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Cell lineage and segmentation in the leech

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[Plate 1]

Segments in the leech arise by the proliferation of longitudinally arrayed bandlets of blast cells derived from ten identifiable embryonic stem cells, two M, two N, four O/P and two Q teloblasts. In each bandlet, older blast cells lie ahead of those born later. By using microinjected cell lineage tracers it was shown previously that the teloblasts give rise to characteristic cell patterns made up of segmentally iterated complements of progeny designated as M, N, O, P and Q kinship groups. When a teloblast is injected after it has begun generating blast cells, a boundary is observed later in development between anterior, unlabelled progeny of blast cells produced before injection and posterior, labelled progeny of blast cells produced after injection. We have examined such boundaries in detail to establish the precise relationship between blast cell clones and segments, with the following conclusions: (i) in the M, O and P cell lines, one blast cell generates one segmental complement of progeny, but serially homologous blast clones intermix so that no segment boundaries can be defined based on primary blast cell clones; (ii) in the N and Q cell lines, two blast cells are required to generate a complete segmental complement of progeny; (iii) in the process of forming the germinal plate, cells derived from the N and Q teloblasts move past those derived from the M and O/P teloblasts, so that consegmental blast cell clones do not come into register until well after the establishment of segmentally iterated units within each bandlet.

Introduction

It is generally accepted that the variously limbed and winged arthropods evolved from vermiform ancestors which also spawned, and probably resembled, the modern annelids. A major feature common to all of these groups is the organization of the body into repeating units called segments. Thus, the related questions of how segmentally iterated cells arise from the egg during embryogenesis and how segmentation in more complex forms has evolved from processes operating in simpler forms may be approached by studying such invertebrates. The relationship between cell lineage and segmentation in insect development has been extensively investigated. Here we describe studies of cell lineage and segmentation in an annelid, the glossiphoniid leech *Helobdella triserialis*. The comparisons to be made are doubly interesting because, although the segments of annelids are phyletic homologues to those of insects, they arise by very different cellular mechanisms (Anderson 1973).

In insects, the fertilized egg undergoes several rounds of karyokinesis without cytokinesis. A few thousand syncytial nuclei are formed, most of which migrate to the periphery of the egg, forming a syncytial blastoderm; cellularization ensues and the embryo proceeds to gastrulate.

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Evidence coming mostly from investigations of *Drosophila* suggests that the syncytial nuclei are of equal developmental potential before migration; however, by the time cellularization occurs, commitment has progressed to the extent that groups of neighbouring embryonic cells have become specified to act coordinately in the formation of repeating units of the mature body pattern (Lawrence 1981). These founder cell groups were first postulated because their descendants remain spatially segregated throughout subsequent development, thus giving rise to distinct compartments of the mature insect body (Garcia-Bellido *et al.* 1973; Lawrence 1973; Crick & Lawrence 1975). It seems likely that these same groups of coordinately specified cells are distinguished in the early *Drosophila* embryo by spatially periodic patterns of transcription (Hafen *et al.* 1984). There is some doubt as to whether the initial repeating unit corresponds to the traditionally defined body segment (Martinez-Arias & Lawrence 1985), but in any case segmental periodicity is thought to arise by the partitioning of a large expanse of cells into multicellular regions. This view of segmentation is consonant with studies of vertebrate embryogenesis, where the mesoderm is partitioned into somites by the separation of groups of cells from a single large mass (Hamilton 1969).

In contrast to the insect mode, annelid embryogenesis proceeds from the start through holoblastic cleavages. Individual blastomeres can be identified by various criteria including size,

Description of plate 1

Figure 1. (*a*) Anterior boundary of the O pattern in a stage 10 leech embryo viewed from the ventral surface. In this embryo HRP was injected into an O/P teloblast which had already begun blast cell production, and the anterior boundary of the labelling pattern reflects a boundary between unlabelled and labelled blast cell clones. Compare with figure 5. (*b*) Anterior boundary of an HRP-labelled M pattern in a stage 10 embryo viewed from the side, with ventral to the right. The arrow at the right marks the anterior edge of the labelled musculature within the germinal plate. Label is also evident within the muscle fibres of the provisional integument; however, the full complement of these provisional muscle fibres is not labelled until several segments more posteriorly (left arrow). The dark material that lies anterior to labelled the musculature is botryoidal tissue, which stains during HRP reaction even in unlabelled embryos (Weisblat *et al.* 1984). (*c*) Anterior boundary of an M pattern labelled with rhodamine–dextran and viewed from the ventral surface by fluorescence microscopy. The anteriormost pair of labelled connective muscles (c.m.) lie in the ganglion anterior to the anteriormost labelled cluster of M-derived central neurons (m.n.). (*d*) Lateral body wall of a stage 10 embryo viewed with Nomarski optics. Cell florets 3 and 4 in two consecutive segments are visible as islands of rounded cell segments. The nucleoli of the surrounding squamous epidermal cells are visible as refractile dots. (*e*) Fluorescence micrograph of the same field, revealing the anterior edge of a rhodamine-labelled M pattern. Note that the anteriormost labelled muscle fibres run along the posterior edge of the cell florets. (*f*) Sibling stage 10 embryos in which pairs of contralateral teloblasts had been injected with HRP partway through blast cell production. Arrows mark the anterior edge of each labelling pattern, although dark botryoidal tissue can be seen more anteriorly. The N pattern is labelled on the left in all three embryos, with its boundary lying near the anterior end of the body. In the embryo at the left the O pattern is labelled on the right, and its boundary is located in a much more posterior segment than the contralateral N boundary. In the middle embryo the P pattern is labelled on the right, and its boundary is also located in a segment much farther posterior than the contralateral N boundary. In the embryo at the right the Q pattern is labelled on the right, and displays a boundary quite close to that of the contralateral N boundary. The differential location of these various boundaries is consistent with the finding that it takes twice as many n or q blast cells as o or p blast cells to generate the same number of body segments. (*g*) Fluorescence micrograph of a stage 7 embryo in which ipsilateral p and q bandlets were labelled with fluorescein–dextran by simultaneous injection of their parental teloblasts 24 h previously. The anteriormost labelled blast cells in each bandlet lie close to one another in the posterior portion of the germinal band. (*h*) Sibling embryo to the one shown in (*g*), photographed 48 h after injection. Note that the labelled p blast cells still lie in the posterior portion of the germinal band, while the anteriormost labelled q blast cells have slid past the p bandlet and now lie on the other side of the egg in a much more anterior portion of the band. The same embryo could not be used for both photographs because the necessary excitation of the fluorescent dye leads to damage of the labelled cells (Shankland 1984). Scale bar; 50 μm in (*a*), (*b*), (*g*) and (*h*); 10 μm in (*c*), (*d*) and (*e*); 100 μm in (*f*). Anterior is up in all panels.

