Developmental Biology **224,** 339–353 (2000)
doi:10.1006/dbio.2000.9781, available online at http://www.idealibrary.com on IDEAL®

Leech Segmental Repeats Develop Normally in the Absence of Signals from either Anterior or Posterior Segments

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We have investigated whether the development of segmental repeats is autonomous in the embryo of the leech *Helobdella robusta.* The segmental tissues of the germinal band arise from progeny of five stem cells called teloblasts. Asymmetric divisions of the teloblasts form chains of segment founder cells (called primary blast cells) that divide in a stereotypical manner to produce differentiated descendants. Using two distinct techniques, we have looked for potential interactions between neighboring blast cell clones along the anterior–posterior axis. In one technique, we prevented the birth of primary blast cells by injection of DNase I into the teloblast, thereby depriving the last blast cell produced before the ablation of its normal posterior neighbors. We also ablated single blast cells with a laser microbeam, which allowed us to assess potential signals acting on either more anterior or more posterior primary blast cell clones. Our results suggest that interactions along the anterior–posterior axis between neighboring primary blast cell clones are not required for development of normal segmental organization within the blast cell clone. We also examined the possibility that blast cells receive redundant signals from both anterior and posterior neighboring clones and that either is sufficient for normal development. Using double blast cell laser ablations to isolate a primary blast cell clone by removal of both its anterior and its posterior neighbor, we found that the isolated clone still develops normally. These results reveal that the fundamental segmental repeat in the leech embryo, the primary blast cell clone, can develop normally in the apparent absence of signals from adjacent repeats along the anterior–posterior axis. © 2000 Academic Press

Key Words: **leech; annelid; segmentation; cell fate; cell autonomous; anterior–posterior; laser ablation; *Helobdella robusta.***

INTRODUCTION

It is currently unknown whether the last common ancestor of annelids and arthropods was segmented along its anterior–posterior (A/P) body axis (Davis and Patel, 1999). If segmentation is homologous in these two phyla, one would expect some degree of conservation in the developmental mechanisms utilized to make segmental repeats. The generation of a segmented body plan is best understood at a mechanistic level in *Drosophila melanogaster* (reviewed in Lawrence and Struhl, 1996), and a key feature of segment formation in the fruitfly embryo is cell interactions occurring along the A/P axis that cross both the segmental and the parasegmental borders (Heemskerk and DiNardo, 1994; Lawrence *et al.*, 1996). One of the critical molecular components of this signaling pathway is the segment polarity gene *engrailed* (*en*), and highly conserved *en* expression patterns suggest that this particular step of the segmentation process is characteristic of a variety of arthropod taxa (for example see Patel, 1994). It is not known if cell interactions are required to properly pattern the developing segmental repeats of annelid embryos or larvae. We have taken a direct experimental approach to this question and ablated single cells to ascertain whether inductive cell interactions along the A/P axis are required for the normal formation and patterning of segmental repeats in the ectoderm of an annelid, the leech *Helobdella robusta.*

The leech embryo has an invariant pattern of cell division. The segmented mesoderm and ectoderm on each side of the embryo arise from five stem cells called teloblasts,

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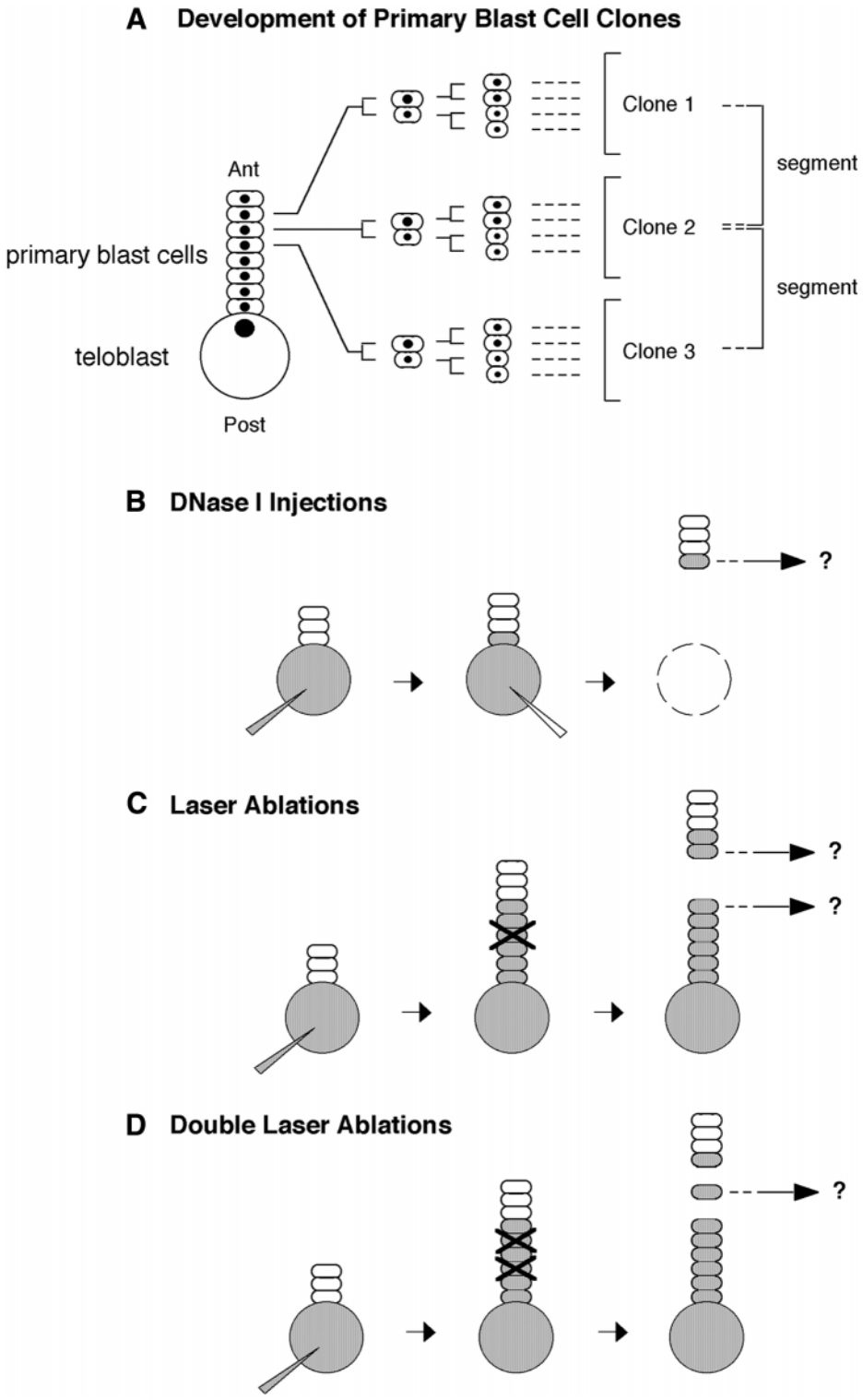

FIG. 1. (A) Development of primary blast cell clones in the O and P lineages. The teloblasts undergo asymmetric divisions to produce a chain of primary blast cells. Primary blast cells represent the fundamental repeating unit of the segmental body plan and undergo stereotyped cleavages that are unique for each teloblast lineage. Each primary blast cell within a teloblast lineage produces the same segmentally repeated set of descendants. Although the descendants from a single primary blast cell represent a segmental complement, the clone of differentiated cells spans parts of two segments (see also Figs. 2C and 2D). (B, C, and D) Methods utilized to remove primary blast

each of which divides asymmetrically to produce a column of progeny cells called primary blast cells. The chains of primary blast cells from the five teloblasts then merge to form a germinal band on each side of the embryo. The left and right germinal bands later fuse to form a germinal plate, which differentiates into the segmented ectoderm and mesoderm. The firstborn blast cells in each teloblast lineage contribute to the most anterior body segments, and later born blast cells contribute to progressively more posterior segments.

