Origin of segmental identity in the development of the leech nervous system

MARTY SHANKLAND1,†, MARK Q. MARTINDALE1,*, DENISE NARDELLI-HAEFLIGER1, EUAN BAXTER2 and DAVID J. PRICE2

1Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115, USA
2Department of Physiology, University Medical School, University of Edinburgh, Edinburgh EH8 9AG, UK

* Present address: Department of Organismal Biology and Anatomy, University of Chicago, Chicago, Illinois 60637, USA
† Author for correspondence

Summary

The leech embryo develops its segmental body plan by means of a stereotyped cell lineage. Each hemilateral segment arises from a small set of embryonic blast cells via a comparable sequence of formative cell divisions, and for the most part, lineally homologous cells manifest similar patterns of differentiation in the various hemisegments. Nonetheless, some identified central neurons undergo segment-specific or laterally asymmetric patterns of neuropeptide expression and/or cell death. Certain aspects of this regional diversification result from competitive cell interactions which occur at the level of the postmitotic neuron. However, the neuron's segmental identity is lineally determined, being inherited from its blast cell progenitor over several intervening rounds of mitosis. To learn more about the molecular basis of this phenomenon, we have isolated and begun to characterize leech homeobox genes which are related to the genes that govern segmental identity in other organisms.

Key words: leech embryo, cell lineage, segmental identity, neuronal differentiation, homeobox gene.

Introduction

Among segmented animals, annelids are the only phylum that utilizes an invariant cell lineage throughout the entirety of embryonic development. The most detailed analyses of annelid embryogenesis come from the glossiphoniid leeches (Sandig and Dohle, 1988; Weisblat and Shankland, 1985; Bissen and Weisblat, 1989), whose large embryos are good subjects for single cell injection so that individual cell lineages can be labeled and traced for extended periods of development (Fig. 1). This combination of lineal stereotypy and experimental accessibility makes the leech a favorable organism for the cellular analysis of embryonic pattern formation, and studies of leech segmentation have offered many insights into the mechanisms that govern the periodicity, number and ultimate diversification of segments (for a recent review, see Shankland, 1991).

In this article, we will examine the role played by cell lineage in the process of segmental diversification. Each segment has a developmental identity, in that it manifests certain unique characteristics which correlate precisely with its location along the anteroposterior body axis. Among these segment-specific characteristics are some very precise patterns of neuronal differentiation within the central nervous system (CNS), which will be the focus of our discussion. We will first present an abbreviated survey of the segmentation process, and then review experimental studies which have elucidated the developmental origin of the differences between segments, as well as the development of lateral asymmetries between right and left hemisegments. Finally, we will describe the identification and incipient characterization of leech homeobox-genes which are related to the genes that control segmental identity in other organisms.

Generation of segmental founder cells

Leeches have 32 bilaterally symmetric body segments which are generated during a single phase of embryogenesis. The main features of this body plan derive from the spatiotemporal patterning of the earliest cell divisions. Symmetry is established at the fourth round of cleavage, and subsequent cleavages produce a set of 5 bilaterally paired stem cells, the M, N, O, P and Q teloblasts, which will together give rise to the segmented tissues of the adult. The ipsilateral teloblasts can be uniquely identified by their cell lineage histories (with one exception, Weisblat and Blair, 1984), and injection of vital cell lineage tracers has shown that each one
generates a distinctive subset of the mature tissues (Weisblat and Shankland, 1985; Kramer and Weisblat, 1985). Ablation and transplantation studies offer insight into the means by which the various teloblast lineages become specified to their differential fates (Blair, 1984; Weisblat and Blair, 1984; Shankland and Weisblat, 1984), but we will here focus only on the anterior differentiation within these cell lineages.

