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Segmentation in leech development

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Summary

Segments in glossiphoniid leeches, such as *Helobdella triserialis*, are the products of stereotyped cell lineages that yield identifiable cells from first cleavage. Cell lines generating segmental tissues are separated from those generating prostomial tissues early in development. Segments arise from five bilateral pairs of longitudinal columns of *primary blast cells* that are generated by five bilateral pairs of embryonic stem cells called *teloblasts*. There are four ectodermal cell lines (N, O, P and Q) and one mesodermal cell line (M) on each side of the embryo. In normal development, each cell line generates a segmentally iterated set of identified definitive progeny comprising a mixture of cell types. In the M, O and P cell lines, each blast cell generates one segment's worth of definitive progeny (segmental complement). But the clones of blast cells in each of these three cell lines interdigitate longitudinally with cells of the adjacent clones from the same line, so that the clone of an individual m, o and p blast cell is distributed across more than one segment. Thus, there is no simple clonal basis for morphologically defined segments. In the N and Q cell lines, two blast cells are required to produce one segmental complement of definitive progeny; in each of these two cell lines, two classes of blast cells (nf and ns, qf and qs) are produced in exact alternation. Primary n and q blast cells are about the same size and are produced at the same rate as blast cells for the o and p bandlets, but the longitudinal extent of their clones is roughly half that of the o and p blast cells' clones. During division of the blast cells, the n and q bandlets become compressed relative to the o and p bandlets, so that the segmental complements of the different cell lines can come into register. This compression movement is manifest as a movement of n and q bandlets relative to o and p bandlets in the posterior portion of the germinal band. The number of true segments in leech is fixed at 32; the counting mechanism is not known, but several hypotheses have been disproved. Segmentation in annelids and arthropods differs extensively at the cellular level, yet these phyla are presumed to share a common segmented ancestor. One strategy to identify homologous processes in annelid and arthropod segmentation is to compare the patterns of expression of evolutionarily conserved, developmentally important genes. Preliminary observations using a cross-reacting antibody that is thought to recognize a highly conserved region of a *Drosophila* segmentation gene, *engrailed*, labels nuclei of some blast cells early in development and, later, some neurones in the differentiating suboesophageal ganglion.

Key words: leech, segmentation, cell lineage, primary blast cell, teloblast.

Introduction

Segmentation in annelid development is of interest because the segmented body plan is such a prominent feature of this phylum. In addition, the fact that segmentation occurs in several phyla raises questions about the phylogenetic origins of this developmental process and how it has changed during evolution. It is generally held that the most recent common ancestor of chordates and arthropods was not segmented and that segmentation has therefore arisen separately during the evolution of these two groups (Dobson & Dobson, 1985). On the other hand, annelids and arthropods, along with Onychophora and related phyla, are thought to share a common segmented ancestor, so segmentation in these phyla should be homologous. If true, these assumptions suggest that we are more likely to find common elements when comparing the segmentation processes of annelids and arthropods than when comparing the segmen-

tation processes in either of those groups with chordates. But first, it is essential to describe segmentation in the organisms of interest in sufficient detail so that meaningful comparisons can be made. Here, we describe segmentation in leech development. Most of the observations are from *Helobdella triserialis*, a glossiphoniid species.

Segmental tissues arise by stereotyped lineages from five bilaterally paired cell lines

Development of glossiphoniid leeches, first described over 100 years ago by C. O. Whitman (1878), proceeds through a series of cell divisions that are largely invariant from embryo to embryo. A series of unequal cleavages (stages 0–6) generates five bilateral pairs of embryonic stem cells called M, N, O/P, O/P and Q *teloblasts*. Throughout these stages of development, there is no evidence of segmentation. The first manifestations of segmentation come about in stage 7, as each teloblast makes a series of several dozen highly unequal divisions, producing a coherent column of segmental founder cells called *primary blast cells* (Fig. 1). Within each column, or *bandlet*, the birthranking of the primary blast cells is maintained; older cells lie more distal (future anterior) to the teloblast (future posterior). The five bandlets on each side, designated m, n, o, p and q, are arranged in stereotyped order into a curving ridge of cells known as the *germinal band*. The left and right germinal bands connect *via* their distal ends just ventral to the animal pole of the embryo. As more primary blast cells are produced, the germinal bands lengthen and move across the surface of the embryo, eventually coalescing rostrocaudally along the ventral midline during stage 8, like the two halves of a zipper, forming the *germinal plate*.