Each of the five teloblast lineages makes a distinct and stereotyped contribution to the segmental tissues (Weisblat and Shankland, 1985). In both the O and the P teloblast lineages, the primary blast cell is the fundamental repeating unit of the segmented body plan, and segmental periodicity is therefore manifest in the production of the primary blast cells. Each o or p primary blast cell represents a lineage-specific segmental founder cell, i.e., it gives rise to one segmental repeat of the teloblast's descendant tissues (Fig. 1A). In doing so, each primary blast cell undergoes a series of stereotyped cell divisions (Bissen and Weisblat, 1989) and produces a descendant clone of roughly 70 terminally differentiated cells (Shankland and Weisblat, 1984) (Figs. 2A and 2B). However, the primary blast cell clone does not map to a single anatomically defined segment; rather, each blast cell clone straddles the boundary between two segments (Weisblat and Shankland, 1985) (Figs. 2A and 2B). Thus, within a single hemilateral segment there are contributions from two successive primary blast cell clones that intermingle to form the full pattern of O teloblast descendants. The same is also true for the P lineage (Weisblat and Shankland, 1985). Therefore, the ultimate distribution of an o or p blast clone is not truly segmental (Weisblat and Shankland, 1985), nor is it "parasegmental" by the definition used for *Drosophila* embryos (Martinez-Arias and Lawrence, 1985). The intermingling of cells from two adjacent primary blast cell clones occurs fairly late during morphogenesis (Figs. 1A, 2A, and 2B), long after the manipulations performed here.

The blast cell clones of the O and P lineages have been characterized in detail through injection of lineage tracers (Shankland, 1987a,b). The terminally differentiated components of each clone can be uniquely identified as individual cells or small clusters of cells, cumulatively referred to as O or P pattern elements (Figs. 2C and 2D). In both the O and the P lineages, the primary blast cell clone gives rise to various ectodermal cell types, including squamous epidermis, central neurons, glia, peripheral neurons, and epidermal specializations called cell florets. The o blast cell clone also contributes one cell to the nephridium, the segmental organ of urinary excretion. Although the O and P lineages give rise to most of the same histotypes, their blast cell clones contain distinct sets of pattern elements that can be easily distinguished by their stereotyped positions (compare Figs. 2A and 2B).

Previous studies have demonstrated that some cell fate decisions made during leech embryogenesis are specified in a cell-autonomous manner (reviewed in Shankland, 1991). For example, segment identity of the blast cell clone appears to be established through a cell-intrinsic mechanism that does not depend on segmental location (Gleizer and Stent, 1993; Martindale and Shankland, 1990; Nardelli-Haefliger *et al.*, 1994). However, segmental identity and segmental periodicity are not necessarily coupled, as has been demonstrated by genetics in *Drosophila* (Struhl, 1981), and we are interested in how the periodicity and polarity of segments are established in the leech embryo. Because a periodic pattern is evident in the production of primary blast cells by the teloblast, it is possible that divisions of the teloblast establish repeating units, i.e., segmental periodicity, and that the execution of the normal developmental program by each repeating unit are autonomous. Alternatively, cell–cell interactions could still be critical for the establishment of repeated segmental units, as has been proposed for the stereotyped and segmentally iterated cell lineages of certain crustacean embryos (Dohle and Scholtz, 1988; Scholtz *et al.*, 1993). Cell interactions along the A/P axis are known to play an important role in *Drosophila* segmentation, both across segmental boundaries (Heemskerk and DiNardo, 1994) and across parasegmental boundaries (DiNardo *et al.*, 1988).

To investigate potential interactions between adjacent segmental repeats along the A/P axis in the leech embryo, we ablated either o or p primary blast cells and then examined whether anterior and posterior neighboring clones produced their normal complements of differenti-

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cells. With all three techniques, the development of primary blast cell clones is monitored by injection into the teloblast of lineage tracer, which is inherited by the progeny chain of primary blast cells. After experimental manipulation, the embryos are allowed to develop until stage 9, when the primary blast cells have undergone many cell divisions to produce terminally differentiated descendants. (B) In the first method, the teloblast is injected with DNase I approximately 1 h after injection of lineage tracer. During this time interval, the teloblast produces on average one labeled primary blast cell. DNase I prevents further divisions of the teloblast, resulting in an absence of primary blast cells posterior to the labeled blast cell (note that the teloblast also dies). This technique allows assessment of cell interactions that normally occur between a blast cell and its posterior neighbors. (C) In the second method, after injection of lineage tracer, the teloblast is allowed to produce a chain of labeled primary blast cells. A single primary blast cell is then ablated with a laser microbeam. Using the laser ablation technique, one can assess the normal influence of a blast cell clone on both its anterior and its posterior neighbors. In D, two primary blast cells were ablated with the laser microbeam. This manipulation results in the isolation of a single primary blast cell clone from direct contact with neighboring clones on both its anterior and its posterior side. Ant, anterior; Post, posterior.