Leech segmentation originates through the patterned cell divisions of the individual teloblasts. Each teloblast undergoes a rapid series of repetitive divisions, thereby generating a chain or bandlet of much smaller daughters, known as primary blast cells (Fig. 2A). These primary blast cells serve as segmental founder cells, in the sense that they form segmentally repeated subunits of the teloblast's descendant lineage. Each blast cell gives rise to a clone of roughly $10^2$ terminal differentiated descendants, and blast cells within the same bandlet generate clones which are nearly identical in composition and situated in successive segments along the body's length (Zackson, 1982; Weisblat and Shankland, 1985). Thus, the linear array of blast cells within each bandlet becomes translated into a longitudinally periodic array of descendant cell phenotypes (Fig. 2B,C).

During normal development, there is a precise correlation between a blast cell's birth rank in the stem cell lineage of its parent teloblast and the segmental location of its descendant clone. The exact relationship varies for the progeny of different teloblasts, and further complicated by the fact that the blast cell clones do not themselves define a segment boundary (Shankland, 1991). However, the most salient points of this relationship can be appreciated from the simplified diagram depicted in Fig. 2. For the M, O and P teloblasts, the firstborn blast cell generates a descendant clone which takes part in the formation of the most anterior body segments, the second blast cell generates a similar clone which is situated one segment posteriorly, and so forth along the body's length (Weisblat and Shankland, 1985). The N and Q teloblasts differ only slightly from this scheme, in that they each produce 2 blast cells/segment (Bissen and Weisblat, 1987). Thus, segmental differences in the development of the leech embryo can be traced to the diversification of segmentally repeated blast cell clones, and these differences correlate precisely with the cell lineage, i.e., the birth rank, of the ancestral blast cells.

**Segmental diversification within the CNS**

The leech CNS is subdivided into segmental ganglia which are linked by connective nerves (Fig. 3A), except in the head and tail where compound ganglia arise from the fusion of several segmental neuromeres. Each hemilateral ganglion or neuromere contains approximately 200 postmitotic neurons (Macagno, 1980), a substantial fraction of which have been uniquely identified on the basis of physiological and/or biochemical criteria (surveyed in Muller et al., 1981). Many of the identified neuronal phenotypes are represented by sets of bilateral homologues which are found in most or all body segments, as expected from the bilaterally symmetric and segmentally iterated character of the organism's embryonic cell lineage.

Lineally homologous neurons tend to express the same general constellation of differentiated characteristics, but also display a reliable pattern of regional differences. These often involve relatively minor modifications of a common developmental program, but are in some cases so pronounced that it becomes
Fig. 1. Fluorescence micrograph of a leech embryo (Hirudinella triserialis) containing a single rhodamine-labeled blast cell clone, seen in red. Nuclei are stained with Hoechst 33258, and appear as blue dots. The embryo was dissected away from its yolk-mass and flattened. Anterior is towards the top. Prominent features include the invaginating mouth or stomadeum (st), and the segmental ganglia (sg) of the CNS, which form a chain along the midline. A single o blast cell was injected with rhodamine-dextran 4 days earlier in development, and has given rise to a clone of several dozen labeled descendants. This clone lies to one side of the body midline, and is roughly one segment in width. Scale bar, 50 μm.

Fig. 3. Fluorescence micrographs of leech (Thelomyzon rude) embryo nerve cords which have been stained with anti-SCP by indirect fluorescein-immunofluorescence. Anterior is towards the top. (A) The mature RAS neurons (r) stain intensely, and are restricted to a domain of 4 contiguous body segments. This domain includes the three most anterior midbody ganglia, which are numbered, as well as the most posterior neuromere of the fused head ganglion. The mature RAS neuron is unpaired, and shows a strong tendency to alternate right-and-left sides in adjacent segments. Scale bar, 50 μm. (B) At an earlier stage in development, antibody staining reveals a bilateral pair of faintly immunoreactive RAS neurons situated at the anterolateral corners of each ganglion. In the segments of the RAS domain, one of these two homologues will become the mature RAS neuron, while the other cell will lose all detectable immunoreactivity by the end of embryonic life. Scale bar, 20 μm.
prohibitive to assign neuronal homologies solely on the basis of cell phenotype. Many of these regional differences correlate with the identity of the segment in which the neuron differentiates. Identified leech neurons have been shown to develop segment-specific patterns of axonal projection (Shafer and Calabrese, 1981; Glover and Mason, 1986; Gao and Macagno, 1987a,b), synaptic connectivity (Wittenberg et al. 1990) and biochemical differentiation (Loer et al. 1986; Glover, 1987; Shankland and Martindale, 1989). There are also some neurons whose bilateral homologues undergo asymmetric patterns of neuropeptide expression or cell death on the two sides of a single segment (Stuart et al. 1987; Shankland and Martindale, 1989).