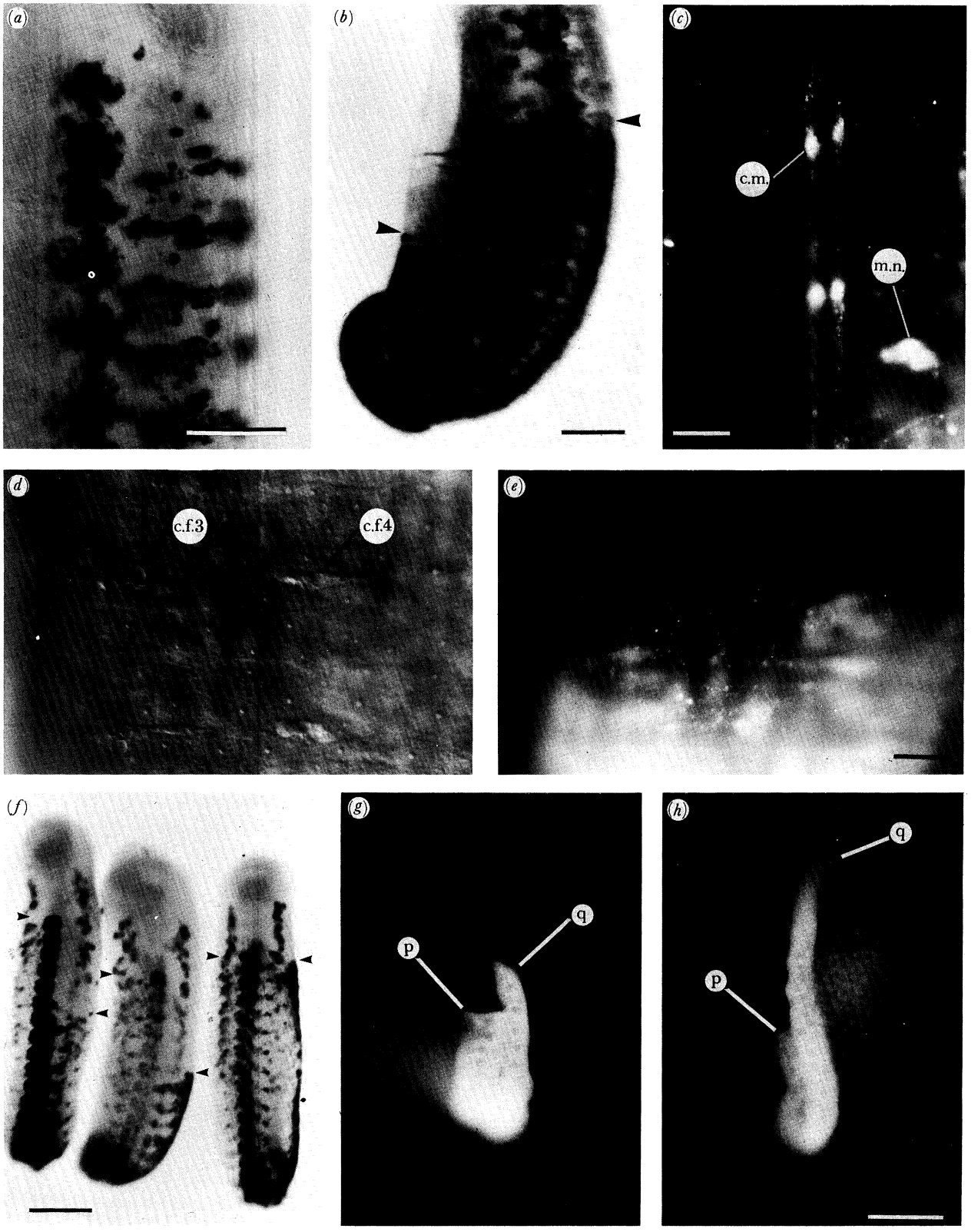

FIGURE 1. For description see opposite.

(*Facing p.* 40)

position, birth order and cytoplasmic specializations (figure 2). This process has been best studied in the embryos of glossiphoniid leeches (Whitman 1878; Stent *et al.* 1982). In such embryos, segmental tissues arise from bilateral sets of five large blastomeres, the M, N, O/P, O/P and Q teloblasts. By a series of several dozen iterated unequal divisions, each teloblast generates a bandlet of much smaller primary blast cells, which are designated by lower case letters corresponding to the teloblast of origin. Ipsilateral bandlets merge to form left and right germinal bands, and the two bands migrate over the surface of the embryo to form a sheet of cells, the germinal plate, along the future ventral midline. Within the germinal plate, the clones of older blast cells in each bandlet lie anterior to those of blast cells born later. Segmental tissues arise by differentiation of blast cell clones.

By using microinjected cell lineage tracers to mark the progeny of selected teloblasts, it has been shown that each teloblast normally gives rise to a particular pattern of segmentally iterated progeny, to which we will refer as the M, N, O, P and Q cell lines. Each cell line includes both neural and non-neural cells (Weisblat *et al.* 1984). In accord with the spatiotemporal arrangement of blast cells within the bandlets, when a teloblast is injected with lineage tracer after it has begun making blast cells, a boundary is observed between anterior, unlabelled segments, containing progeny of blast cells born before the injection, and posterior, labelled segments, containing progeny of blast cells born after the injection (figure 1, plate 1). At first glance, such boundaries are rather sharp, suggesting that there is little longitudinal intermixing of blast cell clones. This observation is consistent with Penner's (1924) suggestion that individual m blast cells might found hemilateral segmental complements of mesodermal tissue. Extending this line of thought to encompass the ectodermal lineages as well, each segment in the leech could be envisioned as a polyclone comprising all the progeny of one or more blast cells from each bandlet. This hypothesis was attractive because of the obvious homology to segmentation in insects. In the work reported here, we have generated boundaries in all the five teloblast lines and examined them in detail. This analysis has allowed us to characterize the definitive segmental distribution of the progeny of the primary blast cells in each bandlet and to determine that the leech segment is in fact *not* a compartment founded by primary blast cells.

Materials and methods

Embryos of the glossiphoniid leech *Helobdella triserialis*, were obtained and maintained as described previously (Blair & Weisblat 1984). The nomenclature used for these embryos is that used by Whitman (1878), as modified by Stent *et al.* (1982) and Weisblat & Blair (1984); the staging system used to assess the developmental progress of the embryos is as described previously (Stent *et al.* 1982). A schematic summary of the developmental features relevant here is provided by figure 2. The use of the enzyme horseradish peroxidase (HRP) and fluorescent molecules as microinjected cell lineage tracers have also been described elsewhere (Weisblat *et al.* 1978, 1980*a*; Gimlich & Braun 1985). For these experiments, selected teloblasts were injected with lineage tracer during stages 6–7, at varying times after the onset of blast cell production. The patterns of labelled descendants near the boundaries were observed at stage 10, by which time segmental structures are well differentiated. HRP was detected by reaction with benzidine in glutaraldehyde-fixed tissue. Fluorescent dextrans were viewed under the fluorescence microscope, either in living embryos anaesthetized with 0.2% (by volume) chlorobutanol or in

