**Lox2**, a putative leech segment identity gene, is expressed in the same segmental domain in different stem cell lineages

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**Summary**

The segmented tissues of the adult leech arise from a set of five, bilaterally paired embryonic stem cells via a stereotyped sequence of cell lineage. Individual segments exhibit unique patterns of cell differentiation, and previous studies have suggested that each stem cell lineage establishes at least some aspects of its own segmental specificity autonomously. In this paper, we describe a putative leech segment identity gene, *Lox2*, and examine its expression in the various stem cell lineages. Both sequence analysis and the segmental pattern of *Lox2* expression suggest a specific homology to the fruitfly segment identity genes *Ubx* and *abdA*. In situ hybridization reveals a cellular accumulation of *Lox2* RNA over a contiguous domain of 16 midbody segments (M6-M21), including postmitotic neurons, muscles and the differentiating genitalia. *Lox2* transcripts were not detected at the stage when segment identities are first established, suggesting that *Lox2* gene products may not be part of the initial specification process. Individual stem cell lineages were labeled by intracellular injection of fluorescent tracers, and single cell colocalization of lineage tracer and hybridization reaction product revealed expression of *Lox2* RNA in the progeny of four different stem cells. The segmental domain of *Lox2* RNA was very similar in the various stem cell lineages, despite the fact that some stem cells generate one founder cell/segment, whereas other stem cells generate two founder cells/segment.

**Key words:** cell lineage, homeobox gene, leech embryo, segment identity, stem cell.

**Introduction**

In both insect and vertebrate embryos, segmentation does not depend upon a fixed cell lineage, but results instead from the positional specification of spatially defined cell groups (Akam, 1987; Wilkinson and Krumlauf, 1990). Segmental differences arise secondarily because distinct cell groups express segment identity genes in differing combinations or patterns depending upon their position along the anteroposterior body axis (Peifer et al., 1987; Duncan, 1987; Akam, 1989). In contrast, the leech develops its segmental body plan through a stereotyped cell lineage, with cells of previously specified segment identity coming together over large distances to assemble into segmental primordia (Martindale and Shankland, 1990b; for review, see Shankland, 1991). Both insects and vertebrates use complexes of Antennapedia-like homeobox (HOM/HOX) genes to specify segment identities (Akam, 1989; Graham et al., 1989), and we are interested in learning to what extent the leech embryo employs homologous genes, and the nature of the developmental events that regulate their expression.

The adult leech has 32 body segments (Fig. 1A) which are conventionally demarcated in accordance with the segmental ganglia or neuromeres of the central nervous system (CNS). The four most rostral neuromeres fuse to form a single subesophageal ganglion, and the associated segments are designated as R1 - R4 in anteroposterior order. The twenty-one midbody segments retain their individual unfused ganglia, and are designated as M1 - M21. The seven most caudal segments fuse to form the rear sucker and associated terminal ganglion, and are designated as C1 - C7.

Glossiphoniid leeches have large embryos in which the developmental fate of particular embryonic cells has been extensively characterized by the intracellular injection of vital lineage tracer compounds (Weisblat et al., 1978; Gimlich and Braun, 1985). The early embryonic cleavages produce five bilaterally paired stem cells, collectively known as teloblasts, which are individually designated as cells M, N, O, P and Q. Lineage tracer injection reveals that each teloblast gives rise to a distinctive set of descendant tissues which are segmentally arrayed along the anteroposterior axis (Weisblat and Shankland, 1985). Together, these five teloblasts give rise to the segmented mesoderm and ectoderm for all 32 body segments. The endoderm and the anteriormost region of the head arise from other, unsegmented cell lineages (Weisblat et al., 1984).

The segmental periodicity of the ectoderm and mesoderm arises from the iterated stem cell divisions of the teloblasts.
A given teloblast buds off a linear chain (or bandlet) of much smaller primary blast cell daughters, which serve during subsequent development as segmentally homologous precursor cells (Fig. 1B). In the M, O and P lineages, each blast cell produced by the teloblast gives rise to a similar clone composed of roughly $10^2$ terminally differentiated descendants (Weisblat and Shankland, 1985; Shankland, 1987a,b). The primary blast cell clones within each teloblast lineage are tandemly arrayed in successive body segments, with the firstborn blast cells — which come to lie furthest from the teloblast — contributing to the most anterior segments. The primary blast cells divide to produce multicellular descendant clones. In the M, O and P lineages, there is one blast cell clone/segment, whereas in the N and Q lineages there are two blast cell clones/segment. Although segmentally arrayed, the individual blast cell clones cross segment boundaries and intermingle in a stereotyped fashion to form the segmented tissues of the adult leech (Weisblat and Shankland, 1985).