The proliferation of blast cells within the germinal plate (stages 9–10) gives rise to the definitive segmental tissues of the leech. During this process, the germinal plate lengthens and expands laterally.

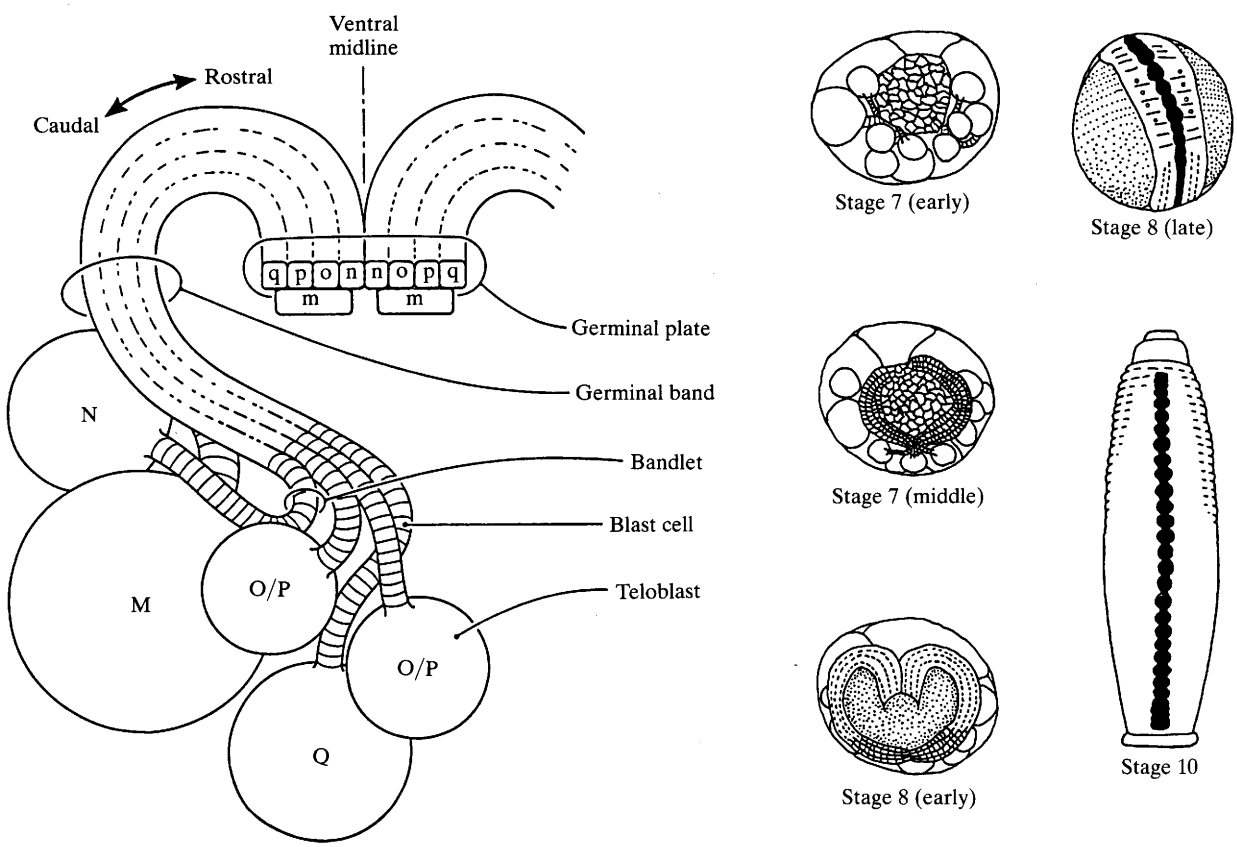

Fig. 1. Schematic representation of late stages of development in *Helobdella triserialis*. Left: Hemilateral arrangement of teloblasts and their primary blast cell bandlets within the germinal band and germinal plate. Near right: Dorsal views of three embryos: early stage-7 embryo in which the teloblasts have begun to produce blast cell bandlets; mid stage-7 embryo in which the bandlets have merged to form germinal bands; and early stage-8 embryo showing the heart-shaped germinal bands that have begun to coalesce into the germinal plate. Far right: Ventral views of two embryos: late stage-8 embryo showing the germinal plate on the ventral midline, with the nascent ventral nerve cord and its ganglia and ganglionic primordia indicated by filling; stage-10 embryo in which the chain of ganglia, shown by filling, already closely resembles the adult nerve cord.

Eventually, its outer edges zipper together along the dorsal midline, which closes the body tube of the leech.

Additional details of this developmental process have been obtained in the last decade, using microinjected cell lineage tracers in conjunction with a variety of other techniques (e.g. see Weisblat *et al.* 1984). This work builds on anatomical and neurobiological studies of leeches begun by Retzius (1891) and continued by Nicholls and others since the 1960s (reviewed in Nicholls, 1987 and Muller *et al.