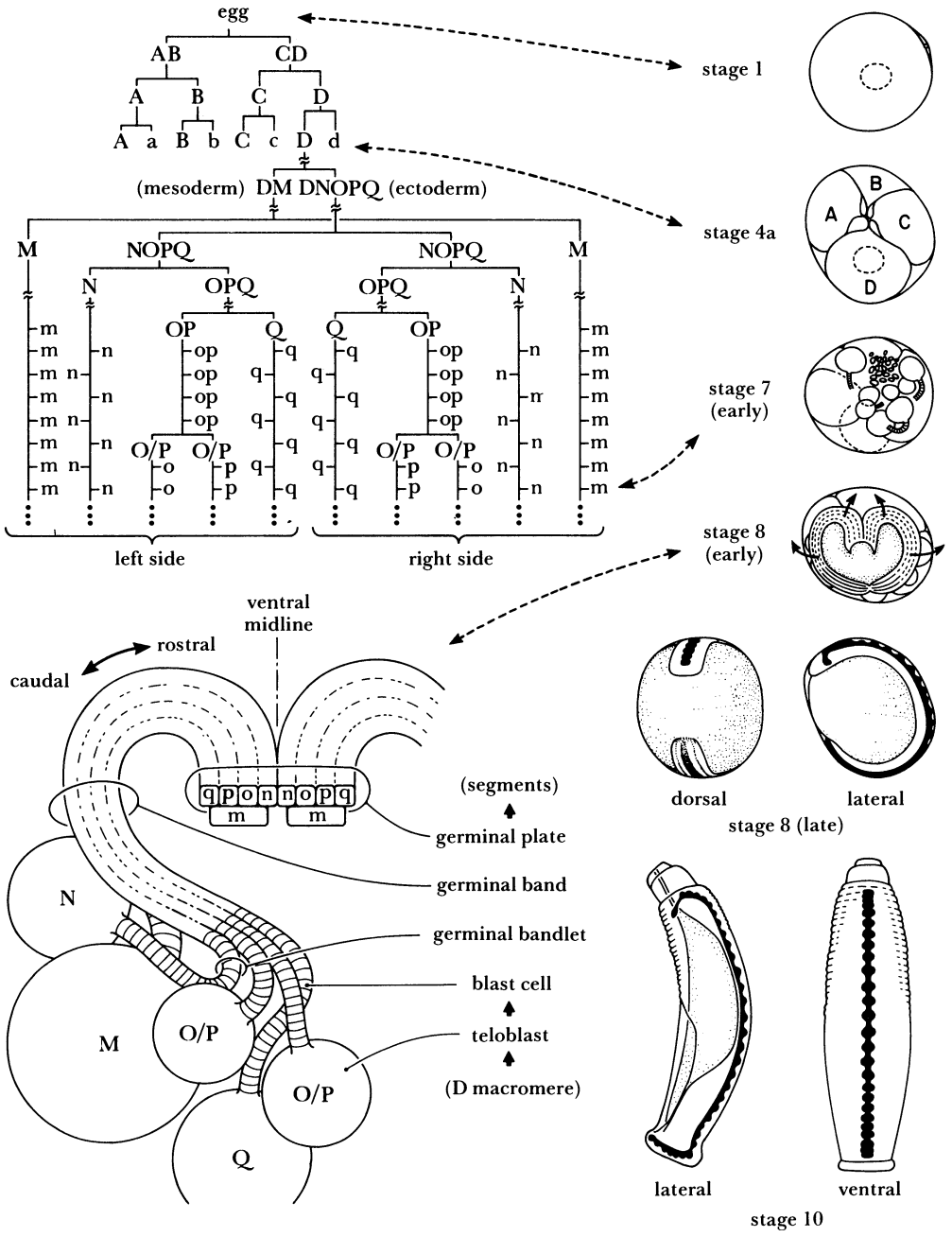

FIGURE 2. Schematic summary of the development of *Helobdella triserialis*. Upper left: cell pedigree leading from the uncleaved egg to the teloblasts M, N, O/P, O/P and Q, and the primary blast cell bandlets. Breaks in the lineage indicate points where additional micromeres are produced (R. Ho, unpublished). Lower left: hemilateral disposition of teloblasts and bandlets in the germinal band and germinal plate. Right margin: diagrammatic view of embryos at various stages. The dashed circle in the uncleaved egg (stage 1) represents specialized yolk-deficient cytoplasm which is passed on selectively via cell D (stage 4a) to the teloblasts. In the stage 7 embryo, the many small contours on the upper portion represent the micromere cap, which gives rise to the provisional integument. The larger circles represent the teloblasts, which are just starting to generate bandlets of primary blast cells. By early stage 8, the bandlets have come together on either side to form a heart-shaped pair of germinal bands, which migrate over the surface of the embryo as indicated by the arrows. Provisional integument is indicated by stippling. In late stage 8, the germinal plate has formed on the ventral midline, with ganglionic primordia indicated in black, and the provisional integument covers the entire dorsal surface. In the stage 9 embryo (not shown) the germinal plate begins to expand dorsally, and in the stage 10 embryo the two edges of the plate fuse along the dorsal midline. Here, stippling indicates yolky remnants of teloblasts and cells A, B and C, now enclosed in the gut. The chain of ganglia linked via connectives now closely resembles the adult nerve cord.

handcut sections, roughly 100 μm thick, of embryos fixed in paraformaldehyde and embedded in glycol methacrylate.

The basis of the analysis of the label boundaries is as follows. If a single blast cell in a bandlet generates all the cells for one segmental complement of the appropriate cell line, then (ignoring segmental specializations) each blast cell in the bandlet is equivalent to its rostral and caudal neighbours. In this case, the blast cells would constitute a simple repeating pattern (that is, aaaa . . .) and the boundary between unlabelled anterior cells and labelled posterior cells should always be at the same position relative to the border of the segment. On the other hand, if two or more blast cells in the bandlet generate a segmental complement of progeny, then adjacent blast cells may not be equivalent within the bandlet and would form a more complex repeating sequence of cells (for example, abab . . ., abcabc . . ., etc.). In this latter case, the position of the boundary between labelled and unlabelled cells relative to the segment border would vary with the identity of the first labelled cell. Thus, a number of different classes of boundary patterns could be observed, equal to the number of primary blast cells in the repeating unit.

Results from the application of this analysis to the n bandlet cells have been published previously (Weisblat *et al.* 1980*b*) and are included here for purposes of comparison. Zackson (1982, 1984) examined the m and q bandlets by a different technique and obtained results that are entirely consistent with our conclusions. Because our results indicate that the repeating units established from primary blast cells interdigitate and spread across more than one segment's length in the leech, we make no attempt to define segments on the basis of their developmental history. Rather, we adopt the convention in which segments are defined as anatomical units associated with the individual ganglia of the ventral nerve cord (Mann 1953).

Results

Segmental anatomy

The definitive anatomy of *Helobdella* is largely established at stage 10 of embryogenesis. By that point in development there has been a secondary fusion of the four most anterior segments and of the seven most posterior segments. The 21 intervening abdominal segments show relatively little variation in structure. Because the left and right halves of a segment are symmetrical in structure and in ontogeny, only a hemisegment is shown in the figures. The arabic numerals used to designate segments here refer to the 21 abdominal segments only, with segment 1 being the most anterior of these.

At the ventral midline in each segment lies the ganglion, one of the chain making up the central nervous system of the leech. Three large peripheral nerves, the AA, MA and PP nerves, project from the ganglion into the body wall on either side, and there course circumferentially to the dorsal midline. Each nerve becomes associated with one of the three circumferential annuli (anterior, central and posterior) that subdivide each abdominal segment in the *Helobdella* adult; these annuli are not yet visible at stage 10. A fourth major nerve, the DP nerve, runs dorsally through the body cavity.