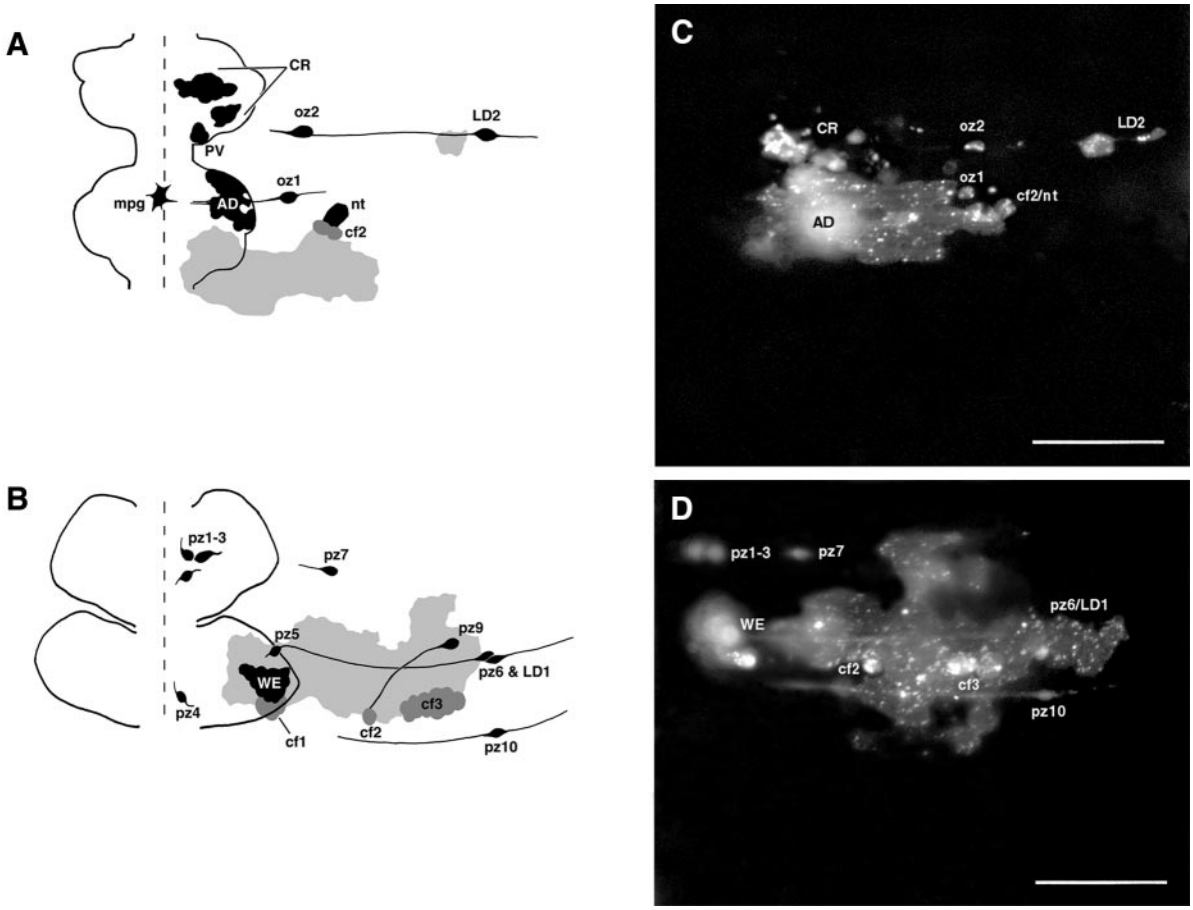

FIG. 2. Tracings of primary blast cell clones for the O and P lineages, and fluorescent micrographs of embryos in which either an o or a p primary blast cell clone developed in the absence of neighboring posterior blast cells. All images are from stage 9 embryos in which primary blast cell clones have produced differentiated descendants and show blast cell clones on the left side of the body viewed from the ventral surface. In all images anterior is up and medial is to the left. (A) Tracings of normal descendants from a single o primary blast cell in a stage 9 embryo. (B) Tracings of descendants from the p primary blast cell of a stage 9 embryo. The tracings in A and B display a small artifactual curvature of the peripheral nerves due to dissection and mounting. (C and D) O or P teloblasts were injected with rhodamine dextran and later injected with DNase I to prevent production of additional primary blast cells. Note that the labeled pattern elements are distributed in three dimensions, and as a consequence some pattern elements are out of the plane of focus or covered by other labeled cells. (C) A labeled o primary blast cell clone which developed in the absence of more posterior o blast cells. All of the normal O pattern elements are present, but the squamous epidermis has spread anteriorly and as a consequence covers the AD neuron cluster. (D) A labeled p primary blast cell clone which developed in the absence of more posterior p blast cells. Note that there is some minor spreading of the epidermis posteriorly into the region of the missing blast cell clones, but otherwise the labeled clone is normal. Among the descendants of both the O and the P lineages are a variety of ectodermal cell types, including epidermis (light gray), central neurons (for O, the cell clusters CR (crescent), PV, AD; for P, pz1-3, pz4, and the cell cluster WE (wedge)), glia (mpg), peripheral neurons (for O, oz2, LD2, oz1; for P, pz7, pz5, pz6, LD1, pz9, pz8, pz10), and epidermal specializations called cell florets (for O, the cell cluster cf2; for P, cf2 and the cell clusters cf1, cf3). The O lineage also contributes a cell to the nephridium (nt). Each pattern element can be uniquely identified by position and cell morphology. Abbreviations of pattern elements are shown next to the corresponding cell/cluster of cells. Anterior is to the top. Scale bars, 50 μm.

ated descendants. In previous studies in which one or more blast cells were removed from a teloblast lineage, only gross morphological characters were scored (Gleizer and Stent, 1993; Ramirez *et al.*, 1995; Shankland, 1984). In contrast, we have performed a detailed analysis of a number of differentiated descendants to ascertain whether a primary blast cell clone immediately adjacent to the ablation developed normally or experienced changes in cell fate that might be subtle and not affect all of its descendants or the overall morphology of the clone. It should be noted that the ablation of a primary blast cell removes potential interactions between segmental repeats throughout the entire

development of the segment, from the stage when it is one cell wide through the production of terminally differentiated cell types. Cell fate changes resulting from a loss of cell interactions at all ages should be cumulative in the cellular composition of the terminally differentiated descendant clone.

There are several possible outcomes one might expect if these ablations do in fact prevent necessary interactions along the A/P axis between neighboring blast cell clones. For instance, a primary blast cell clone that develops in the absence of a neighboring clone might undergo a change of A/P polarity. A second possible outcome is that one or more of the sublineages within the clone may *trans*-fate into a different sublineage, with the result that some descendant pattern elements of the blast cell clone would be duplicated while other pattern elements would be missing. Another possibility is that all of the pattern elements may be properly specified, but have abnormal positions within the clone. Finally, it is possible that blast cell clones situated next to the ablation may undergo compensatory regulation and replace some or all of the missing pattern elements that would normally have arisen from the ablated blast cell.

We used two distinct techniques to examine A/P interactions between primary blast cell clones. In one approach, we prevented the formation of posterior primary blast cells by injection of DNase I into the teloblast after several anterior blast cells had already been born (Fig. 1B) (Blair, 1982). The last blast cell produced before the DNase I injection never experiences more posterior blast cells within the same lineage. In our second approach, we removed potential cell interactions by ablating single blast cells within the blast cell chain using a laser microbeam (Fig. 1C). This latter technique has the advantage of allowing us to examine what—if any—effect the absence of a blast cell clone has on both anterior and posterior clones. We performed each of these experimental manipulations separately in two of the teloblast lineages, the O and the P lineage. We report here that primary blast cell clones can produce an appropriate complement of descendant cells in the absence of segmentally homologous clones on either the anterior or the posterior side. Thus, interactions between successive blast cell clones are not required for the proper patterning of segmental cell fates within the segmental repeat.

MATERIALS AND METHODS

Animals

Embryos of *H. robusta* were obtained from a laboratory colony maintained at the University of Texas at Austin. The colony was established with animals collected from Shoal Creek in the Austin area. Adults were maintained at room temperature in 1% artificial seawater and fed physid snails three times a week. Embryos were removed from adults and raised in buffered saline medium as described by Torrence and Stuart (1986) to desired stages. Embryonic stages and nomenclature are as described in Stent *et al.* (1992).

DNase I Ablations

Teloblasts from the O or P lineage were injected in early stage 7 embryos with the lineage tracer tetramethylrhodamine dextran amine (Molecular Probes) (100 mg/ml in 0.2 M KCl) in a 1:1 dilution with 4% Fast Green FCF (Sigma) in 0.2 M KCl. Teloblast injections were performed as previously described (Weisblat *et al.*, 1978, 1980). After 1–2 h the same teloblast was reinjected with a 2:1 solution of 4% fast green in 0.2 M KCl and 1% DNase I Type IV (Sigma) in 0.15 N NaCl. DNase I is a toxic enzyme that prevents the teloblast from undergoing further divisions and does not directly affect adjacent cells (Blair, 1982). DNase I injections performed 2 h after the lineage tracer injection resulted on average in one or two rhodamine-labeled primary blast cells. This was confirmed in each experimental embryo by counting the number of labeled blast cell clones at later stages. Embryos were reared until stage 9 at 24°C in buffered saline supplemented with antibiotics (50 μg/ml tetracycline, 100 U/ml each penicillin and streptomycin) and then fixed overnight in 4% formaldehyde in 50 mM Hepes-buffered saline, pH 7.4, +2.5 μg/ml Hoechst 33258 (Sigma). After dissection away from the yolk, the embryos were mounted ventral side up in buffered 80% glycerol with 4% *n*-propyl gallate and viewed by epifluorescence using a Nikon E800 microscope. Images were captured digitally with a Spot CCD camera (Diagnostic Instruments, Inc.). Tracings of pattern elements in the O and P lineages were generated from combined digital images taken at multiple dorsal–ventral focal planes.

Laser Ablations

A pulsed nitrogen laser (Laser Science, Inc.; VSL-337 with dye laser module 337110) using Coumarin 440 laser dye (Sigma) was used to ablate individual cells on a Zeiss Axioskop under a 40× triple immersion lens (numerical aperture 0.9) (Blair *et al.*, 1990). The laser beam was directed into the epi-illuminator port of the microscope via a microscope coupler (Laser Science, Inc.) and focused to the plane of visual focus. The diameter of the focused laser beam on the specimen is 1–2 μm, approximately $\frac{1}{3}$ of the shortest dimension of a primary blast cell (5 μm).

To perform blast cell ablations, an O or P teloblast was injected with a 2:1 solution of 4% fast green in rhodamine dextran at early stage 7 and embryos were allowed to produce a chain of labeled primary blast cells. The fast green remains as particulate staining in the cytoplasm of the labeled cells and was used to identify labeled cells under the microscope using bright-field illumination. In addition, fast green absorbs light at the laser beam wavelength and increases the efficiency of the laser ablations. Primary blast cells in *H. robusta* are fairly transparent and it was more difficult to ablate cells that did not contain fast green.