The invariant correlation between a blast cell’s birth rank and the segmental location of its descendant clone raises the possibility that some of the segmental differences observed in the mature leech could derive from segment identities specified at the time of blast cell formation. Support for this idea has come from transplantation experiments in which one of the ten teloblast lineages is ‘slipped’ out of register with the other segmented tissues. Following slippage of the right or left N lineage, the displaced blast cells incorporate into ectopic segments, but generate neuronal phenotypes that are appropriate for their own lineage history, independent of the identity of the host segment (Mar-
Blast cells that are going to contribute their descendants to the same adult segment are generated at diverse positions and times, and the slippage experiments demonstrate that the blast cells already possess segment identities several days before their descendants come together to form spatially coherent segmental primordia. This suggests that each teloblast lineage establishes its own segmental specificity autonomously, and teloblast ablation experiments conform to this idea. If one or more teloblasts are eliminated prior to blast cell formation, the absence of their descendant lineages does not abolish the segmental specificity of the remaining cell lineages (Martindale and Shankland, 1990a). Such findings have led to the hypothesis that each teloblast effectively ‘counts’ its cell cycles, and establishes the segment identities of its blast cell progeny accordingly in anteroposterior succession (Shankland et al., 1991).

The developmental independence of the various teloblast cell lineages raises questions about the potential role of segment identity genes in this organism. Do different teloblast lineages utilize the same regulatory molecules to establish segment identities, and do they express regulatory molecules in similar or differing segmental domains? If a segment identity gene were expressed in the same domain of segments in all five teloblast lineages, it would mean that the domain of gene expression would be compressed anteriorly in the N and Q lineages (Fig. 3B).

To address these questions, we have examined the spatiotemporal expression of a putative leech segment identity gene, Lox2, in relationship to various steps of the segmentation process. This homeobox gene was originally isolated in the hirudinid leech Hirudo medicinalis (Wysocka-Diller et al., 1989), and later described from the glossiphoniid leech Helobdella robusta (Shankland et al., 1991).

Sequence analysis indicates a close phylogenetic relationship between Lox2 and the HOM/HOX genes Utrarabilitho -rux (Ubx) and abdominal-A (abda), which serve a segment identity function in the fruitfly Drosophila (Duncan, 1987; Peifer et al., 1987). The Helobdella embryo is amenable to lineage tracer analysis, and we have used this latter technique in conjunction with in situ hybridization to compare the pattern of Lox2 expression in the progeny of different teloblasts.

**Materials and methods**

**Animals**

The leeches used in this study were taken from a laboratory breeding colony maintained at Harvard Medical School, and established with individuals collected in Sacramento, California (Shankland et al., 1992). The colony is kept at 24°C in 0.5% artificial sea water supplemented with 1 mM CaCl₂ and fed thrice weekly on pond snails. Embryos were isolated from the maternal parent 1-2 days after egg deposition, maintained in a buffered saline medium (Torrence and Stuart, 1986) and staged according to Stent et al. (1982).

**Polymerase chain reaction (PCR)**

The Lox2 homeobox was initially isolated by PCR amplification. Amplifications were carried out on 1.2 µg of H. robusta genomic DNA, diluted in 50 µl volume with reagents from the GeneAmp kit (Perkin Elmer Cetus) supplemented with 1 mM MgCl₂ and 1 µg of each primer. Reaction temperature was controlled on an MJ Research programmable thermal cycler using the following conditions: 4 minutes at 95°C; 35 cycles of 1 minute at 95°C, 2 minutes at 40°C and 2 minutes at 72°C; 5 minutes at 72°C.

PCR was performed with two different pairs of degenerate oligonucleotide primers corresponding to highly conserved

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Fig. 3. Hypothetical patterns of segment identity gene expression within the M, N, O, P and Q cell lineages. Rectangles represent blast cell clones, numbered according to the birth rank of the ancestral blast cell in the stem cell lineage. (A) If a segment identity gene is expressed in a similar domain of segments in all lineages, the N and Q lineages would differ from the rest in terms of the relationship between blast cell birth rank and gene expression. (B) If blast cells within a particular range of birth ranks express the segment identity gene regardless of their teloblast of origin, then the segmental domain of gene expression would be compressed anteriorly in the N and Q lineages.
Fig. 4. Nucleotide and deduced amino-acid sequence of the Lox2-Hro cDNA. Characterization of genomic DNA restriction fragments yielded an identical sequence from the known splice site (hollow arrowhead) to the beginning of the poly(A) tail (solid arrowhead). The homeodomain is boxed, and the YPWM sequence and polyadenylation signal sequences are marked. The deduced amino-acid sequence of the Hirudo Lox2 gene [Hme] is shown for comparison (Wysocka-Diller et al., 1989), with identical amino-acids marked by dashes. Note that the two predicted protein products have a stretch of 78 identical amino-acids spanning the homeodomain, but diverge in the C-terminal region. This sequence has been deposited in the EMBL Library database (accession number Z14313).
regions of the homeobox. The first amplification was performed with primers SMS6 \([5'-CA(A/G)TGGA(A/G)-(C/T)TGGA - 3']\) and SMS8 \([5'-TT(T/G)AG(A/G)AA(A/G)TGAT(T/C)TT - 3']\), expected to yield homeobox sequences of 110 bp (corresponding to Nt 691 - 800, Fig. 4). A second amplification was performed with primers SMS11 \([5'-GA(A/G)(C/T)-TGGA(A/G)AA(A/G)GA(A/G)TT - 3']\) and SMS8, expected to yield homeobox sequences of 110 bp (corresponding to Nt 681 - 800, Fig. 4).