Developmental commitments governing neuronal specificity

Because of the fixed relationship between a blast cell’s birth rank and the segmental location of its descendant clone, one could envision that segmental differences in neuronal differentiation might be specified at any time from the production of the ancestral blast cell until the terminal differentiation of the postmitotic neuron, an interval which spans most of embryonic life. This problem has been approached experimentally by confronting neurons — or identified neuronal progenitors — with altered segmental environments in order to test the persistence of their normal, lineage-specific cell fates. A number of neuronal phenotypes have been examined in this manner, and in this article we will focus on one particular cell, the Rostral Alternating SCP-immunoreactive (RAS) neuron, whose development exemplifies certain general principles of neuronal differentiation.

The mature RAS neuron stains prominently with antibodies to Small Cardioactive Peptide (SCP) (Evans and Calabrese, 1989; Shankland and Martindale, 1989). In glossohoriid leeches, immunoreactive RAS neurons are only found in a domain of four contiguous segments towards the animal’s front end (Fig. 3A). Within each of these segments, there is a single RAS neuron whose cell body is located on either the right or left side of the ganglion, and the immunoreactive cells show a pronounced tendency to alternate right-and-left sides in successive segments. Experimental studies reveal that this pattern of RAS neuron differentiation results from two discrete patterning events which occur at disparate stages of neurogenesis (Martindale and Shankland, 1990a,b).

Spatially coordinated patterns of neuronal competition

Embryological investigations have shown that the four mature RAS neurons arise by selective differentiation from a widely distributed set of bilaterally paired homologues, referred to collectively as nz4 neurons, which are descended from the right and left N teloblasts (Shankland and Martindale, 1989). The nz4 neurons begin to express SCP-like immunoreactivity a few days after their birth, at which time they have already formed extensive axonal projections. At first, the right and left homologues express this immunoreactivity at comparable levels (Fig. 3B), but over the following 1–2 days they develop a pronounced asymmetry which results in one homologue becoming the persistently immunoreactive RAS neuron, while the contralateral cell ceases to exhibit any detectable antibody staining. The ultimate fate of this transiently immunoreactive homologue is unknown, and its apparent loss of neuropeptide expression could either reflect a dramatic change in neurotransmitter phenotype or be a prelude to cell death.

The asymmetry of RAS neuron differentiation results from competitive cell interactions that occur at the level of the postmitotic neuron. In the four segments of the RAS domain, there is an equal likelihood that the right or left homologue will take on the RAS phenotype during normal development. However, either cell can be reliably forced to take on this phenotype by ablation of the contralateral homologue neuron or one of its progenitors (Martindale and Shankland, 1990a; Blair et al. 1990). Thus, both the right and left nz4 neurons have the potential to become RAS neurons, and during normal development there are cell interactions that force one of these cells (i.e. the transiently immunoreactive homologue) into an alternative developmental pathway. These interactions are seemingly unaffected by ablation of other neuronal cell lineages, suggesting that the nascent RAS neuron may be directly conveying its contralateral homologue signals which exclude that cell from following the same developmental pathway. Thus, in a formal sense these interactions represent a type of neuronal competition. This competition is clearly postmitotic, since nz4 neurons can still alter their developmental pathway several days after their birth (Martindale and Shankland, 1990a).