* 1981). One result of this large body of work, which has concentrated primarily on the hirudinid species *Hirudo medicinalis*, is that many cells, especially neurones and glia, are known to be individually identifiable from segment to segment and animal to animal in various leech species.

Lineage-tracing techniques have shown that in normal development, although cell lines are not specialized for the exclusive production of a particular cell *type*, individual, identified cells arise invariantly from a particular cell line (M, N, O, P or Q). Moreover, although the complete lineages for definitive progeny are not yet known, it seems likely that they arise from stereotyped cell lineages, as in the nematode *Caenorhabditis elegans* (Zackson, 1984; Shankland, 1987).

When a teloblast is injected with lineage tracer after it has already begun producing primary blast cells (stage 7) and the embryo is examined at stage 10, a boundary is seen between anterior, unstained definitive progeny derived from blast cells produced prior to injection and posterior, stained cells derived from blast cells produced after the injection. By examining the position of the boundaries in such embryos, it is possible to infer the *number* of blast cells in each bandlet needed to generate one hemisegmental complement of progeny and also the spatial distribution of the clone descended from an individual blast cell (Weisblat *et al.* 1984; Weisblat & Shankland, 1985). These inferences, summarized in Fig. 2, have been confirmed by other procedures (Zackson, 1984; Shankland, 1987). Several features of the segmentation process in *Helobdella* can be discerned from this lineage analysis and associated experiments.