Labeled primary blast cells that had moved into the germinal band were ablated by delivering single pulses of the laser microbeam to selected cells under bright-field illumination. Targeted cells were monitored by visual inspection for a few minutes following the laser pulse. The laser beam was aimed at a fast green granule within the cytoplasm of the targeted cell, and this granule usually disappeared following firing of the laser. We also frequently observed cytoplasmic movements at the time of the pulse, followed by the development of a more granular appearance of the nucleus. Often, the integrity of the cell was lost and cytoplasm was observed to flow out from the cell. If cells neighboring the targeted cell showed visual signs of damage, the embryo was discarded. Embryos that showed no visible changes in the targeted cell were also discarded. The short-term development of the ablated blast cell was

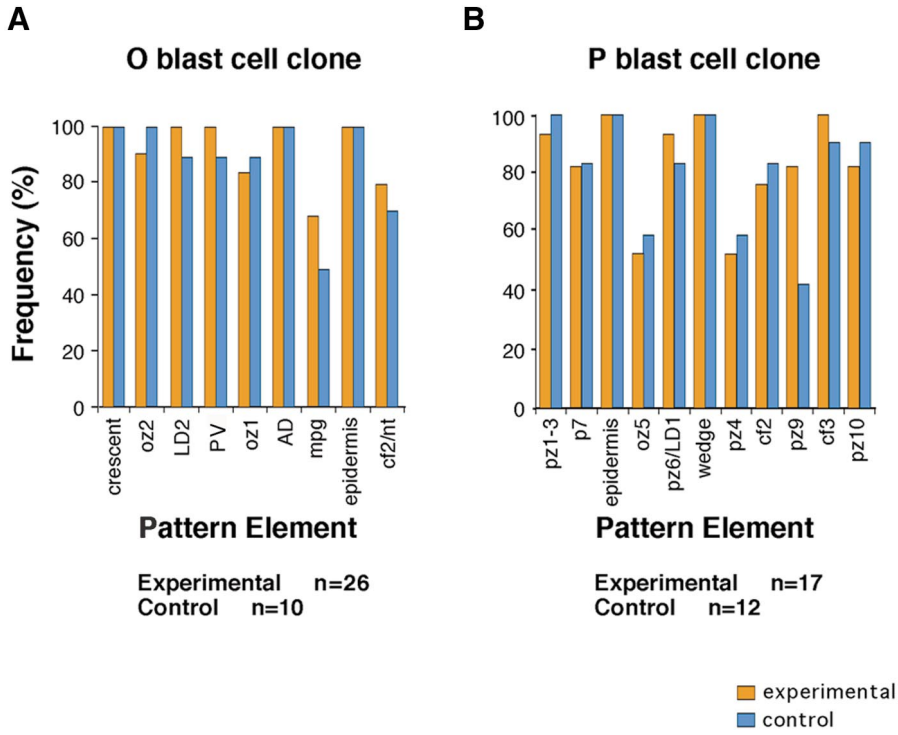

FIG. 3. DNase I manipulations. The ability of o and p primary blast cells to produce the normal segmental complement of descendants is not dependent upon interactions with the primary blast cell clone immediately posterior to it. The *x* axis lists pattern elements characteristic for each teloblast lineage. In some embryos, the teloblast produced two primary blast cells before injection of DNase I; the anterior labeled clone farthest from the ablation site was scored as a control. For each experiment, controls are shown as blue histogram bars and experimentals are shown as orange bars. (A) Frequency of pattern elements observed in embryos when an o blast cell clone developed without a neighboring posterior primary blast cell clone. The frequencies of pattern elements that could be unambiguously identified in the experimental clones did not differ significantly from the frequencies observed in control clones. (B) Frequency of pattern elements observed in embryos in which p primary blast cell clones developed in the absence of a neighboring posterior blast cell clone. All pattern elements characteristic of the P lineage were observed at frequencies similar to those of control p primary blast cell clones which developed with a primary blast cells on their posterior side. Note that pz6/LD1 are counted as present even if only one cell body can be visualized.

not followed and we observed persistent labeled debris at stage 9 in only 4/37 embryos. Embryos in which single blast cells had been successfully ablated were reared to stage 9 and prepared for analysis by fluorescence microscopy as described above.

Statistical Analysis

Differences in the presence of pattern elements between experimental and control groups were analyzed using Pearson's χ^2 test. A cumulative χ^2 value was calculated for all pattern elements in each experiment. To be sure that any significant differences in frequency between experimental and control groups that affect some but not all pattern elements (cell fate transformation of part of the clone) would be detected, a χ^2 value was also calculated for individual pattern elements that had the largest differences in frequency between experimental and control groups for each experiment. We did not detect any significant differences between experimental and control pattern elements when pattern elements were considered individually ($P > 0.05$); thus all reported values are cumulative values.

RESULTS

To determine whether signaling occurs between primary blast cell clones along the A/P axis, we experimentally removed primary blast cells from developing embryos and then examined whether the neighboring primary blast cell clones produced their normal complement of differentiated descendants or pattern elements. We investigated potential signaling along the A/P axis in two of the four ectodermal lineages, the O lineage and the P lineage.

After performing experimental manipulations on o and p blast cells, we raised the embryos to late stage 9 of embryogenesis, by which time the remaining primary blast cell clones had produced differentiated descendants. Nine distinct pattern elements were scored for experiments on the O lineage and 11 pattern elements were scored for experiments on the P lineage. Even in unoperated embryos, not every pattern element can be identified with 100% accu-

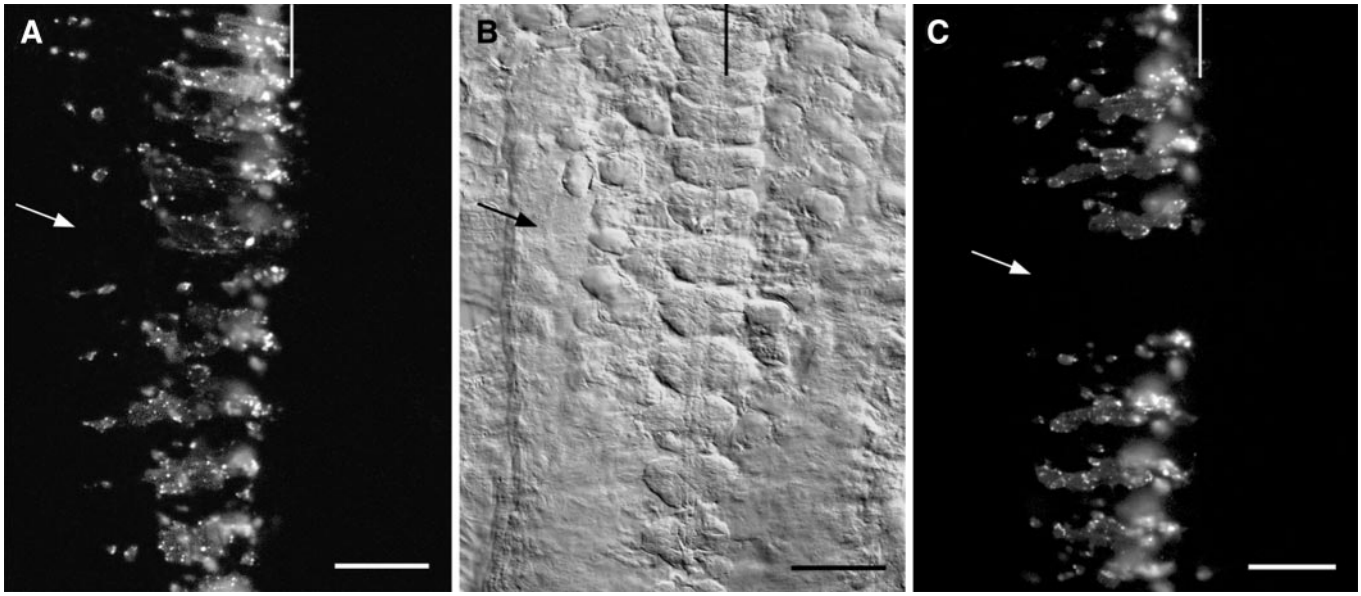

FIG. 4. Stage 9 embryos in which the O teloblast lineage had been labeled with rhodamine dextran and a single labeled blast cell ablated with the laser microbeam at stage 7. The serial repetition of differentiated cells along the A/P axis is apparent in the pattern of labeled cells in A and C and in the segmental ganglia in B. Anterior is to the top and the vertical lines mark the midline. (A) In this embryo, ablation of a single primary blast cell resulted in a gap corresponding to a single blast cell clone. Note the missing pattern elements at the site of the ablation (arrow). Because of the interdigitation of adjacent clones, when a single clone is missing and there is no slippage, the apparent gap in labeled tissues appears to be less than one segment in length. (B) Corresponding differential interference contrast image for A showing the segmentally repeated ganglia of the CNS. Arrow is positioned identically. Clones immediately adjacent to the site of the ablation appear normal at a gross level (A and C) and have all pattern elements characteristic for the O lineage. (C) In some embryos, the gap observed at the site of the laser ablation (arrow) widened as a result of "bandlet slippage" (Shankland, 1984). Primary blast cell clones adjacent to the site of the ablation in embryos in which slippage occurred developed indistinguishably from blast cell clones in embryos in which slippage did not occur. Scale bars, 50 μm.

racy in every clone. This is largely due to the inherent difficulty of observing a specific labeled cell in a region of the nervous system or body wall that contains many other labeled cells. As a consequence, some pattern elements are inherently more difficult to score than others (see controls, Figs. 3 and 5). We scored pattern elements as present only when they were unambiguously distinguishable from other surrounding labeled cells and also scored labeled control clones in each experimental embryo to normalize for labeling intensity and overall health.