The final pattern of RAS neuron asymmetries is not random, as there is >95% likelihood that mature RAS neurons will lie on alternate right and left sides in adjacent ganglia (Evans and Calabrese, 1989; Shankland and Martindale, 1989). Ablation of single nz4 neurons has revealed interganglionic interactions which bias the asymmetry of the intraganglionic competition in neighboring segments (Blair et al. 1990), with the result that patterns of asymmetry develop in a spatially coordinated fashion over several consecutive segments. Thus, each individual nz4 neuron develops the RAS and non-RAS phenotypes in response to a complex array of neuronal interactions (Fig. 4). Some of these interactions have a variable outcome, and the final pattern of RAS neuron differentiation is therefore an emergent property of the interacting network. A similar sequence of events has also been observed for several other identified leech neurons (Macagno and Stewart, 1987; Stuart et al. 1987; Shankland and Martindale, 1989).

In the case of the nz4 neurons, this particular patterning mechanism seems to be restricted to those four segments that will harbor mature RAS neurons. In other segments, both the right and left nz4 neurons
cease to display detectable antibody-staining at an early stage in their differentiation, and no mature RAS neurons are formed (Shankland and Martindale, 1989). The following section describes experiments which show that this segmental difference results from developmental commitments occurring at a much earlier stage of neurogenesis.

Fig. 4. The mature RAS neuron distribution results from a patterned array of postmitotic cell interactions. Initially, homologue neurons (grey circles) situated over a span of 4 consecutive segments have the potential to become RAS neurons. These immature neurons compete for the RAS phenotype (bidirectional arrows), both with their contralateral homologues in the same ganglion, as well as with homologues in neighboring ganglia. The typical outcome is for mature RAS neurons (black circles) to develop on alternate right-and-left sides in successive ganglia. These mature RAS neurons suppress the peptidergic phenotype (unidirectional arrows) in the other homologues.

Neuronal cell lineage and segmental identity
The primary blast cells of the leech embryo appear to have intrinsically determined segmental identities which influence the differentiation of their descendant neurons. This conclusion comes from experiments in which one blast cell bandlet is effectively transplanted out of segmental register with the other nine bandlets (Martindale and Shankland, 1990b; Gleizer and Stent, 1990). Misalignment is brought about by ‘bandlet slippage,’ a morphogenetic abnormality which occurs following certain precisely localized embryonic cell lesions (Shankland, 1984). The lesion itself is restricted to the anteriormost blast cells of a single bandlet, and causes the trailing portion of that bandlet to be frameshifted posteriorly with respect to the other bandlets (Fig. 5). Thus, blast cells in the slipped portion of the lesioned bandlet take part in the formation of segments that are inappropriate for their cell lineage history.

Slipped blast cells generate descendant clones of the appropriate size and bandlet-specific composition, and these clones are readily integrated into their host segments. However, examination of several segment-specific neuronal phenotypes has revealed that the slipped blast cells produce descendants that are for the

Fig. 5. The blast cells of one bandlet can be selectively relocated by a process known as bandlet slippage (Shankland, 1984) into segments that are inappropriate for their cell lineage history. Numbers represent blast cell birth ranks. (A) Blast cells destined for the same segment come together during the normal course of morphogenesis, but abnormal alignments can be induced by photoabating a small number of cells at the point where the bandlets merge. (B) The lesion produces a gap which widens as development proceeds, such that blast cells within the trailing fragment of the broken bandlet fail to keep up with the flanking bandlets. Thus, blast cells in the trailing fragment slip posteriorly with respect to the other segmented tissues, and take part in the formation of segments which are inappropriate for their birth ranks.
most part appropriate for their segment of normal destiny (Martindale and Shankland, 1990b; Shankland and Martindale, 1992), even when those phenotypes are inappropriate for the host segment. This phenomenon has been studied most extensively for the n bandlet, i.e. the progeny of the N teloblast. The n bandlet gives rise to a number of central neurons – including the nZ4 (RAS) neuron – which normally undergo segment-specific expression of peptide or monoamine neurotransmitters. Following unilateral slippage of the n bandlet, these neuronal phenotypes are observed in abnormally posterior body segments (Fig. 6), and each of the slipped blast cells gives rise to neuronal phenotypes which are for the most part characteristic of its normal fate (Fig. 7). This experiment does not alter the segmental specificity of neuronal differentiation in the other, unslipped teloblast lineages – including the contralateral n bandlet – further indicating that the observed changes in the segmental specificity of neuronal differentiation are a direct result of relocating the ancestral blast cells.