At stage 10 of embryogenesis each abdominal hemisegment has six epidermal specializations distributed circumferentially between the dorsal and ventral midlines in the body wall. The cells in each specialization are distinguished from surrounding squamous epidermal cells by their rounded, bud-like form. We call these structures cell florets and refer to them from ventral

to dorsal as cell florets 1–6 (Shankland & Weisblat 1984). The florets lie in the body wall which becomes the central annulus of the segment in the mature leech, but their relation to the specializations of the adult skin is for the most part unknown. In those segments containing nephridia, however, the nephridial tubule connects to the inner surface of the ventrolaterally located cell floret 2, thereby marking it as the future site of the excretory pore. The nephridium itself is a coiled mass of tubules lying against the lateral body wall, with segment-specific differences in its occurrence and situation that will be described in a later section.

Number of ectodermal blast cells to generate one segmental complement

N and Q teloblasts

As described previously (Weisblat *et al.* 1984) the great majority of the progeny of the N teloblasts are found within the segmental ganglion. In addition, there is a very small number (1–2 at stage 10) of N-derived squamous epidermal cells just over the ganglion and two or three peripheral neurons outside the posterior half of each ganglion. When an N teloblast is injected after blast cell production has begun, the resultant stage 10 embryos fall with equal probability into either of two classes with respect to the pattern of the frontmost labelled segment. In one class all the N-derived cells in the ipsilateral hemiganglion are labelled; in the second class, only the caudal portion of the hemisegment contains labelled cells (figure 3).

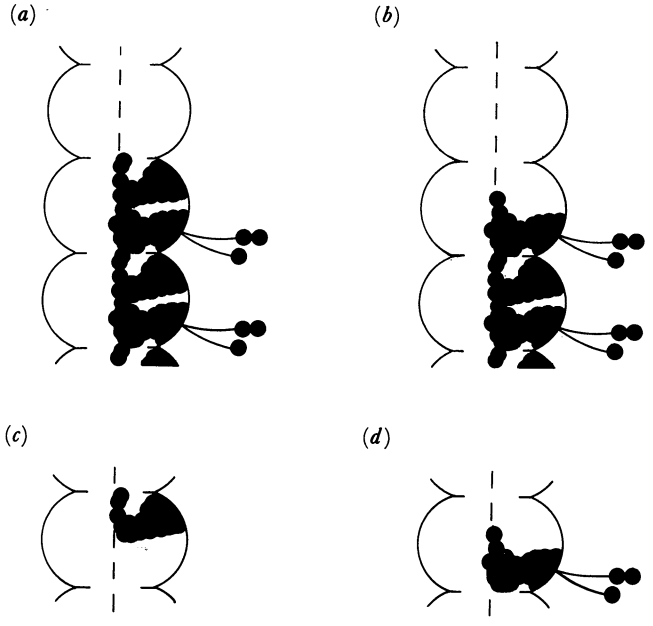

FIGURE 3. Two primary n blast cells generate one segmental complement for the N cell line. In this and all subsequent figures anterior is up, the ventral midline is indicated by a dashed line through the centre of the segmental ganglia and the representation is that of an animal which has been opened along the dorsal midline and laid out flat. (*a*) and (*b*) Schematic drawing of the boundaries between labelled and unlabelled segments in stage 10 embryos obtained after injection of N teloblasts early in stage 7. Three complete segments are shown. Two classes of boundary are seen. In (*a*) all cells in the N kinship group are stained in the first labelled segment, including three peripheral neurons and a small patch of epidermis which lies just ventral to the ganglion and is thus not shown. In (*b*) only the cells in the posterior half of the first labelled segment are stained, including the peripheral neurons. The inferred contributions of the first labelled blast cell in these two classes are shown in (*c*) and (*d*), respectively.

The bandlets derived from the Q teloblasts come to lie at the lateral edges of the germinal plate. Accordingly, progeny of the q bandlets lie primarily in the dorsal half of the body wall, contributing squamous epidermal cells, cell florets and peripheral neurons (figure 4). A few ventral neurons and glia do derive from the Q teloblast, however, migrating from the lateral edge of the plate toward the ventral midline (Weisblat *et al.* 1984) in an apparently unprecedented example of invertebrate neuroblast migration (Hopkins & Brown 1984).

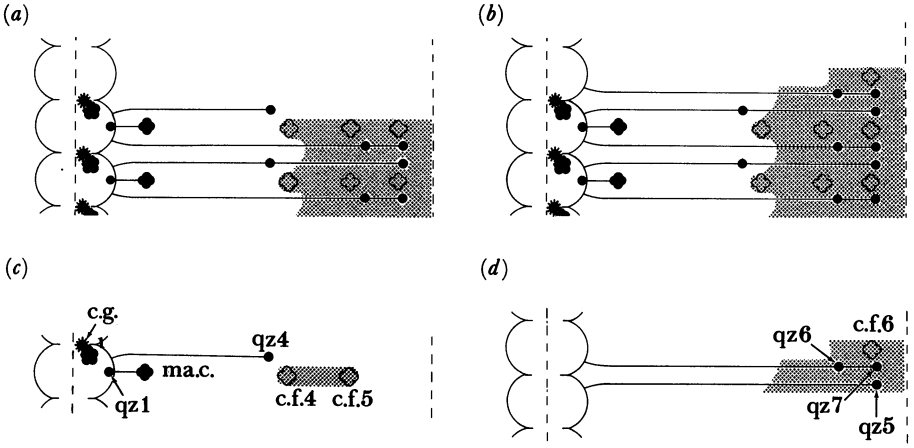

FIGURE 4. Two primary q blast cells generate one segmental complement for the Q cell line. In this figure and the next two, only the right side of the embryo is shown, and the dorsal midline is indicated by a dashed line at the right side of each panel. Stippled areas indicate domains of labelled epidermis, whose shape has been idealized for the purposes of representation. Labelled cell florets are indicated as lobed shapes within the stippled area. The cell bodies of neurons and glia are shown in black, as are the afferent axons of the peripheral neurons. (*a*) and (*b*) Schematic drawing of the boundaries between labelled and unlabelled segments in stage 10 embryos after injection of Q teloblasts early in stage 7. Three complete segments are shown. Two classes of boundary are observed. In (*a*) the anterior edge of the labelled dorsal epidermis (including cell florets 4–6) extends circumferentially in line with the middle of the ganglion and one labelled peripheral neuron, qz4, with a process in the AA nerve, is clearly visible over unlabelled epidermis. In the other class of boundary (*b*) there is an additional patch of epidermis extending ahead of the first labelled ganglion, including cell floret 6 in the next anterior segment, neuron qz5 in the AA nerve and neurons qz6–7 in the PP nerve. The inferred contributions of the first labelled blast cell in these two classes are shown in (*c*) and (*d*), respectively. Abbreviations: cell florets, cf; connective glia, c.g.; cluster of neurons on MA nerve, ma.c.