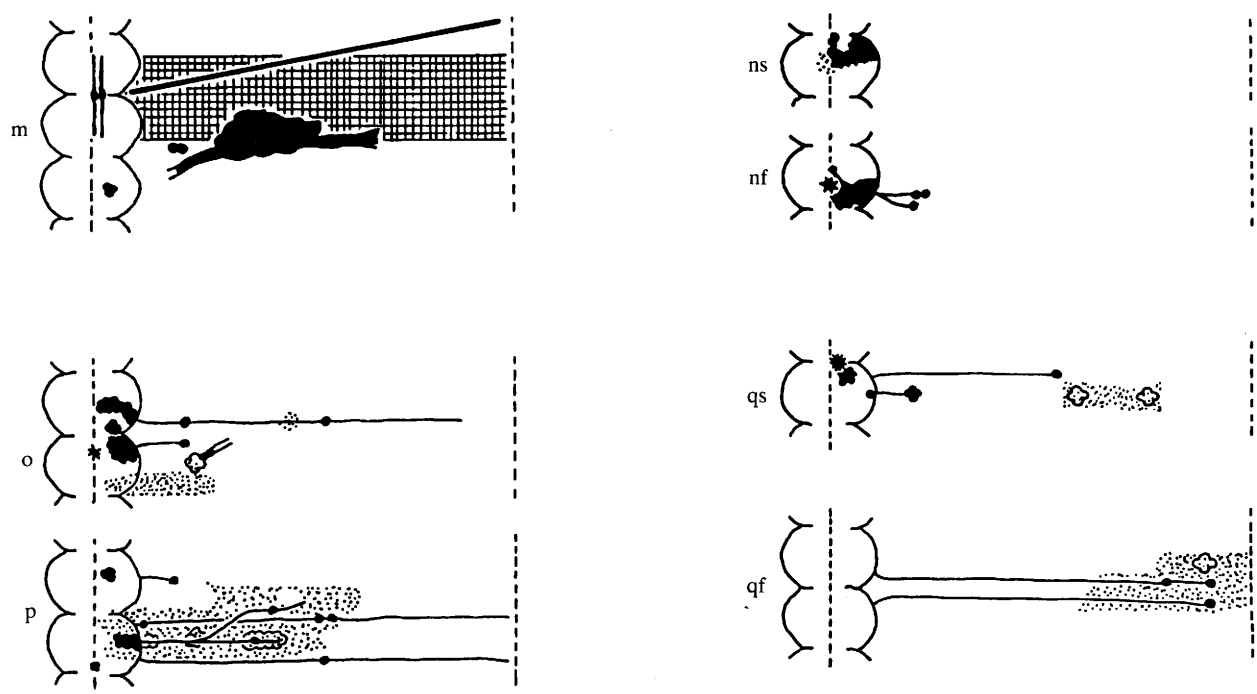

Fig. 2. Schematic representation of the seven primary blast cell clones in the stage-10 *Helobdella* embryo. Not all cells are shown for the m clone; cross-hatching indicates the extent but not the disposition of muscle cells; the diagonal line represents the dorsoventral muscles; spindle-shapes between the first and second ganglia represent muscles associated with the interganglionic nerve; in the third ganglion, the filled contour represents M-derived neurones; the small filled contour outside the ganglion represents presumptive gonoblasts; the large filled contour represents the nephridium. For each ectodermal clone (o, p, ns, nf, qs, qf) central and peripheral neurones are represented as unlabelled filled circles (individual neurones) or multilobed contours (clusters of neurones), glia are represented as filled stars, and epidermal cells by stippling and by unfilled contours. In the o clone, an O-derived cell at the distal tip of the nephridial tubule is shown just anterior to the medial patch of epidermis. For each clone, anterior is up; ganglia are shown in outline; dashed lines through the centres of ganglia and to their right indicate ventral and dorsal midlines, respectively. (Adapted from Weisblat & Shankland, 1985.)

(A) *There are seven basic classes of primary blast cells*

The boundary analyses show that, in the m, o and p bandlets, each primary blast cell generates a complete *segmental complement*, i.e. one set of the definitive progeny from the cell line in question is found in a left or right half segment. And accordingly, each m, o or p blast cell normally undergoes a stereotyped set of further mitoses that is characteristic of its bandlet (Zackson, 1984).

For the N and Q cell lines, by contrast, two blast cells are needed to generate one segmental complement of progeny. In the N cell line, for example, one primary blast cell generates ganglionic neurones and epidermal cells primarily in the anterior portion of the segment, while the primary blast cell just behind it in the bandlet generates a small set of peripheral neurones, a glial cell and posterior ganglionic neurones (Weisblat & Shankland, 1985; Bissen & Weisblat, 1987). The distinction between the progeny generated by the two primary q blast cells needed to make one segmental complement of definitive progeny is similarly clear. In addition to these differences in definitive fates of the two n and two q blast cells, alternate blast cells in the n and q bandlets exhibit differences in the timing and symmetry of their first mitoses (Zackson, 1984); and these differences serve to predict the two different fates (Bissen & Weisblat, 1987). Ablation experiments have so far failed to reveal any plasticity in the fates of cells in the n and q bandlets. So, in contrast to the m, o, and p bandlets, each of which comprises a single class of blast cell, the n and q bandlets each comprise two distinct classes of blast cells (called nf and ns, qf and qs) that arise in exact alternation. Thus, a total of seven classes of primary blast cells can be defined by their intermediate lineages (e.g. the timing and symmetry of their first mitosis) and by their definitive fates (the phenotype and spatial distribution of their definitive progeny). This analysis ignores the important issue of segment-specific differences in the phenotype and occurrence of definitive progeny (Macagno, 1980; Glover & Mason, 1986; Jellies *et al.* 1987).