Products of amplification were separated by polyacrylamide gel electrophoresis and visualized with ethidium bromide. For each reaction, a visible band of the appropriate length was isolated and purified by electrophoresis, and cloned into Bluescript KS+ (Stratagene). Recombinant plasmid was transformed into \(E. \ coli\) strain GT1 by the method of Chung et al. (1989). In total, we screened 3\times10^4 transformants. The most prevalent clones were characterized by random isolation and dideoxy sequencing (Sequenase kit; US Biochemical). Other, less prevalent clones were detected by using \(^{32}P\)-labeled DNA synthesized from a combination of the prevalent insert sequences to screen at high-stringency nylon lifts of 10^11 bacterial colonies. Sixty colonies that did not hybridize with this combined probe were also subjected to plasmid purification and dideoxy sequencing.

This analysis led to the isolation of nine distinct amplified sequences, six of which were clearly derived from homeobox genes, including \(Lox2\) (Shankland et al., 1991). We obtained one clone of \(Lox2\) from the first amplification and three from the second amplification, all showing the same DNA sequence in the region amplified between the two primers.

**Library construction and screening**

We used the 110 bp amplified \(Lox2\) sequence to screen 8\times10^6 recombinant clones of an \(H. \ robusta\) genomic DNA library constructed in \(\lambda\) phage EMBL3a by E. Baxter and D. J. Price, University of Edinburgh. Phage plating and duplicate filter lifts were carried out according to standard procedures (Sambrook et al., 1989). Prehybridization was carried out in 0.75 M Na2HPO4 (pH 7.2) with 1% SDS at 65°C, and hybridization was performed in the same buffer containing 30 ng of \(^{32}P\)-labeled probe DNA (10^6 cts/minute/\(\mu\)g). Filters were washed three times 1 hour in 0.03 M Na2HPO4 (pH 7.2) with 1% SDS at 65°C. Positive clones were plaque purified, and the \(\lambda\) DNA isolated on DEAE-cellulose. The purified DNA was digested with a panel of restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon (Amershams) and hybridized with the \(Lox2\) probe as described above. A hybridizing 5 kb \(HindIII\) fragment was subcloned into Bluescript KS+ and partially sequenced.

Two separate \(H. \ robusta\) cDNA libraries were constructed in \(\lambda\)-ZAP according to manufacturer’s instructions (Stratagene). Total RNA was isolated respectively from stage-10 embryos and adults (Chomczynski and Sacchi, 1987), and the poly(A) fraction purified on oligo(dT) cellulose (Sambrook et al., 1989). We screened 10^6 independent clones from both libraries as described above using a 395 bp PCR-generated \(Lox2\) genomic fragment (Nt 633 - 1,027, Fig. 4). A single positive clone was obtained from the embryonic library; none from the adult library. The plasmid replicative form (Bluescript SK-) was rescued by in vivo excision, and the cDNA insert completely sequenced in both orientations. Sequence compressions were resolved by replacing dGTP with dITP (Sequenase kit; US Biochemical).

**In situ hybridization**

The cellular distribution of \(Lox2\) message was examined by non-radioactive whole-mount in situ hybridization. Our technique was based on the Genius RNA labeling and detection kit (Boehringer-Mannheim) as modified by R. Harland, UC Berkeley. In short, leech embryos were fixed for 30 minutes in 4% formaldehyde, washed and stored in methanol, rehydrated in PBS [130 mM NaCl + 10 mM phosphate buffer, pH 7.4] and digested for 20-25 minutes in 0.5 mg/ml Pronase E (Sigma) in 50 mM Tris and 5 mM EDTA (pH 8.0). Digestion was stopped with 2 mg/ml glycine in PBS. Digested embryos were transferred to 1 ml of 0.1 M triethanolamine buffer (pH 8) and acetylated by two sequential 5 minutes treatments with 3 µl acetic anhydride. Acetylated embryos were returned to PBS, and post-fixed for 30 minutes with 4% formaldehyde in PBT [PBS + 0.1% Tween-20], followed by washing and storage in PBT.