When a number of Q-injected embryos are examined, two classes of boundaries are again observed, differing not within the ganglion but rather as to which of the peripheral derivatives are stained in the first labelled segment (figure 4*a*, *b*). This finding is consistent with studies of blast cell dynamics described later in this paper which show that for the Q cell line, as for N, each segmental complement arises from a consecutive pair of primary blast cells. Of these, one gives rise to both some dorsal and ventral progeny: a ventral cluster of central neurons and the connective glia; an isolated central neuron (qz1) at the base of the MA nerve; a cluster of six or seven peripheral neurons situated ventrolaterally on the MA nerve, including a previously identified dopamine-containing neuron (Blair 1983); an isolated peripheral neuron (qz4), located on the AA nerve; and a strip of epidermis including cell florets 4 and 5 (figure 4*c*). The second blast cell gives rise to exclusively dorsal progeny: one peripheral neuron (qz5) on the AA nerve; two other peripheral neurons (qz6 and qz7) on the PP nerve; and dorsal epidermis, including cell floret 6, which extends over the length of one segment, but not in register with the ganglion (figure 4*d*). In contrast to the results obtained with the N cell line,

the two categories of Q boundary were not obtained with equal frequency. The frontmost labelled clone was of the purely dorsal type significantly less than half the time, an observation we are at present unable to explain, since successive mitotic cycles of the Q teloblast appear to be equal in length (Wordeman 1982).

The foregoing observations suggest that, for the N and Q cell lines, one segmental complement of progeny arises from two consecutive primary blast cells, each of which gives rise to a distinct set of cells. This conclusion is consistent with the observation that the secondary lineages of alternate blast cells in these bandlets are also distinct on the basis of timing and symmetry of their early mitoses (Zackson 1984).

O/P and M teloblasts

Ipsilateral O/P teloblasts give rise to distinct sets of progeny, referred to as the O and P patterns. Both O and P patterns include a number of central and peripheral neurons, epidermal cells and, in the case of O, cells of the nephridial tubule (figure 5). Most of these O and P pattern elements have already been identified (Weisblat & Blair 1984; Shankland & Weisblat

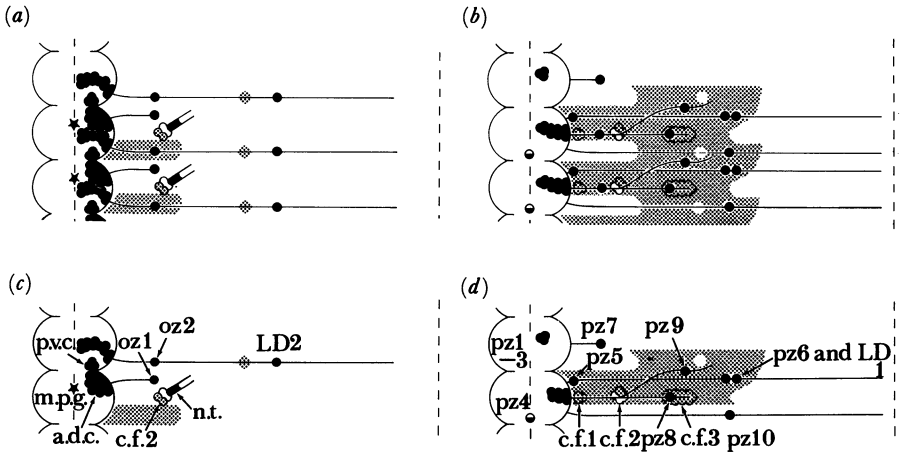

FIGURE 5. One primary o/p blast cell generates one segmental complement for either the O or the P cell line. (*a*) and (*b*) Schematic drawing of the boundaries between labelled and unlabelled segments for the O and P cell lines, respectively. Only a single class of boundary was obtained for either the O or the P pattern. Certain pattern elements are unlabelled in the boundary segment, indicating that each o/p blast cell contributes progeny to more than one segment. (*c*) and (*d*) The inferred trans-segmental distribution of single blast cell clones for the O and P cell lines, respectively. Abbreviations: anterior dorsal cluster of neurons, a.d.c.; medial packet glia, m.p.g.; posterior ventral cluster of neurons, p.v.c.; nephridial tubule, n.t.; cell floret, c.f.

1984; Kramer & Weisblat 1985). The parent teloblasts share a common designation because their primary blast cells have a common developmental potential, and take on different fates only as the result of a hierarchical, position-dependent interaction (Weisblat & Blair 1984; Shankland & Weisblat 1984). Apart from this ambiguity as to which labelling pattern will be obtained, however, when an O/P teloblast is injected after its production of primary blast cells is already under way only a single class of boundary is observed in the frontmost labelled segment (figures 1*a* and 5*a*, *b*).

The mesodermal bandlet, derived from the M teloblast, lies beneath the four ectodermal bandlets on each side of the embryo. The m blast cells give rise to a large number of cells in each segment, including most, if not all, of the segmentally iterated muscle cells, the nephridia,

mesenchymal cells and a few neurons in each ganglion as well. Examination of the boundary pattern obtained with M teloblasts were injected with lineage tracer after they had begun making blast cells (figure 6*a*) revealed no systematic variation in the pattern beyond that ascribable to segmental differences in the distribution of nephridia and presumptive gonadal tissue (figure 7).

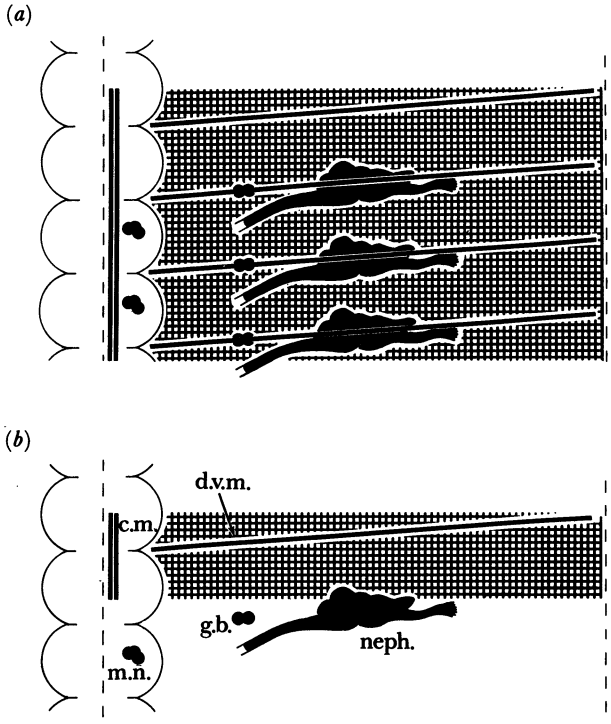

FIGURE 6. One primary m blast cell generates one segmental complement for the M cell line. (*a*) Schematic drawing of the boundary between labelled and unlabelled segments for the M cell line. Not all cells derived from the M teloblast are shown; in particular, crosshatching indicates merely the extent, not the actual disposition of body wall muscles, and the contractile fibres of the larval integument are not shown. Only one class of boundary was observed, but certain pattern elements are unlabelled in the first two segments, indicating that each m blast cell contributes progeny to three or more segments. (*b*) The inferred trans-segmental distribution of cells identified thus far of a single m blast cell clone. Abbreviations: connective muscles, c.m.; dorsoventral muscles, d.v.m.; presumptive gonoblasts, g.b.; nephridia, neph.; M-derived neurons, m.n.