A single n blast cell produces approximately 70 descendant neurons (Kramer and Weisblat, 1985), and segmental identity must, therefore, be passed down to the postmitotic neuron over roughly 6 intervening rounds of mitosis. Segmental identity has little influence on the blast cell's pattern of mitosis (Zackson, 1984; Shankland, 1987a, b; Bissen and Weisblat, 1989), or on the number of postmitotic neurons it produces (Kramer and Weisblat, 1985). Nonetheless, the nZ4 neurons which arise from certain blast cells inherit a propensity to become RAS neurons, and are able to do so even if relocated into inappropriate body segments. On the contrary, nZ4 neurons derived from other blast cells will invariably choose the non-RAS developmental pathway, even if relocated into segments of the normal RAS domain.

These findings indicate that there are two discrete
certain n blast cells have the potential to generate blast cells. We here examine the hypothesis that only This finding supports the idea that the formation of observed and predicted locations of the most posterior account the number of segments the n bandlet had been embryo could be accurately predicted by taking into account the number of segments the n bandlet had been slipped. This histogram shows the disparity between the observed and predicted locations of the most posterior immunoreactive RAS neuron for a total of 73 embryos in which either the right or left n bandlet had been slipped posteriorly by 1–7 segments. In 87% of these embryos, the location of this cell was consistent with the measured slippage, within the experimental error of the technique. This finding supports the idea that the formation of immunoreactive RAS neurons is largely determined by the segmental identity of their ancestral blast cell.

steps in the spatial patterning of RAS neuron differentiation. Segmental specificity is passed down to the neuron from its ancestral blast cell, and thus there are eight nz4 neurons (four on each side) whose cell lineage endows them with the potential to take on the RAS neuron phenotype. Competitive interactions ensure that only one of the two nz4 neurons within a ganglion will actually take on this phenotype, and thereby determine the spatial patterning of differentiation within the lineally defined domain.

Establishment of blast cell identity

The slippage experiments demonstrate that the segmental specificity of the n blast cell's descendant clone is already established, at least in part, at the level of the primary blast cell, since the blast cells are repositioned before they undergo subsidiary divisions or come into register with other segmental tissues. Recent findings suggest that segmental diversification is also established at an early stage in the cell lineages derived from the other teloblasts. Slippage of the p bandlet causes a corresponding shift in the segmental distribution of another SCP-immunoreactive neuron (M. Q. Martindale, unpublished data), and slippage of the mesodermal m bandlet alters the segmental distribution of nephridial differentiation (Gleizer and Stent, 1990).

These studies raise the important question of how blast cells within a bandlet obtain their differential identities. The principal issue is to distinguish whether primary blast cells are distinct at the time of their birth, or if their identities are specified by cell interactions at some time during their 20h cell cycle (Zackson, 1984). There are several lines of evidence that argue against instructive cell interactions. Interaction with the other bandlets appears to be ruled out by the slippage experiments, and by the persistence of segmental identity following ablation of various teloblast lineages (Blair, 1983; Shankland and Martindale, 1989). Nor does blast cell identity seem to be determined by instructive signals passing along the bandlet's length. Ablating the N teloblast during blast cell production does not alter the segmental identity of its last daughter cell (M. Shankland, unpublished data), indicating that the establishment of this identity does not require instructive signals emanating from more posterior blast cells. Blast cell identity is also unaffected by ablation of more anterior blast cells (Martindale and Shankland, 1990b; Shankland and Martindale, 1992), although it is not feasible to perform the latter experiment for roughly 15h after the blast cell is born, and we cannot exclude the possibility that instructive signals have already been received from more anterior blast cells during that time. Finally, studies of the teloblast cell cycle suggest that the two alternating types of segmental blast cell which constitute the n and q bandlets are distinct from one another at the time of their birth (Bissen and Weisblat, 1987).