Hybridization was performed overnight at 59°C in a 1:1 mixture of deionized formamide and 5x SSC buffer, with 0.2 µg/µl tRNA, 2% 50x Denhart’s solution, 0.1 µg/µl heparin, 0.1% Tween-20 and 0.1% CHAPS (Sigma). The plasmid containing the \(Lox2\) CDNA was linearized at a restriction site upstream of the insert, and a full-length digoxigenin-labeled antisense riboprobe was synthesized using T7 RNA polymerase. To improve tissue penetration, this probe was hydrolyzed into shorter fragments by heating for 45 minutes to 60°C in a solution of 40 mM sodium bicarbonate + 60 mM sodium carbonate. Optimal staining was obtained with hybridization solution containing approximately 1 ng/µl digoxigenin-labeled RNA. Labeled embryos were washed at 60°C with several changes each of hybridization solution without probe and 2x SSC with 0.3% CHAPS. To remove unhybridized probe, embryos were treated for 30 minutes at 37°C with 0.5 µg/µl RNase A (Sigma) in 2x SSC, followed by additional washing at 60°C.

Washed embryos were transferred to room temperature PBT, and the bound probe visualized by immunohistochemistry. Non-specific binding was reduced by a 1 hour incubation with 10% heat-treated (55°C) sheep or goat serum. Embryos were then washed and incubated overnight at 4°C in a 1:5000 dilution of alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments (Boehringer-Mannheim). Unbound antibody was removed with several washes, totalling 5 hours, in room temperature PBT. Embryos were then transferred to AP reaction buffer [100 mM NaCl + 100 mM Tris + 50 mM MgCl2; pH 9.5] with 0.1% Tween-20 and 1 mM levamisol. The AP reaction was performed in the dark at room temperature using the NBT and X-phosphate color reagents provided in the Genius kit. Optimal staining was obtained with reaction periods ranging from 15 hours to 3 days, with color reagents replenished daily. Reaction was terminated with PBT, followed by PBS.

Intact embryos were cleared and photographed with a 10× objective using bright-field optics. Clearing was accomplished either in 80% glycerol, or by dehydration in ethanol and propylene oxide, followed by infiltration with Polybed plastic embedding medium (Polysciences). Other specimens were dissected in PBS so as to remove the yolk-filled midgut, and the body wall flattened under a coverslip in 80% glycerol. Some of the specimens embedded in Polybed were cured, cut into sections of approx. 0.1 mm thickness using a handheld razor blade and mounted under a coverslip in Fluoromount (Biomedical Specialties; Santa Monica, CA). Dissected and sectioned specimens were viewed with 40× or 63× objectives using Nomarski optics.

To determine the specificity of hybridization staining, embryos of various stages were processed with various alterations in the protocol: (i) sense and antisense RNA probes taken from other leech homeobox genes; (ii) absence of digoxigenin-labeled RNA probes; (iii) absence of anti-digoxigenin antiserum and (iv) pre-treatment of 1 hour at 37°C with 20 µg/ml RNAase A (Sigma) in 0.5 M NaCl + 1 mM EDTA + 10 mM Tris-Cl, pH 8.0, to digest cellular RNA.
Fluorescent lineage tracers

To ascertain the lineage history of cells that express Lox2 transcripts, identified embryonic blastomerses were pressure injected with 50 mg/ml tetramethylrhodamine-dextran-amine (RDA) in 0.1 M KCl and 2% fast green FCF. The RDA used in this study was obtained from Molecular Probes (Eugene, OR; catalog no. D-1817). Injected embryos were monitored by fluorescence to ensure that the labeled cell lineage was developing normally, and were fixed at the appropriate ages and hybridized for Lox2 RNA as described above. Double-labeled embryos were dissected, mounted under cover slips and examined with a 63× objective on a Bio-Rad MRC-600 confocal microscope. The AP reaction product was co-visualized with Nomarski optics using a transmission detector linked by fiber optic to a second channel photomultiplier.

For experiments involving two lineage tracers, one embryonic blastomere was injected with RDA, and a second with a fluorescein-dextran-amine (FDA; Molecular Probes; D-1820).

Results

Isolation and sequence analysis

The Helobdella Lox2 gene was initially isolated from genomic DNA by PCR amplification using redundant oligonucleotide primers corresponding to homeobox sequences highly conserved among the HOM/HOX genes (see Methods). Amplified DNA of the expected length was cloned into the Bluescript plasmid and individual clones sequenced, leading to the discovery of several distinct homeobox fragments (Shankland et al., 1991). One of the homeoboxes obtained in this manner has a conceptual translation product identical to that of the Hirudo gene Lox2 (Wysoccka-Diller et al., 1989). Where distinction is necessary, we will refer to the Hirudo medicinalis gene as Lox2-Hme and to the Helobdella robusta gene as Lox2-Hro.