The observations of only a single class of boundary in the O, P and M patterns suggests that there is only one type of blast cell clone in each of these cell lines. This is consistent with the finding that in these bandlets, adjacent blast cells undertake their secondary lineages with the same timing and symmetry of mitoses (Zackson 1984), and suggests that each blast cell clone makes an integral number of segmental complements. That each blast cell clone makes a *single* segmental complement (rather than two or three) follows from the observation that the boundary can fall in *any* segment (rather than only in every second or third segment), from experiments in which single o or p blast cells are directly injected with tracer and their descendant clones characterized (Shankland & Stent 1985) and, for the M line, from experiments in which single blast cell clones were differently labelled by timed, sequential injection of two different fluorescent lineage tracers (Zackson 1982).

Trans-segmental distribution of primary blast cell clones

In the course of the study of the M, O and P patterns, it became obvious that certain pattern elements in each line were invariably missing from the first labelled segment (figure 1*a–c*). This deficiency occurred without regard to the identity of the segment in which the boundary fell. Examination of sectioned embryos has confirmed that the cells corresponding to the missing pattern elements are in fact present, but unlabelled. We conclude that this observation can not be explained by segment-specific differences in the composition of individual blast cell clones and that the unlabelled cells in the most anterior stained segments must derive from more anterior blast cells, produced by the teloblast before the injection of lineage tracer. It follows that the clone of cells descended from a single m or o/p blast cell is not confined to a single segment, but rather becomes distributed in a highly stereotyped fashion over two or more segments. By determining in which segment the first labelled cell of each phenotype occurs, it is possible to map the clone of a single o, p or m blast cell and show its trans-segmental distribution. In the case of O and P, these maps have been confirmed directly by observing the trans-segmental distribution of clones derived from single blast cells individually injected with lineage tracer (Shankland & Stent 1985). The complementary clones of two adjacent n blast cells easily fall within one segmental repeat, and the complementary clones of two adjacent q blast cells overlap segmental boundaries only slightly. Therefore, these groups are not discussed in this section.

The o *blast cell clone*

A primary o blast cell contributes descendants over two consecutive body segments (figure 5*c*). In the more posterior segment, it gives rise to: an anterior dorsal cluster of neurons in the ganglion; at least one identified packet glia; a large patch of ventral epidermis; a peripheral neuron in the AA nerve (oz1); a pair of cells at the distal end of the nephridial duct; and two rounded epithelial cells associated with cell floret 2. In the more anterior segment, the primary blast cell gives rise to middle and posterior ventral neurons of the ganglion, two peripheral neurons in the PP nerve (oz2 and LD2), plus a small patch of lateral epidermis. Note that, as in the composition of the teloblast-derived cell lines themselves, there is no evident segregation of cell types between the segmental contributions of the blast cell; it contributes central and peripheral neurons and epidermis to both segments. Moreover, it is impossible to redefine the segment boundaries so as to coincide with observed clonal boundaries, because serially homologous clones interdigitate with each other. Thus, in any given segment the ventral patch of O-derived epidermis comes from one o blast cell clone, while the other O pattern elements which lie in the same annulus, including oz2, which lies immediately under this epidermis, come from the next posterior o blast cell clone.

The p *blast cell clone*

A primary p blast cell also contributes to two consecutive segments (figure 5*d*). In the more posterior segment it gives rise to: most of the P kinship groups central neurons, whose cell bodies lie in a mediolateral strip on the ventral aspect of the ganglion; an unpaired central neuron (pz4) which lies posteromedially on the ventral aspect of the ganglion and is only seen in one half of the p blast cell clones examined (Kramer & Weisblat 1985); six peripheral neurons (pz5, 6, 8–10 and LD1), distributed in the AA, MA and PP nerves; cell florets 1–3; and a band

of epidermis which lies anterior to the cell florets and extends from the ventral midline to the future lateral edge of the embryo. In the more anterior segment this same blast cell contributes three central neurons (pz1–3), a peripheral neuron (pz7) located in the MA nerve and a patch of lateral epidermis, from which a narrow strip often extends medially. As is the o blast cell clone, the p blast cell clone is distributed over more than one segment. This trans-segmental distribution is particularly evident in the peripheral nervous system, where a single p blast cell contributes one neuron (pz7) to the central annulus of one segment and another neuron (pz10) to the posterior annulus of the next posterior segment. Thus, one p blast cell contributes progeny over five annuli (a length of one and two-thirds segments), and the P kinship group neurons within a single MA nerve arise from two different p blast cells.

The m *blast cell clone*

For both the O and P kinship groups, it appears that each blast cell contributes progeny to two segments and that, conversely, two blast cells contribute progeny to each segment. In the case of the M kinship group, the situation seems to be more complex still, with at least three blast cells contributing progeny to a single segment. This conclusion is based on the observation that, in embryos obtained after stage 7 injections of an M teloblast, the full pattern of identified progeny is not present in the first two segments which contains labelled cells.

Because the mesodermal derivatives are many, and include a number of poorly defined cell types, our description of the M pattern is not intended to be complete (figure 6*b*). One of the most obvious contributions of the m blast cell is a circumferential band of body muscles, extending from the posterior edge of the cell florets in one segment to the posterior edge of the cell florets in the next posterior segment (figure 1*d*, *e*) and from ventral midline to dorsal midline. This band includes circular, oblique and longitudinal muscle fibres. We frequently observe labelled longitudinal fibres which extend over the anterior edge of the band. This is consistent with the observation that, in adult *Haementeria ghilianii*, longitudinal muscle fibres interdigitate extensively and do not obey segmental boundaries (Cline 1986). In these anterior segments, the m blast cell also gives rise to two muscle cells coextensive with the longitudinal muscles in the band, but located in the interganglionic connective nerve (figure 1*c*). The nuclei of these muscle cells lie at the anterior edge of the more posterior ganglion lapped by the band of body muscles. The m blast cell also gives rise to dorsoventral muscle fibres in register with the body wall contribution.

The same m blast cell also gives rise to at least three definitive structures associated with yet more posterior segments: a cluster of three or four central neurons in the ganglion posterior to that containing the nuclei of the connective muscles (figure 1); the main mass of the nephridium, whose exit duct connects to cell floret 2 of the segment containing the neurons; and, in segments 8–18, a pair of cells in the same septum as the nephridium, whose location and segmental distribution suggest that they are rudiments of testes (Bürger 1902). There are only 15 segmental pairs of definitive nephridia in *Helobdella*, associated with the septum at the posterior edge of segments 2–5 and with the septum at the anterior edge of segments 8–18. As noted before, the nephridial tubule connects to the surface of cell floret 2, in the future central annulus of the segment, and thus runs anteriorly in segments 2–5 and posteriorly in segments 8–18 (figure 7).

In addition to the structures described above, each m blast cell generates a set of circumferential contractile fibres underlying the epithelium of the provisional dorsal integument

(Weisblat *et al.* 1984). The provisional integument, including its contractile fibres, disappear once the body tube of the leech has closed at the end of stage 10, and are thus not properly considered as elements of the definitive segments. However, these cells are segmental in that they arise in iterated sets from m blast cells. Most of these provisional contractile fibres do not lie in register with their clonally related definitive muscle fibers, but rather occupy a position several segments posterior (figure 1*b*).