Given the persistent correlation of blast cell birth rank and segmental identity following various experimental manipulations, one must entertain the notion that each individual blast cell already possesses some degree of segmental identity at the time of its birth. In this scenario, primary blast cells would receive ordinal identities in accordance with the stem cell lineage of the parent teloblast, and these ordinal identities would then translate into segmental identities due to the geometry of leech morphogenesis (Fig. 8). For instance, one could envision that the teloblast endows its blast cell progeny with different regulatory factors in a process that is closely linked to its repetitive cell cycle. Regulatory factors might be heterogeneously localized within the teloblast cytoplasm, and be differentially parcelled out to successive blast cell daughters by an orderly process of cell cleavage. Alternatively, the teloblast might express differing amounts or combinations of regulatory factors at each successive cell cycle. In this latter model, the teloblast would effectively 'count' its cell cycles (Fig. 8), and its blast cell daughters would obtain their unique identities by sampling the chemical composition of the teloblast's cytoplasm at different points in time.

The blast cell slippage experiments must be interpreted with an element of caution. While it is clear that there is some degree of anteroposterior differentiation within the newly formed bandlet, the experimental results obtained to date do not necessitate that every primary blast cell has its own unique identity.
These experiments have relied upon only a small number of segmental markers, each of which is normally expressed over a domain of several body segments. By analogy with *Drosophila* segmentation (Akam, 1987), one could imagine that the bandlet is first subdivided into multisegmental domains that are several blast cells in length, and that the blast cells within a given domain do not receive unique segmental identities until some later stage. Additional experimentation will be necessary in order to resolve this issue.

**Regulatory genes and segmental diversification**

Another, more molecular approach to understanding segmental diversification is to identify gene products that play a specific role in the establishment and/or maintenance of segmental identity. The genetics of segmentation is best understood in the fruitfly *Drosophila*, which possesses a set of eight 'homeotic' genes that are primarily responsible for the diversification of its individual segments (Akam et al. 1988; Morata et al. 1990). Molecular analysis has revealed that these particular genes encode transcription factors whose DNA binding region – the 'homeodomain' – is a structural motif shared by the products of a functionally diverse array of regulatory genes (Scott et al. 1989).

The structure of the homeodomain has remained very highly conserved throughout phylogeny, as has the DNA sequence (or 'homeobox') which encodes it. As a result, it has been straightforward to isolate and characterize related genes from a wide variety of animal species. For instance, different vertebrate species possess one or more homeobox gene clusters which are homologous to the homeotic genes of the fruitfly, indicating that they arose from a primordial gene cluster that antedates the phyletic divergence of chordates and arthropods (Graham et al. 1989). In addition, there is experimental evidence that some vertebrate homeobox genes may have a role in segmental diversification (Wright et al. 1989; Kessel et al. 1990). Such findings have led us to imagine that homologous genes may also be involved in the segmental diversification of the leech embryo, and we have therefore undertaken to identify related homeobox genes from the glossiphoniid leech species *Helobdella robusta* (Shankland et al. 1992).

**Leech homeobox genes**

Homeobox sequences were amplified from *H. robusta* DNA by the polymerase chain reaction (PCR). For some of these sequences, longer fragments of the gene were then isolated by screening a genomic DNA library, constructed by E.B. and D.J.P. The details of this procedure will be described elsewhere, and in this paper we will present the deduced amino-acid sequence for four of the leech homeobox genes we have encountered (Fig. 9). Each of these four leech genes shows a pronounced sequence similarity to one or two of the *Drosophila* homeotic genes.

(i) The leech homeobox gene *Lox5* appears to be a specific homologue of *Antp*. The sequence of the *Lox5* homeodomain supports a general homology to the *Drosophila* homeotics, but does not allow it to be identified with any single homeotic gene. However, there is a stretch of the predicted translation product downstream from the homeodomain in which *Lox5* is identical to *Antp* at 10/16 residues (with two gaps). Comparison of *Lox5* with other *Drosophila* genes reveals minimal similarity outside the homeodomain.