Posterior Clones Are Not Required for Normal Segmental Development

We initially examined potential interactions between adjacent primary blast cell clones by injecting DNase I into the teloblast after it had already produced several blast cells. This prevents further cell divisions, thus eliminating the birth of any additional primary blast cells. Using this technique, we removed potential signals being transmitted from one primary blast cell clone to another in the posterior-to-anterior direction. When DNase I is injected into either the O or the P teloblast approximately 1 h following injection of rhodamine dextran lineage tracer, a single labeled primary blast cell is produced, and this blast cell will develop in the complete absence of other blast cells from that same lineage on its posterior side (Fig. 1B).

In the O lineage, a primary blast cell clone that develops under these conditions (n = 26) produces its normal complement of descendants (Figs. 2C and 3A). Control clones were scored in those embryos in which two labeled primary blast cells had been produced before the DNase I injection, with the control being the more anterior of the two labeled clones, i.e., the one farther from the ablation. The frequency with which we could unambiguously identify O lineage pattern elements in the experimental clones (immediately anterior to the ablation) is nearly identical to that of the corresponding pattern elements in control clones (n = 10) (Fig. 3A). In addition, we saw no evidence that any of the normally occurring pattern elements had duplicated in the experimental clone to replace the missing tissue, nor did we observe the formation of any differentiated cell types that are not normally produced by an o blast cell clone. All of the pattern elements were properly positioned, and the boundaries of the clones were essentially normal, indicating that descendants of the labeled blast cell did not migrate

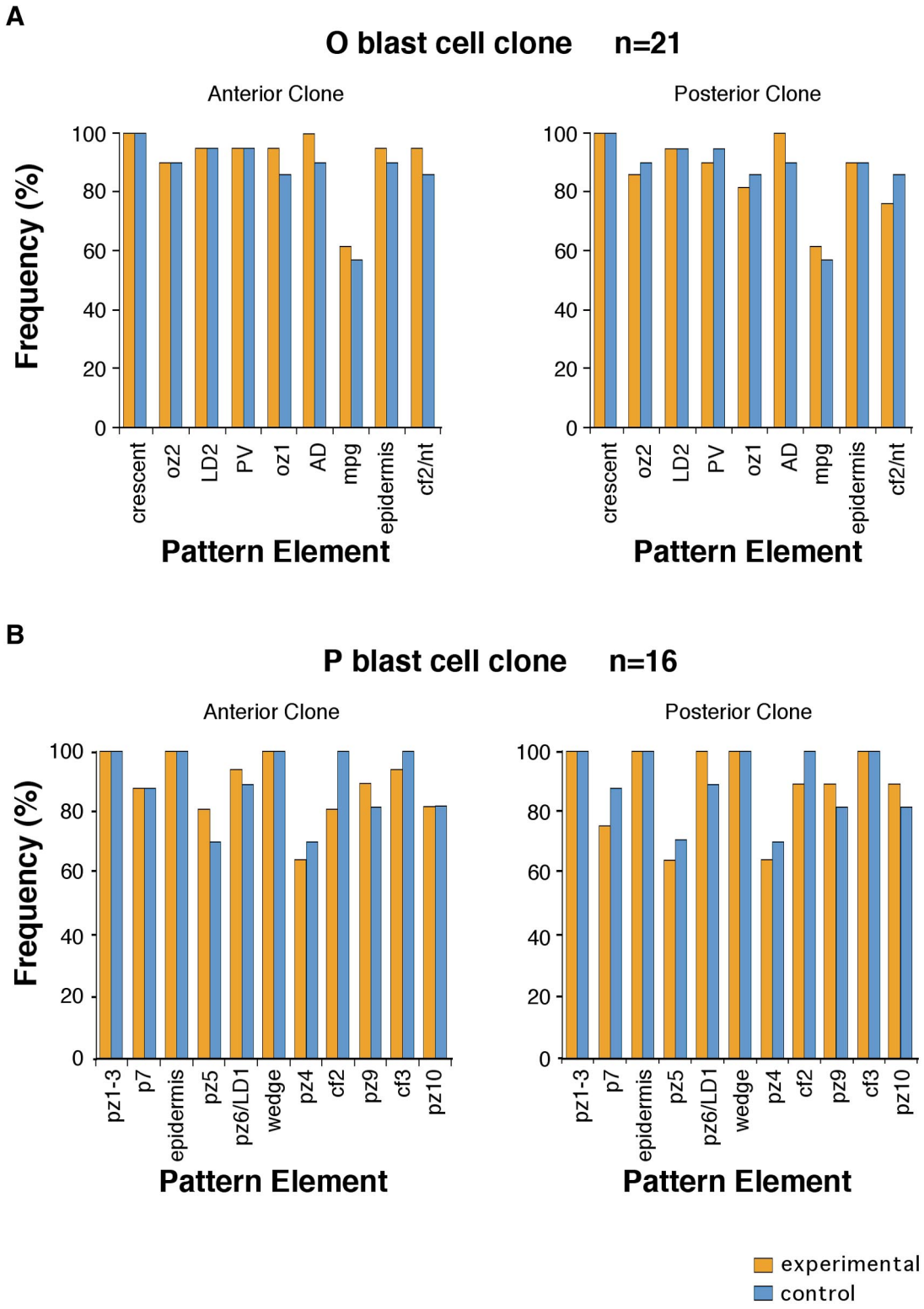

FIG. 5. Laser ablation of primary blast cells reveals that A/P signaling between adjacent primary blast cell clones is not required for normal segmental development. Single primary blast cells were ablated with a laser microbeam, and the development of neighboring anterior and posterior primary blast cell clones was assessed by scoring for the presence of differentiated cells or pattern elements at embryonic stage 9. Pattern elements characteristic for each lineage are listed. In every experimental embryo, a labeled control clone was scored two

into the region of the ablation. Hence, there was no apparent change in segment polarity. Therefore, it appears that the ability of a primary blast cell from the O lineage to produce its full complement of normal descendants is not dependent upon interactions with the primary blast cell clone immediately posterior to it. In only 1 of a total of 26 embryos did we observe a single labeled pattern element (neuron LD2) positioned in the region of the missing blast cell clones. It appears to have arisen by a relatively rare occurrence of compensatory regulation since there was not a corresponding loss of this pattern element from the next anterior segment.

In a separate set of experiments, DNase I was injected into the teloblast of the P lineage. The primary blast cell born just before DNase I injection ($n = 17$) produces all the differentiated cell types characteristic of the P lineage (Figs. 2D and 3B) with approximately the same frequencies as control clones ($n = 12$). Similar to what we observed in manipulations of the O lineage, DNase I injections into the P teloblast did not produce any abnormalities affecting the overall morphology of the clone, duplications, or improper positioning of pattern elements. We did observe minor spreading of labeled epidermis into the site of missing blast cell clones (compare Figs. 2B and 2D), and it has been previously observed that epidermis often spreads to fill in an epidermal deficit produced by teloblast ablation (Blair and Weisblat, 1984). We did not observe any labeled neurons, cell florets, or glia at the site of the missing blast cell clones. Thus, our results from the DNase I injections suggest that primary blast cell clones do not require cell–cell interactions with the next posterior clone in order to develop normal segmental periodicity or polarity in either the O or the P lineages.

