in a variety of arthropod taxa (Patel et al., 1989b; Manzanares et al., 1996; Damen et al., 1998; Gibert et al., 2000), and the pattern of *wg* expression is also conserved in non-dipteran insects (Nagy and Carroll, 1994) and – to a lesser extent – some crustaceans (Nulsen and Nagy, 1999). This is in contrast to the pair-rule step of the *Drosophila* segmentation mechanism, which diverges significantly from the *Drosophila* model in certain other insect groups (Patel et al., 1992: Dawes et al., 1994). The fact that segment polarity specification shows a relatively high degree of conservation within the arthropods suggests that it is a good candidate for conservation in other phyla with homologous segmentation.

The expression of the *en* gene in the leech *H. triserialis* is similar in several regards to that seen in arthropods (Wedeen and Weisblat, 1991). Embryonic expression begins at a stage when the segmental repeat is only a few cells in length (see Fig. 1A,C), and forms a transverse stripe across the dorsoventral width of each nascent segment (Lans et al., 1993). But there are also some noteworthy differences. In *Helobdella* the onset of *en* expression follows the appearance of segmentally iterated cell divisions, which may indicate that it is simply a downstream marker of segmental periodicity. Furthermore, the *en* stripes of the leech embryo do not define developmental compartments of cell lineage restriction – compare Lans et al. (Lans et al., 1993) with Shankland (Shankland, 1987b) – nor are these stripes restricted to the posterior of the segment – e.g. the *en*-expressing cell p.ap generates neurons and epidermal cells that lie in the anterior half of the anatomical segment (Fig. 1D).

Our present findings reveal other, even more fundamental differences in the mechanism by which leeches and fruit flies establish segment polarity. In the fly embryo, the patterning of AP positional values within the segmental repeat is primarily dependent upon cell interactions occurring along the AP axis (DiNardo et al., 1988; Heemskerk and DiNardo, 1994; Lawrence et al., 1996). Moreover, the en transcription factor plays a pivotal role in initiating and maintaining those cell interactions. In the leech embryo, we find no evidence that cell interactions oriented along the AP axis are required to specify anterior and posterior cell fates within the segmental repeat, and ablation of blast progeny that normally express *en* (cell p.ap) or their lineal precursors (cell p.a; cell o.aa) does not significantly alter the fate of other blast cell progeny within that clone or in more anterior or posterior clones (Seaver and Shankland, 2000). It may be that the en protein is only involved in the specification of cell-autonomous fates during leech segmentation.

Additional evidence of this difference comes from two other studies relating to the role of *en* in leech segmentation. First, blast cells produced by the N teloblast express *en* in a subset of descendants around the time that ganglia form in the central nervous system (Lans et al., 1993). Early reports suggested that these *en*-expressing N teloblast-derivatives might produce the fissures that separate segmental ganglia (Ramirez et al., 1995), but subsequent work indicates that the fissures arise independently of *en*-initiated signals (Shain et al., 2000).

Second, a *hh* ortholog has been isolated from *H. robusta*, and in situ hybridization reveals that its RNA is heavily

expressed in the gut and reproductive organs but undetectable in primary blast cell clones at the time of *en* expression (D. Kang, D. A. Weisblat, D. Li, and M. S., unpublished). We can not rule out low levels of *hh* expression that have escaped detection, nor the possibility that *Helobdella* may possess other, as-yet-uncharacterized members of the *hh* gene family. Nonetheless, the finding that *en*-expressing cells of the leech apparently do not express the gene product that is used to convey segment polarity information in the *Drosophila* blastoderm (DiNardo et al., 1988; Heemskerk and DiNardo, 1994) is entirely consistent with our embryological experiments showing that *en*-expressing cells do not play an organizing role in segment polarity specification.

Evolutionary origin of body axis segmentation

The present study shows that the mechanism by which segment polarity is established in fly and leech embryos is not strictly conserved. One potential explanation is that the last common ancestor of annelids and arthropods was unsegmented, in which case the mechanistic differences seen here could be readily explained by an independent evolutionary origin of the segmentation process.

Attractive as this interpretation may be, it begs an important question – i.e. why would two independently evolved segmentation mechanisms employ outwardly similar patterns of *en* expression? One plausible explanation is that the evolution of morphological periodicity is preferentially likely to employ the *en* gene, and indeed there is evidence supporting that idea. Echinoderms have lost the AP axis that defines the adult body plan of most bilaterian animals, and develop instead into adult forms with multiple ambulacral axes radiating outward from the center (Brusca and Brusca, 1990). The ophiuroid echinoderms (brittle stars) are distinctive in that they have repeating articulations along these ambulacral axes, and unlike other echinoderms the ophiuroids express the *en* gene in a sequence of repeating stripes that predicts the pattern of articulations that will form (Lowe and Wray, 1997). Thus, it seems clear that a developmental relationship between *en* stripes and morphological periodicity has evolved in the Bilateria on more than one occasion, and the possibility that the *en* stripes of arthropods and leech embryos are also homoplasic should not be discounted out of hand.

But while the findings presented in this paper argue against an underlying homology of the segmentation process, they should not be considered conclusive. Homologous developmental mechanisms do undergo significant diversification (Nulsen and Nagy, 1999; Sommer, 2000), and it is difficult to place limits on the amount of mechanistic diversification that might have occurred in the roughly 600 million years since the annelid and arthropod lineages split. What we can say with confidence is that if annelids and arthropods did share a segmented common ancestor, then the segmentation mechanism employed by that ancestor must have undergone a significant derivation in the lineage(s) that gave rise to flies, to leeches, or both. Thus, one important future goal will be to examine segment polarity specification more widely within each phylum, particularly the annelids in which little is known about gene expression patterns or cell interactions outside of the leech.

In summary, our findings show that at least one annelid – the leech *Helobdella* – generates segment polarity by a developmental mechanism that lacks some of the key features of segment polarity specification in arthropods. From this we can conclude that the mechanisms of segment polarity specification employed by leeches and arthropods are either homoplasic or highly diverged.

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