The amplified Lox2-Hro homeobox was used to screen a Helobdella genomic DNA library, leading to the isolation and partial sequencing of a 5 kb HindIII restriction fragment containing the identical homeobox sequence. A portion of this genomic fragment was in turn used to screen a stage-10 embryo cDNA library. Sequence analysis of the single positive cDNA clone revealed extensive identity with both the amplified homeobox and the genomic fragment. Hybridization screening of a second Helobdella cDNA library generated from adult RNA failed to yield any additional positive clones.

The complete sequence of this Lox2-Hro cDNA is shown in Fig. 4. At the 5′ end, this cDNA contains a 1,287 bp open reading frame (ORF) which is in frame with the expected homeodomain coding sequence (Nt 649-828). It seems likely that this cDNA does not include the initiation codon and N-terminal coding sequence, since the first ATG is situated over one hundred residues into the ORF. The ORF is flanked 3′ by a 365 bp non-coding sequence, including a 20 bp poly(A) tail. The nucleotide sequence of this cDNA exactly matches that of the cloned genomic DNA in the region Nt 632 - 1,632, representing the most 3′ exon of this particular Lox2 transcript (Fig. 4). This exon is bounded at the 5′ end by a splice acceptor sequence (CAG/G, in the genomic DNA), and at the 3′ end has a canonical polyadenylation signal (AATAAA) situated 19 bp upstream of the poly(A) tail.

Sequence comparisons

There are extensive similarities in the protein products predicted by the Lox2-Hro cDNA and the Lox2-Hme genomic sequence, although the nucleic acid sequences differ considerably (see below). Both Lox2 genes share the same splice site immediately upstream of the homeobox (Wysoccka-Diller et al., 1989), and they encode a stretch of 78 identical amino-acids which extends from this splice site through the entire length of the homeodomain (Fig. 4). At this point the two amino-acid sequences diverge rapidly, with no detectable similarity near the C terminus. The Lox2-Hro cDNA encodes 100 additional amino acids.

There are also striking similarities between the predicted protein product of the Lox2-Hro cDNA and certain of the fruitfly HOM/HOX gene products. Within the homeodomain, Lox2 shows 85% identity (50/60 amino acids) with both Ubx and abdA. This similarity extends into the coding region flanking both sides of the homeodomain (Fig. 5), a property not shared with any of the other fruitfly HOM/HOX genes. In addition, the next upstream exon encodes the amino-acid sequence YPWM, which is highly conserved among the fruitfly HOM/HOX genes, including Ubx and abdA (Wilde and Akam, 1987; Karch et al., 1990). The remainder of the upstream Lox2 protein sequence showed little similarity to either fruitfly gene (Ubx - 10%; abdA - 8%).

In our single Lox2 cDNA, adjacent exons encode the homeodomain and YPWM sequences. Both Ubx and abdA have a similarly situated exon junction (Fig. 5), but typically include additional exons at this site (Kornfeld et al., 1989; Karch et al., 1990). We have not yet determined whether Lox2 transcripts are subject to alternative splicing.

Taxonomy places Helobdella and Hirudo in distantly related branches of the leech phylogeny, but evolutionary studies have been hindered by an exceedingly sparse fossil record (Sawyer, 1986). To gain insight into the evolutionary history of these species, we examined the accumulation of translationally silent nucleotide changes in corresponding regions (AA 212 - 289; Fig. 4) of the cognate Lox2 genes. (Replacement changes were not considered due to the small number of divergent amino-acid residues available for comparison.) Using the method of Perler et al. (1980) we calculated the percent corrected divergence at silent sites in the sequence, a parameter that increases monotonically with time since separation. Lox2-Hro and Lox2-Hme yield a percent corrected divergence of 191. For comparison, we have calculated that the homeodomain-containing exon of abdA genes from fruitfly and grasshopper (which diverged approx. 300 million years ago) yield a percent corrected divergence of 141 (Tear et al., 1990), and pairwise comparisons of the two leech Lox2 genes and their insect homologues yields percent corrected divergences in the range 143 - 172. The variability in this parameter (Perler et al., 1980) does not permit a precise ordering of evolutionary events. Nonetheless, we feel that the large degree of divergence observed between the two leech genes is consistent with an ancient phyletic separation, and provides general support for Sawyer’s (1986) contention that leeches were established as a distinct lineage no later than the early Mesozoic era (approx. 200 million years ago).
In situ hybridization
To examine the spatiotemporal pattern of Lox2 expression, we hybridized digoxigenin-labeled antisense Lox2-Hro RNA to fixed, permeabilized embryos of various stages (Fig. 6). Bound probe was stained with AP-anti-digoxigenin, and visualized by histochemical reaction. The pattern of Lox2 hybridization was distinct from that seen with other leech homeobox genes (M.S. and D.N.H., unpublished results), and there was essentially no staining of embryos processed without either digoxigenin-labeled RNA or anti-digoxigenin. The observed staining could be eliminated by pretreating embryos with RNAase A. We therefore conclude that the hybridization patterns described here are likely to give an accurate depiction of the cellular distribution of Lox2 RNA.