(B) *Segmental boundaries are not coincident with clonal boundaries*

In the simplest case, it could be imagined, from the results outlined above, that each left or right half segment of the leech would consist of no more and no less than all the progeny of seven primary blast cells, one of each class. This would be consonant with the theory that the segments or parts of segments are *polyclones* comprising all the surviving progeny of a small set of founder cells (Garcia-Bellido *et al.* 1973; Crick & Lawrence, 1975). But the situation is more complex, because there is no simple relationship between morphologically defined segments and primary blast cell clones. In particular, each individual clone derived from an o, p and m primary blast cell in the midbody of the leech extends longitudinally across parts of two or more segments; thus, longitudinally adjacent clones interdigitate so that it is impossible to draw segment boundaries that include all the progeny of just one set of primary blast cells, even if we restrict our analysis to the ectodermal cell lines. In general, any given midbody segment will contain some of the progeny of two o, two p and three m blast cells and all of the progeny of two n and two q blast cells. From the foregoing, and since leeches are not toroidal, the details of the processes by which segments form at the ends of the animal must differ from those by which midbody segments form. For example, there may be cell deaths or missing branches in blast cell sublineages otherwise destined for non-existent segments, and/or there may be modifications of the differentiation or migration patterns of cells so that they remain in the terminal segments.

(C) *Segment founder cells do not come into proper register until after they have already begun dividing*

From various lines of evidence it is known that primary ectodermal blast cells are all about the same size and are all produced at the same rate (Wordeman, 1982). Yet two blast cells from each N and Q teloblast are required to generate one hemisegmental complement, whereas only one m, o or p blast cell is needed per hemisegmental complement. Temporally, this discrepancy is resolved by the simple fact that the N and Q teloblasts continue making blast cells after the other teloblasts have stopped. The spatial aspect of this discrepancy is resolved by compression of the progeny of the n and q bandlets into half of their original longitudinal extent, relative to those of the o and p bandlets. Since the distal (anterior) ends of the bandlets are all fixed, this requires that blast cells in the proximal segments of the n and q bandlets move forwards relative to their neighbours in the adjacent o and p bandlets (Weisblat & Shankland, 1985). In fact, these movements are going on well after the primary blast cells have begun the divisions leading to their definitive progeny. An apparent corollary of these normal movements of ectodermal bandlets relative to one another is the discovery by Shankland (1984) that, if any of the ectodermal bandlets is lesioned within the posterior portion of the germinal band, the blast cells posterior to the break slip backwards relative to their neighbours in adjacent bandlets and assume ectopic positions in more posterior segments. These morphogenetic movements constitute a fascinating, and as yet unstudied, aspect of leech segmentation.

Little is known about how segments are counted out and limited to a fixed number

One difference between segmentation in leeches and in other annelids is that the number of segments in leeches is fixed and constant throughout life. [Another difference is that leeches are apparently unable to regenerate segments (see Sawyer, 1986).] Although the constancy of segment number has been accepted for a long time, there has been dispute over exactly what the number is, because of uncertainty over just what constitutes a segment. Taking the nervous system as a convenient marker for segmentation in leech, there are 21 obvious segments in the midbody, each innervated by one ganglion of the ventral nerve cord. At the ends of the animal, specializations associated with the two suckers complicate matters, but embryological evidence and the occurrence of identifiable neurones homologous to those of the midbody ganglia indicate that the tail brain represents the fusion (more properly, the failure to separate during development) of seven ganglia. From similar evidence, the suboesophageal ganglion of the head brain comprises four segments (Fernandez & Olea, 1982; Yau, 1976).