Laser Ablation of Primary Blast Cells Reveals That neither Posterior nor Anterior Signaling Is Required for Segmental Organization

We also assessed potential cell–cell interactions along the A/P axis by utilizing a focused laser microbeam to ablate single primary blast cells (Fig. 1C). We followed the development of neighboring primary blast cell clones through use of a lineage tracer that was injected into the teloblast prior to the laser ablation. One advantage of the laser ablation technique is that potential changes in cell fate can be examined both anterior and posterior to the missing blast cell clone, and thus cell interactions in both directions can be assayed in the same embryo. A control clone was scored in each experimental embryo and was located two blast cell clones posterior to the ablation. We performed laser ablations of primary blast cells in both the O and the P lineages, a distinct set of experiments for each lineage. We observed similar results for both lineages.

The development of primary blast cell clones immediately anterior to the site of the laser ablation showed no significant alteration in cell fate in either the O or the P lineage (Figs. 4A, 4C, and 5), consistent with the results obtained with the teloblast ablations (see above). The general morphology of both o and p blast cell clones was normal (Figs. 4A and 4C). Furthermore, the blast cell clone retains its normal borders. Pattern elements were found in appropriate locations with the exception of minor epidermal spreading. Closer inspection of descendant clones anterior to the site of the laser ablation revealed that all pattern elements characteristic of the O ($n = 21$) (Fig. 5A) and P ($n = 16$) (Fig. 5B) lineages could be identified at frequencies similar to that observed in control clones (for O, $P > 0.5$; χ^2 test; for P, $P > 0.95$; χ^2 test). These results confirm our observations in which we eliminated production of neighboring primary blast cell clones to the posterior by injecting the teloblast with DNase I. Therefore, it appears that there are no inductive signals necessary for the normal development of primary blast cells passing from posterior to anterior in the O or P lineages.

We also analyzed primary blast cell clones that developed immediately posterior to the site of the laser ablation. The general morphology and boundaries of such primary blast cell clones appear normal (Figs. 4A, 4C, 5A, and 5B). Detailed analysis of the differentiated descendants revealed that the frequency with which particular pattern elements could be unambiguously identified is very similar to that seen in control clones in both the O ($n = 21$) and the P ($n = 16$) lineages (for O, $P > 0.5$; χ^2 test; for P, $P > 0.7$; χ^2 test) (Figs. 5A and 5B). Specifically, all pattern elements characteristic of each lineage were present at expected frequencies, and we did not see duplications of pattern elements. With the few exceptions noted below, we did not observe any changes in overall size or position of individual pattern elements.

In a small proportion of embryos, we observed one or two

segments posterior to the posterior experimental clone. Data taken from controls are shown as blue histogram bars, and data taken from experimental clones are shown as orange histogram bars. The anterior clone histograms represent data from primary blast cell clones whose posterior neighbor had been ablated. Conversely, the posterior clone histograms represent data from blast cell clones whose anterior neighbor had been ablated. (A) Frequency of O pattern elements unambiguously identified in descendant clones in which an o primary blast cell was ablated. In both the anterior clone and the posterior clone, pattern elements are present with a frequency similar to that observed in control clones (in the blast cell clones anterior to the ablation $P > 0.5$; χ^2 test; the blast cell clones posterior to the ablation, $P > 0.5$; χ^2 test). (B) Frequency of P pattern elements identified in embryos in which a p primary blast cell was ablated with the laser microbeam. The frequency of observed pattern elements in both experimental clones (anterior and posterior) is similar to that observed in control clones (the blast cell clone anterior to the ablation, $P > 0.95$; χ^2 test; the blast cell clone posterior to the ablation, $P > 0.7$; χ^2 test).

labeled pattern elements at the site of the blast cell ablation (n = 5/37, 3 cases in the O lineage and 2 cases in the P lineage). These differentiated cells were always identifiable as cell types characteristic of the lineage being analyzed, and in each case there was only one or a few labeled cells in this position, compared to a total of roughly 70 cells in a complete blast cell clone (Shankland and Weisblat, 1984). In two cases, the appearance of a particular pattern element at the site of the ablation corresponded with a loss of that same pattern element from the neighboring clone, suggesting an inappropriate migration of that cell into the gap left by the ablation. In two other cases, the mislocated pattern elements appeared to have arisen by the separation of a cluster of cells, with part of the cluster migrating into the gap left by the ablation. Our analysis does not allow us to detect the loss of one or two cells from a large patch of labeled cells (i.e., in the epidermis). In only 1/37 cases did we observe a normal-sized pattern element (the crescent cluster of central neurons in the O lineage) at the site of the ablation without an obvious and corresponding deficit of that same pattern element from the adjacent segment. In this one case, labeled cells might have arisen by regulation from one of the neighboring labeled clones. In summary, there was little evidence from these experiments that a blast cell clone adjacent to the laser ablation had undergone compensatory regulation or misspecification to generate additional pattern elements that would normally have arisen from the ablated cell.

In the O and P lineages, it is approximately 24 h from the time of birth of a primary blast cell until it undergoes its first division, and we performed the blast cell ablations at several different time points. In early experiments we ablated the primary blast cell soon after it entered the germinal band (approximately 10 h after its birth). However, we saw no difference in the development of the adjacent blast cell clones whether we ablated the targeted blast cells as they entered the germinal band or at a later stage just before the primary blast cell undergoes its first division.

Bandlet Slippage

In approximately half of the embryos in which a primary blast cell was ablated (50% in the P lineage and 55% in the O lineage), a phenomenon called "bandlet slippage" occurred (Shankland, 1984). During normal development the primary blast cells within a teloblast lineage are tightly apposed, but ablation of a single primary blast cell causes a break in the chain that can open up to form a wider gap of up to five segments in width (Fig. 4C). Previous work indicates that slippage does not involve cell fate respecification, but rather a posterior displacement of already specified cells in the posterior fragment of the broken chain (Gleizer and Stent, 1993; Martindale and Shankland, 1990; Nardelli-Haefliger and Shankland, 1993). Slippage usually occurred in the first few hours after the ablation and accentuated the separation of labeled neighboring clones on either side of the ablated cell.

We observed no differences in the development of either o or p primary blast cell clones immediately adjacent to a blast cell ablation whether they had undergone slippage or not (Figs. 4A and 4C). In previous studies, it was observed that blast cells that had slipped along the A/P axis gave rise to their normal patterns of descendant tissues, even with respect to segmental identity (Martindale and Shankland, 1990; Shankland, 1984). Thus, all evidence suggests that the development of blast cell clones that have experienced slippage is not substantially different from that of those that develop *in situ*, and we therefore grouped both categories together in our numerical analysis (Fig. 5). The fact that we did not observe any differences between these two categories further supports our conclusion that neighboring clones are not required for normal development of primary blast cell clones since slippage separates surviving blast cell clones on the two sides of the ablation.

Normal Segmental Organization Develops in the Absence of both Anterior and Posterior Blast Cell Clones

We also investigated the possibility that interactions with a neighboring clone on *either* the anterior or the posterior side is both required and sufficient for normal development. To address this possibility, individual primary blast cell clones in either the O or the P lineage were isolated by ablating the adjacent primary blast cells on both sides (Fig. 1D). In nine experimental embryos, the gaps produced by these two ablations persisted into stage 9. In five other experimental embryos the gap produced by the anterior ablation persisted, but there was no posterior gap visible at stage 9. We believe that the latter outcome arose from closure of the posterior gap (see below) and consequently treated the clone at the posterior edge of the remaining gap as being a transiently isolated experimental clone. In both groups, the first labeled clone produced by the injected teloblast—located one or two segments anterior to the first ablation—was scored as a control.