A sharply defined pattern of cellular staining first becomes evident early in embryonic stage 9 (Fig. 6C), by which time the embryo shows an outwardly segmented morphology and the vast majority of central neurons are undergoing terminal differentiation (Stewart et al., 1986; Stuart et al., 1987). The number and intensity of staining cells increases (Fig. 6E) over the next 2 days of development (the middle of stage 10), and remains stable for a couple of days thereafter. There is a dramatic decrease in staining intensity early in stage 11, which we believe to be an artifact due to changes in tissue penetrability resulting from body wall closure and cuticle secretion. Attempts to perform in situ hybridization on dissected embryos resulted in intense background staining.

The segmental distribution of hybridization staining was similar in different organ systems, but we will describe the CNS and peripheral tissues separately. In the CNS, the earliest detectable Lox2 hybridization is associated with a contiguous domain of segmental ganglia showing a sharp anterior boundary in the seventh midbody ganglion (M7), and gradually decreasing in intensity more posteriorly (Fig. 6C). During early stage 9, staining is restricted to a small cluster of cell bodies situated in the anteromedial region of these ganglia, as well as a single large pair of cell bodies situated more laterally. The medial cell bodies show a uniform, posteriorly decreasing gradient of staining, but the lateral cell bodies are much more intensely stained in segment M7 than in the other segments (Fig. 6C). At this and later stages, Lox2 hybridization was largely restricted to the perinuclear cytoplasm, and did not extend into the neuropil or connective nerves.
As the embryo matures, *Lox2* hybridization is observed in a large number of additional central neurons, and possibly glia, which are distributed through the segmental domain M6 - M21 (Fig. 6A). As with the initial staining, we saw clear segmental differences in the most anterior segments of this domain. In ganglion M6, *Lox2* hybridization is restricted to a bilaterally paired cluster of neurons situated immediately lateral to the root of the posterior connective (Fig. 6B,E). In M7, *Lox2* hybridization is observed in a large number of neuron clusters which are dispersed throughout diverse regions of the ganglion (Fig. 6B). In M8 and more posterior ganglia, most or all neurons exhibit *Lox2* hybridization, leading to a nearly uniform staining of the ganglionic cortex (Fig. 6D,E).

Early in stage 10, there is an anterior-to-posterior gradient of hybridization in the more posterior midbody segments (Fig. 6A). However, by the end of stage 10 ganglia M8 - M17 display superficially indistinguishable hybridization patterns, whereas M18 - M21 show a progressive decrease in the number of hybridizing cells (Fig. 6F). In this species the segmental domain of *Lox2* hybridization has a sharp posterior boundary, and was never seen to extend into the neuromeres of the fused caudal ganglion.

The peripheral tissues show a similar domain of *Lox2* hybridization. Early in stage 9, peripheral hybridization is observed in a reliable array of tissues which outline each of the segmental ganglia that shows neuronal hybridization (Fig. 6C). As the germinal plate spreads out from the ventral midline to envelop the yolky midgut, some of the hybridizing cells are drawn out laterally into spindle-shaped bundles (Fig. 6A,B), which are associated with the septal mesoderm that separates embryonic somites (Fig. 7A,B). By the end of stage 10, it is clear that these hybridizing cell bundles correspond to the dorsoventral flattener muscles, which span the body cavity and constrict the midgut to form grooves between the segmentally arranged crop caeca (Sawyer, 1986). Every segment contains a bilateral pair of dorsoventral muscles, but *Lox2* hybridization is only observed in dorsoventral muscles located in the posterior two-thirds of the midbody (Fig. 6A,B). The ventral end of each muscle is positioned laterally between a single pair of CNS ganglia, and the most anterior dorsoventral muscle to exhibit *Lox2* hybridization is situated at the M6/M7 segment boundary.