Analysis of the supraoesophageal ganglion was more difficult because it resembles the typical midbody ganglia neither in gross morphology nor in the phenotype of individual neurones, and because its embryological origins were also obscure. Then, from cell lineage studies it became apparent that the supraoesophageal ganglion and associated epidermis arises not from the five teloblast-derived cells lines at all, but rather from micromere-derived cell lines that are separated from the teloblast lineages as early as the third cleavage (Weisblat *et al.* 1984). Thus, we can finesse the question of how many segments are in the most anterior part of the leech by excluding this region from the discussion of segmentation altogether and restricting our analysis to the $7+21+4=32$ *true* segments derived from primary blast cells.

These results should be useful in refining the question of how segments are counted during leech development, but little progress has been made in understanding the counting mechanism. However, several key observations have served to eliminate otherwise attractive hypotheses.

(1) Blast cell nuclei are made one at a time, as blast cells are produced (Zackson, 1982). This eliminates the notion that the teloblasts undergo five (M and O/P teloblasts) or six (N and Q teloblasts) rounds of karyokinesis without cytokinesis to generate the precise number of nuclei needed for the subsequent production of the segmental precursor cells.

(2) Ablation of various teloblasts eliminates progeny from segments, but does not affect the number of segments produced (Blair, 1982; Blair & Weisblat, 1982). Such findings eliminate the extreme holistic hypothesis that some interaction between all the teloblasts or all the bandlets is necessary to terminate segmentation normally. But not all possible experiments of this sort have been carried out and there is evidence that interactions between mesoderm and ectoderm are necessary for normal pattern formation (Blair, 1982). The effects of bilateral ablation of mesodermal cell lines should be carefully examined. For example, if the posterior mesodermal precursors are missing, will a normal caudal sucker and tail brain form, truncating the midbody of the leech?

(3) Every cell line, both ectodermal and mesodermal, produces *supernumerary* blast cells that die without contributing to definitive segments (Zackson, 1982). This result eliminates the possibility that the number of segments is limited simply by the number of blast cells produced, although it remains to be proven that the supernumerary blast cells are of normal viability.

(4) Duplicated ectodermal cell lines make twice the normal number of viable blast cells. It was discovered that microinjecting polyadenylic acid (polyA) into newly formed teloblasts or their precursor blastomeres alters the normal cleavage pattern in *Helobdella* so that supernumerary teloblasts, blast cell bandlets and definitive progeny are produced throughout the length of the leech (Ho & Weisblat, 1987). This result runs counter to the hypothesis that some factor that is passively distributed among the teloblasts serves to limit the number of viable progeny and hence the number of segments; if this were the case, then dividing the limiting factor among supernumerary teloblasts should result in its exhaustion after each has made only its anterior complement of primary blast cells. Of course, as with the ablation experiments, it will be important to confirm this result with all cell lines, especially the mesodermal line.

Fernandez & Stent (1982) reported an important observation relevant to the termination of the segmentation process in the hirudinid leech *Hirudo medicinalis*. According to these authors, the developing germinal plate in *Hirudo* can be divided into an anterior part, within which segmentation is evident from the massing of cells into somites and ganglionic primordia, and a posterior 'ribbon' part, within which the columns of blast cells are still largely in parallel arrays. As development proceeds, the anterior part increases in length and the boundary between it and the ribbon part moves posteriorly. Termination of segmentation is evident as a gap in the mesodermal bandlet within the ribbon part, just behind the cells that will form the last definitive segment. Ectodermal cells deprived of mesodermal contact and cells pos-

terior to the gap soon degenerate, while cells anterior to the gap continue dividing to generate the posterior segments.