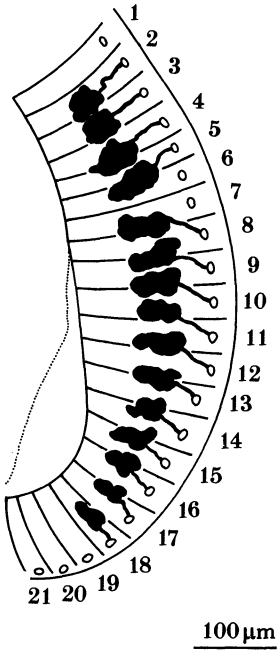

FIGURE 7. Distribution of definitive nephridia and excretory pores in *Helobdella*. Camera lucida drawing of the 21 abdominal segments of an early stage 10 embryo, shown from the side. The lateral edge of the germinal plate is indicated by the solid line on the left side of the embryo and corresponds to the dorsal midline in the anterior half of the embryo. Posteriorly, where dorsal fusion has not yet occurred, the dotted line indicates the dorsal midline. The ventral midline is indicated by the solid line at the right side of the embryo. Cell floret 2 is shown as an oval outline, and is present in every abdominal segment. In segments that contain nephridia, this cell floret is also the site of the excretory pore. Note that in segments 2–5, the nephridium is posterior to its pore, whereas in segments 8–18, the nephridium is anterior to its pore. Segments are demarcated by circumferential boundaries in accordance with the location of ganglia in the nerve cord.

Dynamics of blast cell production: relative motion of n and q bandlets with respect to m, o and p bandlets

Cinematographic analysis and short term, timed injection experiments have both shown that all teloblasts produce primary blast cells at about the same rate, approximately one per hour at 25 °C (Wordeman 1982). Taken with the present results, this means that the M and O/P teloblasts each produce the founders for one segmental complement of cells per hour, whereas the N and Q teloblasts each produce the founders for one segmental complement of blast cells every 2 h. This point is demonstrated by experiments in which two contralateral teloblasts are injected with lineage tracer at the same time in a given embryo, and the positions of the boundaries for the two labelled cell lines are observed after the germinal plate has formed. By repeating this experiment at various times in development for different teloblast pairs, we find, as expected, that later injections result in embryos with progressively more caudal label boundaries. Moreover, when N and Q pairs are labelled, the boundary for the two cell lines

is never separated by more than one segment. Interestingly, even when both N teloblasts in a single embryo are injected within a minute of each other (less than 2% of the cell cycle), their boundaries often vary by a half segment, indicating, in accord with cinematographic observations (Wordeman 1982), that bilaterally homologous teloblasts are not making progeny in precise synchrony. Similarly, when pairwise combinations of M, and the O/P teloblasts are injected, separation between the two boundaries remains small and constant, independent of the time of injection. But when injection of an N or Q teloblast is paired with injection of an M or O/P teloblast, the separation between the two boundaries increases with later injection. The M or O/P boundary progresses caudally at roughly twice the rate of the N or Q boundary (figure 1*f*).

This supports our previous conclusions regarding the rate at which segmental founders are produced in each cell line and further demonstrates that consegmental founder cells are born at different times in different cell lines. Thus, at some period in development, n and q blast cells or their progeny must move past m, o and p blast cells or their progeny so that the founders of a given segment come into register. For the ectodermal bandlets, this relative motion, to which we refer as sliding, occurs after the blast cell bandlets have already entered the germinal bands. This was shown by injecting ipsilateral Q and O/P teloblasts with a fluorescent dextran which could be viewed in the living embryo. Injection was performed during late stage 7. By this time in development, the Q teloblast has produced approximately half of its blast cell progeny, while the O/P teloblast is nearing the end of its blast cell production. When such embryos were examined 24 h after injection, using low light intensity to avoid photodamage (Shankland 1984), it was found that the frontmost labelled blast cells in both these bandlets were near one another at the posterior end of the germinal band (figure 1*g*). However, when the same embryos were examined 48 h after injection, the progeny of the frontmost labelled o/p blast cell were still at the posterior end of the germinal band, whereas the progeny of the frontmost labelled q blast cell, having progressed much further into the band, were now located on the opposite side of the embryo (figure 1*h*).

These observations indicate that cells in the q (and n) bandlets come into register with consegmental cells in the m, o and p bandlets within the germinal band by sliding past cells in those bandlets which are destined for more posterior segments. The stage at which consegmental founder cells in the various bandlets finally come into register is not known exactly, and probably varies for blast cells born at different times, but some cells clearly are not in register until at least 20–30 h after they first enter the germinal band. In contrast, the primary blast cells in the bandlets begin a stereotyped sequence of mitoses within a few hours of entering the band (Zackson 1982, 1984). Thus, the overt expression of segmentation occurs within each individual bandlet long before the progeny of the n and q bandlets come into register with consegmental m and o/p blast cell progeny.

Discussion

Primary blast cells generate segmentally iterated clones

The segmental tissues of the leech arise from five pairs of embryonic stem cells called teloblasts which arise during cleavage. Each teloblast (M, N, O/P, O/P and Q) generates a longitudinal column of primary blast cells and founds a distinct cell line of segmentally iterated progeny. Our results show that these segmentally iterated progeny arise as homologous members of

iterated blast cell clones. In the case of the M, O and P cell lines, sequential blast cells generate identical clones of progeny (ignoring segmental specializations that occur, for example, at the anterior and posterior segments and in clitellar segments); thus we find that there is a single type of blast cell in each of these lines. The remaining cell lines differ only in that the N and Q teloblasts each produce two distinct types of blast cells in an alternating sequence, so that one segmental complement arises from a pair of primary blast cells born at consecutive divisions of the parent teloblast. Thus we find seven types of blast cells generating one segmental complement of tissue on each side (but see Shankland & Weisblat 1984).

These results complement previous studies (Zackson 1982, 1984), in which each bandlet (m, n, o, p and q) was distinguished from the other four bandlets on the basis of the timing, orientation and symmetry of the first mitoses of its primary blast cells. It was found that sequential blast cells within the m, o or p bandlets divide by the same pattern, whereas alternate blast cells within the n and q bandlets follow different mitotic patterns. Further analyses of mitotic patterns within the o and p bandlets support the notion that segmentally iterated, identifiable cells in the leech arise by the same sort of stereotyped lineages that have been described in the nematode (Sulston *et al.* 1983).

Individual blast cell clones are distributed across segment boundaries

Although each m, o and p blast cell makes a segmental complement of cells for its cell line, that complement of cells is spread over at least two adjacent segments; the clones from two (in the case of O and P) or three (in the case of M) blast cells are interdigitated in each midbody segment. Within each segment certain cells in a given line arise from a more anterior blast cell and others from a more posterior blast cell. Moreover, the boundaries of the blast cell clones for different cell lines fall at different places within the segments.

Blast cells are committed to generate a particular type of clone before being committed to a particular segment

Various lines of evidence indicate that cells in the leech embryo are committed to give rise to a particular set of cell phenotypes before and independent of their commitment to contribute to a particular segment or set of segments. We report here that n and q blast cells destined for one segment move past m, o and p blast cells destined for more posterior segments, even though the commitment to make progeny of the N and Q cell lines appears to be set at the time these teloblasts arise (Blair & Weisblat 1982). But we know from ablation experiments that these cells are not *committed* to being in the more anterior segment; when any of the four ectodermal bandlets is broken near its point of entry into the germinal plate, the movement of cells in that bandlet posterior to the breakpoint is retarded so that they become frame-shifted caudally by as many as a dozen segments relative to blast cells in intact neighbouring bandlets (Shankland 1984). These frame-shifted cells seem to follow the fate appropriate to their new location. Finally, in any ablation experiment that results in a boundary between complete segments and those in which the cells of one or another kinship should be lacking, an aberrant trans-segmental distribution of appropriate cells from the segments bordering the deficient zone is detected (Weisblat *et al.* 1980*b*; Shankland 1984).