For the most part, primary blast cell clones develop normally in the combined absence of posterior and anterior neighbors. The experimental clones did not spread beyond their normal boundaries, and we found that isolated clones in both the O (n = 8) and the P (n = 6) lineages produced the appropriate set of differentiated descendants (Fig 6) (for O, $P > 0.95$; χ^2 test; for P, $P > 0.5$; χ^2 test). Similar to the single-cell ablation experiments, we did not observe any consistent changes in cell fate specification, duplication of pattern elements, or mismigration of cells. There was one isolated o blast cell clone in which three of nine pattern elements were visibly abnormal: the crescent cluster was larger than normal, the AD cluster was smaller than normal, and the nephridial tubule was located in the wrong segment. But despite this singular anomaly, it would appear that short-range signals passing in either direction along the A/P axis between primary blast cell clones play little if any

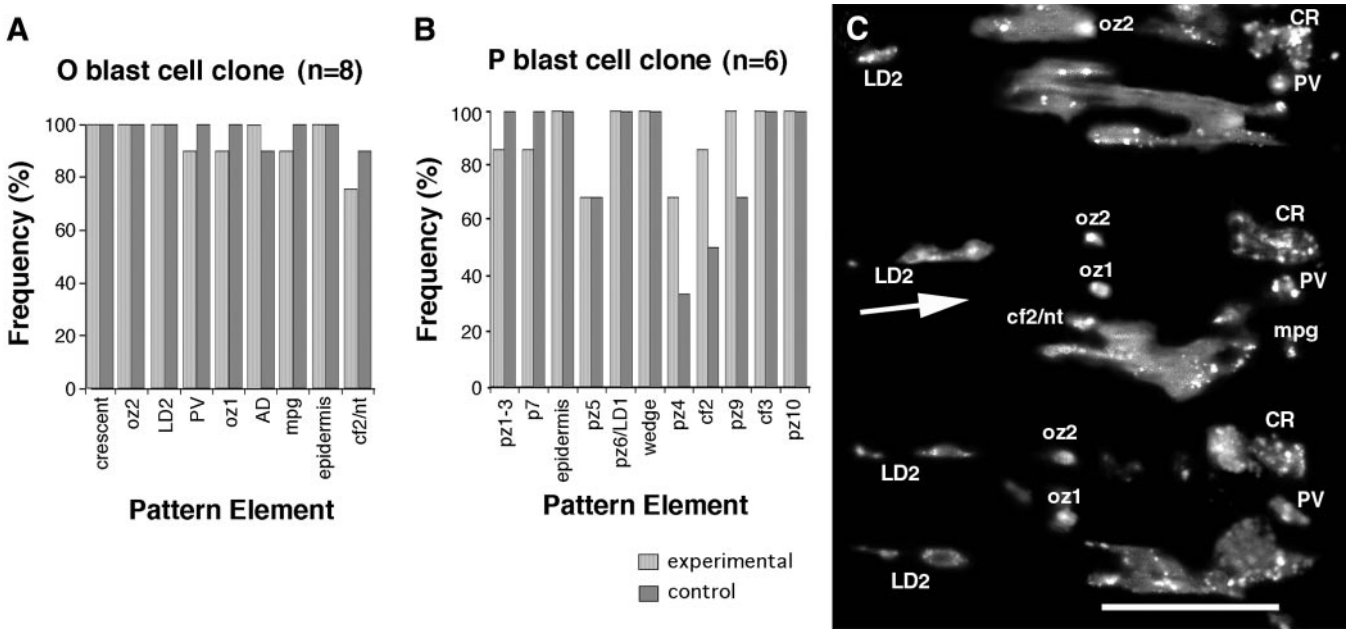

FIG. 6. Primary blast cell clones develop normally in the absence of any neighboring clones from the same teloblast lineage. In these experiments, two primary blast cells were ablated with the laser microbeam, isolating the experimental clone from both of its normal anterior and posterior primary blast cell clones. Histograms showing the presence of pattern elements in experimental (light bars) and control (dark bars) primary blast cell clones in the O lineage (A) and the P lineage (B) are presented. The frequencies with which pattern elements were observed are not significantly different between experimental and control blast cell clones for ablations in both the O lineage ($P > 0.95$; χ^2 test) and the P lineage ($P > 0.5$; χ^2 test). (C) Fluorescent micrograph from an embryo in which two primary blast cells in the O lineage were ablated. In the embryo shown, one blast cell clone is missing on both the anterior and the posterior side of the experimental clone (arrow). Scale bar, 50 μm.

role in specifying the cellular constitution or A/P polarity of those clones.

Similar to what we found with the ablation of single blast cells, the gap produced by the more anterior ablation often grew wider because blast cells situated behind the ablation slip posteriorly in relation to the germinal band as a whole (Shankland, 1984). In contrast, we never observed widening of the gap produced by the posterior ablation, and in 5 of the 14 embryos in which two primary blast cells were laser-irradiated it appeared that the posterior gap had actually closed. We cannot rule out the possibility that the second laser ablation failed in these embryos, but the otherwise reliable success of our ablation paradigm argues to the contrary. There are previous reports for both the M lineage (Gleizer and Stent, 1993) and the N lineage (Ramirez *et al.*, 1995) that a gap produced by ablation of a single blast cell can close during subsequent development, and such closure would seem a likely explanation for the absence of posterior gaps in this subset of our experimental embryos.

DISCUSSION

We have shown that primary blast cell clones produce their normal complement of descendants with a large degree of autonomy during formation of the segmental ectoderm in the leech embryo. We find no evidence for cell interactions between adjacent primary blast cell clones along the A/P axis in either of the two ectodermal lineages examined. This is in contrast to what is known about the establishment of segmental repeats in the *Drosophila* ectoderm, in which cell interactions along the A/P axis play a critical role in the development of segmental repeats (Heemskerk and DiNardo, 1994; Lawrence *et al.*, 1996; Lawrence and Struhl, 1996).

We utilized three distinct approaches to examine the development of primary blast cell clones in the absence of their normal anterior and/or posterior neighbors. First, we removed potential signals from more posterior blast cell clones by preventing the teloblast from producing posterior blast cells through injection of DNase I. Second, we used a laser microbeam to ablate a single primary blast cell within a column of blast cells to examine whether inductive signals are normally transmitted in either the anterior or the posterior direction. Finally, we performed laser ablations of two primary blast cells within the column, forcing the single, intervening blast cell clone to develop in the absence of both its anterior and its posterior neighbor. In all three experiments, we observed normal specification of cell

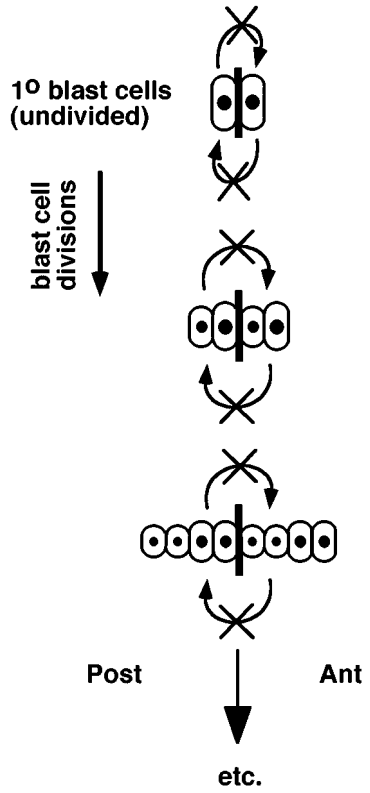

FIG. 7. Schematic illustrating that each blast cell clone develops independent of anterior and/or posterior neighboring clones throughout development. Ablation of primary blast cells reveals that the descendants of primary blast cells do not interact with cells from neighboring clones. For example, even when the primary blast cell has divided and its clone contains two or four cells, there cannot be any cell–cell interactions between neighboring clones that are necessary for normal development or they would have been revealed by primary blast cell ablations. Ant, anterior. Post, posterior.

fates as well as normal positioning of descendant cells in clones immediately adjacent to the ablation.

It should be noted that these ablations eliminate potential cell interactions throughout the entire development of the blast cell clone. For example, a primary p blast cell initially divides to produce a clone of four cells aligned along the A/P axis (Shankland, 1987b). If the posteriormost granddaughter cell were to require a signal from the next posterior p blast cell clone to develop normally, we would expect its fate to change when that posterior blast cell was ablated (Fig. 7). Such a result should be easily detected in our experiments, since 4 of the 11 P pattern elements scored have contributions from the posteriormost granddaughter. Thus, it appears that specification of cell fates occurs independent of interactions with more anterior or posterior clones throughout the time when a primary blast cell develops to produce a clone of approximately 70 differentiated descendants. It should be noted that cell interactions between clones are distinct from cell interactions *within* a blast cell clone, and the latter point will be addressed in a later paper (Seaver and Shankland, in preparation).