This observation suggests that the *initial* signal for the termination of segmentation arises from the mesodermal cell line or that the mesodermal cells are responding to a signal received from the ectodermal lineages or from some other source in the embryo. Support for the notion that the termination signal is unlikely to arise in the ectodermal bandlets comes from similar observations reported for *Helobdella*. In *Helobdella*, the gap in the mesodermal bandlet is seen *before* it merges with the ectodermal bandlets into the germinal band (Zackson, 1982). The results from *Hirudo* and *Helobdella* are not strictly comparable, of course. Moreover, the possibility remains that the ectodermal bandlets in *Helobdella* may fragment independently as well (Marty Shankland, personal communication).

Interphyletic comparisons of segmentation must distinguish between homologous and operationally equivalent processes

Segmentation is unarguably an important aspect of the development of many higher eukaryotic phyla. But in analysing segmentation processes, it is important to remember that simply using the same word, 'segment', to describe the subunits of the body plan in two groups does not guarantee that these structures arise by *homologous* processes. If segmentation arose independently in different groups, then the observed segmentation in, say, frogs and worms may be merely *operationally equivalent* processes, with no underlying mechanistic similarity.

With this caveat in mind, and also the views of phylogeny stated in the introduction, a search for interphyletic common ground might entail comparisons of segmentation in annelids and arthropods. But even here the search for homology is far from trivial, especially since, for historical and technical reasons, we are driven to compare highly derived representatives of the two phyla, such as insects and leeches, rather than more primitive species or apparently intermediate phyla, such as Onychophora. Even ignoring this problem, yet another is that the view of phylogeny stated in the introduction is not universally shared and may not be true. Field *et al.* (1988), for example, conclude on the basis of a molecular phylogenetic analysis that arthropods, chordates and annelids may in fact all have arisen from a common segmented ancestor, and that the arthropods and annelids are not closely related at all. Given this uncertainty, the introduction of molecular phylogenetics and the analysis of conserved gene families like the homeobox-containing genes is a welcome complement to more classical cellular and morphological approaches to analysing the processes we lump together under the rubric of segmentation.

In *Helobdella*, a putative homologue to the *Drosophila* gene *engrailed* appears to be expressed early in development and in differentiating neurones

Unlike early development in leech, which is characterized by holoblastic cleavages, early development in the insect *Drosophila melanogaster* proceeds *via* a syncytial blastoderm, in which the zygote nucleus undergoes 13 rounds of karyokinesis without cytokinesis (Foe & Alberts, 1983). By the last rounds, most of the nuclei migrate to the periphery, where cellularization and then gastrulation occur. Much work has shown that in the final syncytial stages and after cellularization, nuclei differentially express certain members of a class of at least 35 genes that regulate the number, polarity and identity of segments in *Drosophila* (reviewed by Akam, 1987). A number of these genes have been shown to contain similar sequences of about 180 base pairs, called homeoboxes, which encode protein domains of about 60 amino acids thought to mediate DNA binding. Work in *Drosophila* has demonstrated complex regulatory relationships among the various members of this gene family.

It appears that the homeobox sequence has been highly conserved throughout evolution, because genes containing homeoboxes have been identified in most higher eukaryotic phyla, including echinoderms, chordates and annelids (e.g. Carrasco *et al.* 1984; Levine *et al.* 1984; McGinnis *et al.* 1984; Dolecki *et al.* 1986), as well as in arthropods. In *Helobdella*, we estimate that there are at least 18 putative genes that cross-hybridize with various homeobox probes (unpublished results). Given the likelihood that annelids and arthropods evolved from a segmented common ancestor, an analysis of homeobox gene expression in *Helobdella* may bring us closer to understanding evolutionary homologies underlying the radically different cellular processes of segmentation in insects and annelids.