In darkly stained embryos, we observed *Lox2* hybridization in other deep mesodermal structures. This latter staining was faint, and could not be readily ascribed to any particular tissue, but — like the structures described above — was clearly restricted to the posterior two-thirds of the midbody. It should be noted that there are more than a dozen differentiated peripheral neurons in each hemisegment of the leech embryo at the stages examined here (Weisblat et al., 1985), and that if these peripheral neurons express *Lox2* RNA at all, they must do so at a significantly lower level than the central neurons.

In addition to these segmentally repeated structures, segment M6 showed a unique pattern of peripheral *Lox2* hybridization. The dorsoventral muscle which lies at the M6/M7 boundary serves as the focus for an aggregation of mesodermal cells not found in other segments (K. Deisseroth and M. Shankland, unpublished results). The most posterior portion of this mesodermal aggregation begins to show intense *Lox2* hybridization by the middle of stage 9, at first giving the appearance of laterally projecting streaks (Fig. 6A,B; 7C). By the end of stage 10, the dorsal closure of the germinal plate has drawn these two hybridizing struc-

Fig. 7. Mesodermal expression of *Lox2* RNA. (A,B) Paired Nomarski and fluorescence images showing *Lox2* hybridization in the septal mesoderm (sm), which gives rise to the dorsoventral muscles of the mature leech. Images were taken on a confocal microscope from a parasagittal section of a stage-10 embryo in which the left M teloblast had been injected with RDA. Anterior is to the top; ventral, to the right. Intense RDA fluorescence is evident in the septal mesoderm, and the visceral mesoderm (vm) which lines the gut. There is a punctate component to the fluorescent labeling due to sequestration of the intracellular RDA. The injected teloblast was absorbed into the syncytial midgut primordium after completing generation of the segmental mesoderm, and as a result, a small amount of RDA fluorescence can also be seen in the endodermal layer (en) and amongst the yolky contents of the gut. Scale bar, 10 µm. (C) Nomarski image showing *Lox2* hybridization in a portion of the genital primordium (gp), seen in dorsal view of a stage-10 embryo which was dissected following hybridization. Reaction product is also evident in the dorsoventral muscles (dv), and in midbody ganglion 7. Hybridization is not evident in midbody ganglion 6, the salivary glands (sg), or the circular medial portion of the genital primordium. Scale bar, 20 µm. (D) Nomarski image showing *Lox2* hybridization in the genital primordium (gp), seen in dorsal view of an intact early stage-11 embryo. The right and left genital primordia embrace the tubular esophagus (es), which connects anteriorly to the proboscis (pr) and posteriorly to the crop (cr). At this stage in development, the lumen of the crop is filled with yolk platelets, which appear as bright spheres. Scale bar, 50 µm.
tures into a dorsoventral orientation, such that they embrace the midgut near the junction of the esophagus and crop (Fig. 7D). These hybridizing structures seem to represent the most lateral and posterior portion of the genital primordium (Fig. 7C), which gives rise to both the male and female apparatus of the hermaphroditic adult.

Lox2 hybridization of fertilized eggs and cleavage-stage embryos gave uniform staining of all yolk-free cytoplasmic domains, a pattern that was outwardly similar to the background staining obtained with other nucleic acid probes. Hence, on the basis of in situ hybridization there appears to be little or no accumulation of Lox2 RNA prior to embryonic stage 9.

Cell lineage analysis
To investigate the expression of Lox2 RNA in the various teloblast lineages, we injected individual teloblasts with the lineage tracer RDA at early stages of development, and at stage 10 examined the colocalization of RDA fluorescence and in situ hybridization reaction product in descendants of the injected cell. For each lineage, we identified the most anteriorly situated descendant cell which expressed detectable levels of Lox2 RNA, and compared the pattern of expression over the segments M6 - M8. Attention was focused on the anterior end of the Lox2 expression domain because those ganglia exhibit a number of darkly stained cells which are bordered by cells containing little or no reaction product.

M lineage
The M teloblast gives rise to the entire mesodermal layer of the leech germinal band (Weisblat and Shankland, 1985). We have found that the peripheral structures that express detectable Lox2 RNA — the dorsoventral muscles (Fig. 7A,B) and the genital primordium — are both labeled with RDA following injection of the M teloblast. Within this lineage, the anterior boundary of Lox2 expression corresponds to the dorsoventral muscles situated at the M6/M7 segment border (Fig. 8A). The genital tissues that express Lox2 are initially seen in segment M7, and during the course of development move anteriorly to coalesce near this particular muscle.

N lineage
The N lineage gives rise to roughly two-thirds of the central neurons throughout the entire CNS (Weisblat and Shankland, 1985). In this lineage, the anteriormost Lox2 expression was observed in a neuron situated at the anterolateral corner of ganglion M7 (Fig. 8A; 9A,B). Lox2 hybridization product was also observed in several other N-derived neurons within this ganglion. However, the majority of N-derived neurons in ganglion M7 show no detectable staining (Fig. 9A,B), implying that the n blast cell clones that contribute to that ganglion display a mosaic pattern of expression.