(ii) The leech homeobox gene *Lox2* was previously isolated from the non-glossiphoniid leech *Hirudo medicinalis* (Wysocka-Diller et al. 1989). In *H. robusta*, we have discovered a gene whose predicted protein product is identical over the 78 amino-acid domain shown in Fig. 9, and which we therefore believe to be the *Lox2* gene of this species.

*Lox2* shows a pronounced similarity to two *Drosophila* homeotic genes, *Ultrabithorax* (*Ubx*) and *Abdominal A* (*AbdA*), which are themselves closely related. Comparison of the downstream flanking sequence reveals a stretch in which the *Lox2* gene product is identical to *Ubx* at 9/11 residues and to *AbdA* at 4/11 residues (Fig. 9). *Lox2* also shows sequence similarity to *AbdA* in the region immediately upstream from the homeodomain, including the precise location of a splice acceptor site for the exon containing the homeobox. In *Hirudo*, *Lox2* mRNA is expressed in a segment-specific pattern which provides further support for a homology to one or both of these *Drosophila* genes (Wysocka-Diller et al. 1989; D.N.H. and M.S., unpublished results).

(iii) Sequence analysis of the leech homeobox gene *Lox6* suggests that it is a specific homologue of the *Drosophila* homeotic gene *Deformed* (*Dfd*). This conclusion is further supported by comparison with

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**Fig. 8.** Experimental data suggest that the teloblast confers upon each successive blast cell daughter an ordinal identity which accounts at least in part for the segment-specific differentiation of that blast cell's descendant clone. One interpretation of this finding is that the teloblast effectively 'counts' mitotic cycles, and that it endows successive blast cells with different regulatory factors accordingly.
other species. Three different murine homeobox genes have been designated as specific Dfd-homologues based on their comparable location within the phylogenetically conserved homeobox gene cluster (Graham et al. 1989). Fig. 9 compares the sequence of Lox6, Dfd, and its three murine homologues, and shows that there are 6 amino-acid residues which are shared by all of these genes and which distinguish them from Antp. These same residues are also conserved with other putative Dfd-homologues from frog (Harvey et al. 1986) and human (Bonacci et al. 1985).

(iv) The leech homeobox gene Lox7 has only been characterized from PCR-amplified homeobox sequences, but its predicted translation product suggests specific homology to the Drosophila gene labial (lab). Separate PCR amplifications yielded two Lox7 sequences which differed at two bases in codon 46 of the homeodomain (Fig. 9). The two sequences respectively code for a valine or an isoleucine, which are the only amino acids observed at this position within a wide variety of homeodomain proteins – Scott et al. 1989. In the pertinent region of the homeodomain, lab differs from Antp at 7/31 residues, and is identical to the two predicted Lox7 gene products at 4 and 5 of these residues, respectively (Fig. 9). A similar degree of sequence matching was also observed with the murine genes Hox1.6 and Hox2.9, which are believed to be specific lab-homologues based on their position within the phylogenetically conserved homeobox gene complex (Graham et al. 1989; Rubock et al. 1990).

**Evolutionary implications**

Based on sequence analysis, we have tentatively drawn homologies between each of our four leech homeobox genes and one (or, in the case of Lox2, two) of the Drosophila homeotic genes. We imagine that these are specific homologies, by which we mean to imply that each pair of genes is descended from a single ancestral gene which was present in the last common ancestor of the two species. However, our current knowledge of the leech genome is sufficiently fragmentary that one should not exclude the possibility that there may be additional, as yet uncharacterized homologues which may have arisen in the leech genome in the leech genome. As benefits the traditional view of phylogeny, this analysis suggests a more detailed homology between re