One gene that has been especially highly conserved in evolution is *engrailed* (*en*). In *Drosophila*, *en* is expressed in the posterior portion (compartment) of every segment and is required for cells in the posterior compartment to assume their normal identities (Kornberg, 1981; Kornberg *et al.* 1985; DiNardo *et al.* 1985). We have identified an *en* homologue in *Helobdella* (unpublished data). In addition, using a broadly cross-reactive mouse monoclonal antibody (N. Patel,

K. Coleman, T. Kornberg & C. Goodman, unpublished data) that appears to recognize a highly conserved portion of the *en* protein (S. Toole, C. Lehner, personal communications), we have carried out preliminary studies on the expression in *Helobdella* of the epitope recognized by the antibody (in collaboration with Patel and Goodman). Using horseradish peroxidase staining, we found immunoreactivity at three different times of development. In the stage-7 embryo, a small number of nuclei stain, all of which are apparently within a single bandlet (Fig. 3A). In the late stage-8 embryo, a segmentally repeating pattern, apparently a single large cell in the middle of the segment, is observed in the central midbody segments (not shown). And in the stage-9 to -10 embryo, the antibody stains nuclei of many neurones, primarily in the second of the four fused ganglia in the suboesophageal ganglion (Fig. 3B).

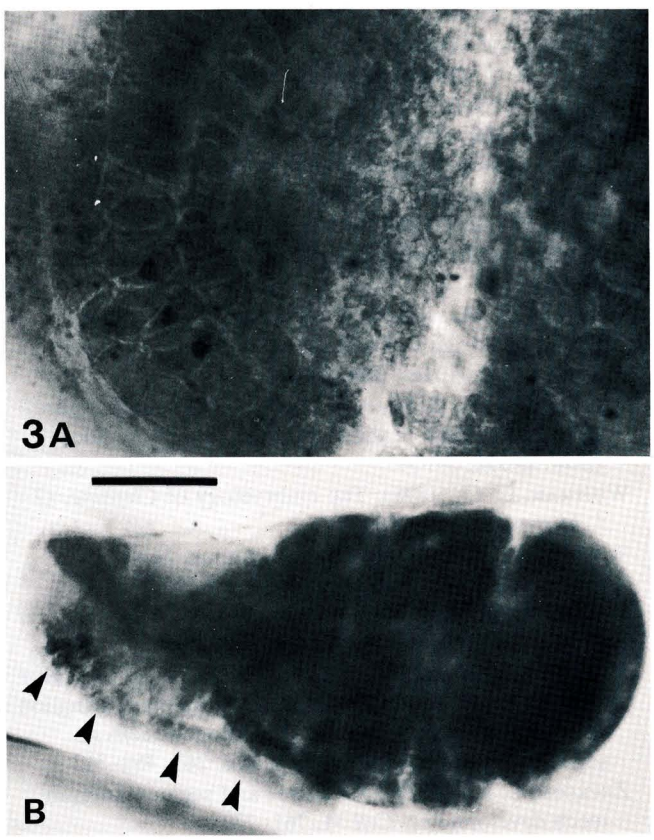

Fig. 3. Photomicrographs of *Helobdella* embryos stained with a monoclonal antibody that was raised to part of the *Drosophila invected* gene product and that recognizes a highly conserved epitope of the *engrailed* gene (N. Patel, K. Coleman, T. Kornberg & C. Goodman, unpublished data). (A) Dorsal view of a late stage-7 embryo. The left germinal band makes a c-shaped curve in this panel, with the proximal portions of the bandlets at the bottom centre and distal portions at upper left. Three blast cell nuclei are labelled in a single bandlet. (B) Lateral view of a stage-9 to -10 embryo; ventral is down, anterior to the left. Nuclear staining is evident in neurones of the suboesophageal ganglion (left arrow) but not in midbody ganglia (right arrows). Scale bar, ca. 50 μm in A; ca. 150 μm in B.

Given the preliminary nature of these results, it is inappropriate to try to make much of a comparison between *Drosophila* and *Helobdella*. But it seems fair to say that the staining patterns we observe are at least vaguely reminiscent of those in *Drosophila*, in that expression is observed early in development in a subset of segmental founder cells, and later in development in a subset of differentiating neurones. More importantly, these results indicate that analysing expression patterns of evolutionarily conserved genes that are putative regulators of development will be useful in studying leech development. By combining a variety of experimental approaches to the analysis of the segmentation problem, it is possible that answers to questions about how segmentation works, how and how often it arose, and how it has been modified during evolution may soon be forthcoming.

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