Comparison of segmentation in leech and insect

On the basis of the theories of segmentation that have emerged from the study of insect development and the phyletic link between annelids and arthropods, a simple relationship between blast cells and morphological segments was anticipated. Namely, in accord with the developmental compartments described in *Drosophila*, it seemed likely that the leech segment would prove to be a polyclone, comprising all the progeny of one set of bilateral pairs of each type of primary blast cell. An outstanding feature of the insect polyclones, indeed their defining characteristic, is that they do not intermingle, but rather remain spatially segregated following their initial specification (Garcia-Bellido *et al.* 1973; Crick & Lawrence 1975). Our present results show that this is clearly not the case in the leech. Despite the fact that m, o and p blast cell clones represent segmental complements of their individual cell lines, sequential clones within these cell lines intermingle along the longitudinal body axis. This finding is in direct contradiction to the idea that sequential blast cells are members of different compartments. Moreover, the fact that the cells within each of these three types of clone are distributed over more than one segment's length means that there is no transverse boundary, either at ganglion's edge or elsewhere in the segment, which is respected by the entire clone. It might be unreasonable to look for a single line of clonal restriction which crosses different organ systems. However, the primary p blast cell contributes peripheral neurons to nerves which span five annuli (one and two-thirds segments, figure 5*d*), and even within the confluent sheet of epidermis all circumferential lines are crossed by the contribution of at least one type of primary blast cell clone (figure 8).

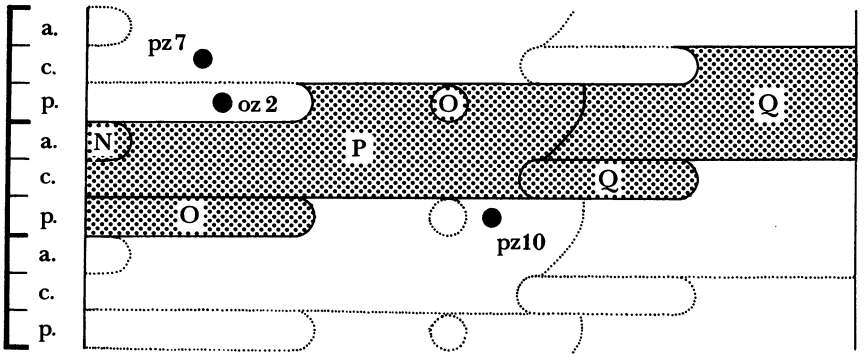

FIGURE 8. Segments are not polyclones founded by primary blast cells. A schematic representation of the right half of the body wall of *Helobdella*. Ventral midline is to the left; dorsal midline to the right. The extent of the anterior (a.), central (c.) and posterior (p.) annuli are shown on the scale at the left. The epidermal domains normally occupied by progeny of individual blast cells are indicated for three segments. In the middle, one segmental domain from each ectodermal cell line has been heavily stippled. Note that the united domains cover parts of five annuli, and that any hypothetical circumferential segment boundary is violated by the epidermal contribution of at least one blast cell. Moreover, this same set of blast cells gives rise to three peripheral neurons which lie outside their epidermal domain.

One way to harmonize these results with the compartment model would be to relax the definition of a compartment so that spatial exclusivity is no longer required. In this case, the segment would be defined developmentally rather than anatomically, as the polyclone comprising all the progeny of the seven pairs of blast cell types; individual cells in one *developmental segment* would be distributed across several *anatomical segments*. Segmentally iterated

structures in the leech arise not because spatially distinct groups of cells are formed which become coordinately committed, but rather because sequential blast cells generate virtually identical clones. Another way to maintain the concept of segment as polyclone in the leech would be to define the set of cells founding the polyclone as those present when no further migration across the chosen segmental boundaries occurs. To do so, however would greatly impoverish the compartment model, however, since it appears that such migrations persist until quite late in the differentiation of segments (J. Braun, unpublished results). Thus, any such delayed compartmentalization would occur after these cell lines have already become subdivided into segmentally repeating units, and hence would not contribute to the establishment of the longitudinal periodicity. We conclude that the process of compartmentalization, which seems so intimately involve in insect segmentation, has no apparent role in establishing the iterated complements of cells that give rise to segmental tissues in the leech.

This brings out a critical distinction between the development of leeches and insects. In insects, it appears that cells become committed to contribute to a particular segment or subsegmental compartment at the blastoderm stage, apparently without becoming committed to generating any particular set of progeny. Once a compartment is established, it seems that any one or more of the committed cells is competent to generate the entire structure. But in the leech, individual blast cells become committed to generating a certain type of clone, without becoming committed to being in any particular segment.

Possible homologies between segmentation in leech and insect

The development of the leech is direct. Thus, the segments and even the individual cells whose appearance we observe during embryogenesis are carried largely unmodified into the adult. In contrast to this, the life cycle of higher insects includes drastically different body forms separated by metamorphosis. Recently it has been proposed that the morphologically defined ectodermal segments in adult *Drosophila* are phase-shifted by 180° with respect to the embryonic–larval counterparts (Martinez-Arias & Lawrence 1985). It could thus be argued that the search for homology between leech and insect segments should be confined to the larval stage. This is interesting in light of the observation that the major contribution of the m blast cell clone in *Helobdella* falls across, rather than within the segment boundaries we have chosen (figure 6*b*). However, the essential differences between leech and insect in the relative timing of segmental commitment and phenotypic commitment hold for either insect stage and for any segment boundary in the leech.

Another place to look for homologies between leech and insect segmentation lies in the enumeration of segments. In the leech, the prostomial segments, including the supraoesophageal ganglion, arise from the micromeres generated during the cleavage phase of embryogenesis, not from the teloblasts (Weisblat *et al.* 1984). This leaves 32 true segmental ganglia. In the insect, because of the much greater degree of segmental differentiation and fusion, counting of segments is more ambiguous. But recently Doe & Goodman (1985) examined ganglionic primordia in the embryo of the grasshopper, before coalescence. Of the 18 ganglionic primordia, they identified homologous neuroblasts in three anterior, three thoracic and ten abdominal segments, but concluded that the most anterior and most posterior clusters of neuroblasts were non-homologous. This suggests that perhaps the primordial number of true segmental ganglia in the insect is 16. Meinhart & Gierer (1980) have proposed a model for the segmentation of the insect blastoderm that invokes a combination of excitatory and inhibitory interactions to

establish periodicity across an initially homogeneous field, followed by a number of other operations which transform the initial periodicity into definitive segments. A key step in this model is one that halves the spatial frequency. Perhaps a step in which the spatial frequency of the segments in insects is halved is one of the modifications built onto a pre-existing process inherited from the annelid-like ancestor.

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FIGURE 1. For description see opposite.