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**Fig. 9.** Comparison of leech homeobox-genes Lox2, Lox5, Lox6 and Lox7 with their apparent Drosophila homologues and certain related mammalian genes. Apparent homologies are marked at the right (see text). The Antp sequence is used as a basis for all comparisons. Dashes represent amino-acids that are identical to Antp. Boxes indicate residues that are conserved between the leech gene and other members of its group, but which differ from Antp. Gaps in the downstream portion of the Lox3 sequence were introduced to improve alignment. For Lox2, Lox3 and Lox6, we here show the amino-acid sequence deduced from DNA sequencing of cloned genomic restriction fragments, beginning at possible upstream splicing sequences and continuing downstream through the regions of apparent homology. An identical Lox2 amino-acid sequence was previously obtained by Wysocka-Diller et al. (1989) for another leech species. The Lox7 sequence was deduced from PCR-amplified DNA, and varies at homeodomain codon 46 in the products of two different amplification procedures. Other sequence data were taken from the following sources: Antp (Schneuwly et al. 1986); Ubx (Weinzierl et al. 1987); AbdA (Tear et al. 1990); Dfd (Regulski et al. 1987); Hox1.4 (Duboule et al. 1986); Hox2.6 (Graham et al. 1988); Hox5.1 (Featherstone et al. 1988); lab (Mlodzik et al. 1988); Hox1.6 (Baron et al. 1987); and Hox2.9 (Rubock et al. 1990).
the homeobox genes of flies and leeches than that which has been described for flies and mice (Graham et al. 1989). It has been proposed that the homeobox gene clusters of flies and mice arose from a primordial cluster that was composed of 6 discrete genes. Five of these ancestral genes are thought to have given rise to single homeotic genes in the present-day fruitfly, including lab and Dfd, and our finding of discrete lab- and Dfd-homologues in the leech is consistent with the notion that these two genes were already distinct at the time when annelids and arthropods diverged. The sixth gene of the primordial complex is thought to have given rise to three of the fly's homeotic genes – Antp, Ubx and AbdA – by duplication and divergence. It has been suggested that these particular duplication events occurred relatively late in phylogeny, and are specific to the arthropods (Akam et al. 1988; Akam, 1989).

However, our data suggest that the leech possesses distinct Antp- and Ubx/AbdA-homologues, which would require that at least one of those duplication events must have occurred prior to the phyletic separation of arthropods and annelids.

Conclusions

The process of segmentation occurs by a quite different sequence of events in the various segmented phyla; all segmented organisms have evolved their own developmental mechanisms for governing the periodicity, number and diversity of segments. We have focused here on the development of segmental diversity in the leech nervous system. Segmental identity originates at the level of the primary blast cell, a segmental founder cell which generates several dozen neuronal descendants. Individual blast cells obtain differing identities in accordance with the rank order of their birth, suggesting a lineage-based mode of determination. However, these early segmental identities do not dictate all aspects of segmental diversification, as some patterns of neuronal differentiation arise at later stages through spatially coordinated cell interactions. We have here discussed only those cell interactions that bring about the diversification of right and left hemisegments, but other studies show that differences between segments can also arise through segment-specific cell interactions (Loer et al. 1987; Gao and Macagno, 1987a,b; Martindale and Shankland, 1988) which elaborate upon the lineally determined differences between blast cell clones.

In addition, this article describes our initial progress towards uncovering the molecular basis of segmental identity in leech development. In Drosophila, this problem has been addressed through genetic analysis, and the discovery and characterization of the homeotic genes must certainly rank as one of the crowning glories of developmental genetics. The extension of those studies to other, less genetically amenable organisms through the molecular identification of closely related genes has now opened the door to understanding how the role of particular regulatory genes has (or has not) changed as different phyla evolved their unique developmental strategies. Comparison of leech and insect should be particularly interesting, for although these animals generate segmentation by strikingly different mechanisms (Shankland, 1991), it is widely believed that they arose from a common segmented ancestor. We hope eventually to develop molecular probes that will allow us to examine the expression of these leech homeobox genes, and to devise strategies for testing their developmental significance by altering patterns of expression. In this way, we may learn what if any role these genes play in the segmental specification of blast cells and/or their neuronal descendants.

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