The degree of developmental autonomy between adjacent blast cell clones is particularly noteworthy when one considers that descendants from one clone intermingle at its anterior–posterior borders with descendants from adjacent clones (Weisblat and Shankland, 1985). Hence, our data not only indicate that adjacent o or p blast cell clones do not interact in the specification of descendant cell phenotypes, but also that the morphogenetic process of clonal mixing is not coordinated by interaction between clones. However, this is not true for all teloblast lineages—in the N lineage, the positions occupied by descendants of one blast cell clone can be influenced by more posterior clones (Blair and Weisblat, 1982).

It should be noted that there are limitations to the laser ablation technique. For instance, we were unable to ablate the primary blast cell until approximately 10 h after its birth—when it first reaches the surface of the embryo—and could not prevent it from sending signals prior to that time. Such signals could be acting on the neighboring primary blast cells in such a way as to specify the cellular constitution and/or polarity of their descendant clones. However, we do feel confident that no segment polarity information is being transferred during this interval in the posterior-to-anterior direction, since DNase I ablation of the teloblast yields results identical to laser ablation of the next posterior blast cell.

Although we find it unlikely, there is a formal possibility that the dying cell or cell debris could transmit molecular signals that affect adjacent blast cell clones. We often observed the phenomenon of slippage (Shankland, 1984), in which the gap between blast cells on either side of an ablation rapidly widens. Blast cell clones immediately adjacent to the ablation developed normally both in embryos in which slippage occurred and in embryos in which it did not. This indicates that the distance between the site of ablation—i.e., anticipated site of cellular debris—and the neighboring blast cell clone is irrelevant and further suggests that there are no essential inductive signals passing between the blast cell clones on either side of the ablation.

In a small proportion of experimental embryos we observed one or two labeled blast cell descendants at the site of an ablation. Although these cases are few, we cannot rule out the possibility that they result from compensatory regulation or misspecification affecting a small percentage of cells in one of the adjacent clones. No examples of compensatory regulation have been reported in leech, although replacement regulation (i.e., *trans*-fating that replaces an ablated cell lineage at the expense of another lineage) has been described (Weisblat and Blair, 1984). There are also reports that one or a few cells can migrate from an otherwise normal blast cell clone into the site of an ablation (Stuart *et al.*, 1989). Because the labeled pattern elements at the site of an ablation varied in identity and occurred rarely

in our experiments, we believe that they represent experimental anomalies rather than evidence of inductive signals that are required for normal development.

Our experiments address only the development of primary blast cell clones in the O and the P lineages. However, generally consistent results have been reported for other teloblast lineages as well. In the ectodermal N lineage, there are two classes of blast cells, n_f and n_s, that occur in alternating order (Bissen and Weisblat, 1987). Together, each pair gives rise to one segmental complement of descendants, which comprise approximately $\frac{2}{3}$ of the CNS. Photoablation of either of these two n primary blast cells results in a loss of regions of the ganglion normally descended from that cell (Ramirez *et al.*, 1995), with other parts of the ganglion appearing morphologically normal. However, single cell fates were not examined, and it is not clear whether blast cells adjacent to n_f or n_s ablations might have experienced changes in segment polarity or respecification of certain sublineages. In studies of the mesodermal M lineage, gaps were introduced by directly injecting DNase I into single primary blast cells (Gleizer and Stent, 1993). Once again, gross morphologies suggested no major change in blast cell fate on either side of the ablation, but only a few pattern elements were examined. Here we show that the autonomy of blast cell clones in the O and P lineages with respect to A/P cell interactions not only is manifest in terms of general morphology or tissue type, but can in fact be extended to essentially all descendant cell phenotypes.

Although our experiments do not reveal any A/P cell interactions that are required to specify the segmental periodicity or polarity of the primary blast cell clone, it is possible that blast cell clones might receive such information from another source, e.g., signals conveyed at least in part through the transverse plane. Primary blast cell clones in the O and P cell lineages are in direct contact with one another, as well as with blast cell clones from the two flanking ectodermal lineages (N and Q) along the dorsoventral axis. Ectodermal blast cell clones are also in contact with underlying blast cell clones of the mesodermal cell lineage (M) and with an overlying epithelium of micromere derivatives (Stent *et al.*, 1992). Ablation experiments have revealed that the mesoderm and ectoderm influence one another's morphogenesis (Blair, 1982). In addition, the differential specification of teloblast identity between the O and the P lineages depends upon an integration of intercellular signals conveyed in the dorsoventral axis between the o and the p blast cells (Shankland and Weisblat, 1984), the q blast cells (Huang and Weisblat, 1996), and the micromeres (Ho and Weisblat, 1987). However, none of the ablation studies performed to date has given any indication that intercellular signaling in the transverse plane plays a role in segment polarity, i.e., the differential specification of anterior and posterior cell fates within a blast cell clone.

It is interesting to compare the apparent absence of A/P signaling between segment repeats in leech with other animals. Malacostracan crustaceans undergo segment formation in a manner that has several parallels with the leech. In malacostracans, postnaupliar segments are generated from ectoteloblasts and mesoteloblasts by stereotyped cell lineages (Dohle and Scholtz, 1988), and the teloblasts divide in stem cell fashion to generate "row cells" that serve as segment founders. Analysis of the *en* expression pattern has led to the suggestion that segment polarity of the crustacean embryo is being specified by cell interactions (Scholtz, 1995; Scholtz *et al.*, 1993). But there are no experimental manipulations of crustacean embryos that directly address whether such interactions are required for normal segmentation.

In *Drosophila*, interactions between cells of adjacent segmental repeats are critical for the formation of segment borders and the patterning of A/P cell fates within each segment. Formation of segments in *Drosophila* is not lineage-based and depends instead upon the subdivision of a field of cells into compartments. This process requires the segment polarity gene *en* (Fjose *et al.*, 1985; Poole *et al.*, 1985), which initiates a sequence of intercellular signals directed along the A/P axis (DiNardo *et al.*, 1988). These intercellular inductive signals are conveyed across both the segmental (Heemskerk and DiNardo, 1994) and the parasegmental borders (DiNardo *et al.*, 1988) and pattern cell fates along the A/P axis of the adjacent segment/parasegment. Despite their lineal stereotypy, it is theoretically possible that the segments of leech embryos might also be generated by stereotyped subdivision of a field of equipotent cells. However, our results demonstrate that primary blast cell clones do not regulate for one another along the A/P axis, nor are interclonal signals required for patterning within the blast cell clone. Thus, it appears that the stem cell divisions of the leech teloblast do in fact generate a sequence of discrete, autonomously specified segment founder cells.

Even though there are clear differences in the cellular basis of segmentation in fly and leech, there could nonetheless be an underlying similarity in the molecular mechanisms employed. In the leech embryo *en* is expressed in a segmentally iterated pattern (Lans *et al.*, 1993; Wedeen and Weisblat, 1991) akin to that seen in *Drosophila*. In the fly, expression of *en* is required to initiate the intercellular signaling that ultimately establishes A/P pattern within any given segment (Heemskerk and DiNardo, 1994; Lawrence *et al.*, 1996). One might have anticipated that expression of *en* during the early stages of leech segmentation could also initiate cell interactions required for normal segmentation. However, our blast cell ablations are performed well in advance of *en* expression, and the results indicate that any *en*-initiated intercellular signals generated during leech segmentation are not required for the normal patterning of the adjacent segmental repeats. The present findings do not preclude the possibility that *en* expression initiates intraclonal cell interactions. Still, the fact that adjacent segmental units do exchange patterning information during *Drosophila* development but do not in leech

emphasizes that there are significant differences in the mechanism by which these species generate segments.

ACKNOWLEDGMENTS

This work was supported by an NIH NRSA (5F32GM19257-02) to E.C.S. and by a NASA research grant (NAG2-1349/98-HEDS-02) and an NSF research grant (IBN-9796063) to M.S.

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Received for publication March 7, 2000
Revised May 12, 2000
Accepted May 15, 2000