It should be noted that the pattern of Lox2 hybridization within the N lineage exhibits stepwise changes in successive body segments. Following N teloblast injection, we did not observe any colocalization of lineage tracer and reaction product in ganglion M6, whereas several N-derived cells were observed to hybridize in ganglion M7. In gan-

Fig. 8. (A) In the M, N, O and Q lineages, the anteriormost expression of Lox2 RNA is situated in either the posterior of segment M6 or the anterior of segment M7. Ganglia of the CNS are shown in outline. The segmental distribution of Lox2 RNA expression was not delineated in the P lineage. (B) Schematic demonstrating the progressive increase of Lox2 RNA expression in successive o blast cell clones. Hemiganglia are shown as grey rectangles, and the O-derived neurons as circles. Each o blast cell clone contributes three clusters of central neurons (CR, PV and AD), which are distributed over two successive ganglia and circumscribed by dashed boxes. The clone that straddles the M5/M6 segment border shows no Lox2 expression, whereas the clone that straddles the M6/M7 segment border shows intense expression (black) in a subset of PV neurons, as well as a lower level of expression (cross-hatching) in some or all AD neurons. The clone that straddles the M7/M8 segment border shows increased expression in the PV neuron cluster, as well as intense staining of some CR cluster neurons.
glion M8, most or all neurons show $Lox2$ hybridization product (Fig. 6E), indicating a significantly greater number of cells that express $Lox2$ RNA within the n blast cell clones.

**O lineage**

The O teloblast gives rise to specific subsets of central and peripheral neurons, as well as portions of the body wall epidermis (Shankland, 1987a; see Fig. 8B). In this study, we found that the most anterior central neurons that express $Lox2$ RNA, i.e. the cluster of hybridizing neurons situated in the posterior of ganglion M6, are derivatives of the O lineage (Fig. 8A; 9C,D).

Previous studies have shown that each o blast cell gives rise to three distinct clusters of central neurons: the crescent (CR) and posteroverentral (PV) clusters within one ganglion, and a separate dorsoventral (DV) cluster situated in the next posterior ganglion (Shankland, 1987a). Colocalization of lineage tracer and hybridization reaction product revealed that the neurons that express $Lox2$ RNA in ganglion M6 represent some, but not all, of the M6 PV cluster (Fig. 9C,D). It should be noted that there was no detectable $Lox2$ hybridization in the M6 CR cluster, although there appeared to be faint hybridization in cells of the M7 AD cluster. Thus, $Lox2$ shows a mosaic pattern of expression within this particular o blast cell clone (Fig. 8B).

As observed with the N lineage, we saw additional neurons expressing $Lox2$ RNA in successively more posterior ganglia (Fig. 8B). In particular, the next posterior o blast cell clone exhibits hybridization not only in the PV and AD clusters, but also in a large cell situated at the medial end of the CR cluster (Fig. 9E,F).

**P lineage**

The P lineage gives rise to a combination of central and peripheral neurons and portions of the epidermis (Shankland, 1987b). We were unable to demonstrate unequivocal colocalization of lineage tracer and $Lox2$ hybridization following injection of the P teloblast, although it appeared that some of the P-derived neurons in ganglion M7 might exhibit low levels of $Lox2$ expression.

**Q lineage**

The Q lineage gives rise to a combination of central and peripheral neurons and portions of the epidermis (Weisblat and Shankland, 1985), and includes an anteroventral (AV) cluster of neurons in each segmental ganglion. Within the Q lineage, the anterior boundary of $Lox2$ expression was situated in ganglion M7 (Fig. 8A), with clear expression of $Lox2$ RNA in at least one of the cells located at the medial edge of this cluster (Fig. 9G,H).

In summary, we have observed detectable $Lox2$ hybridization in four of the five teloblast lineages (M, N, O and Q). The anterior boundary of the expression domain is...
nearly the same in every lineage, with the anteriormost hybridizing cells being situated in either the posterior half of segment M6 or the anterior half of segment M7 (Fig. 8). Thus, Lox2 RNA is expressed over essentially the same segmental domain in teloblast lineages that are composed of either one or two blast cell clones/segment. This finding supports the model depicted in Fig. 3A, and is contradictory to that depicted in Fig. 3B.

Discussion

Although segmentation arises in phylogenetically diverse organisms through dissimilar patterns of cellular specification (Shankland, 1991), some of the underlying regulatory genes are highly conserved, most notably the HOM/HOX genes which govern segment identity (Wilkinson and Krumlauf, 1990; Graham et al., 1989). In this paper, we describe the isolation and sequencing of a cDNA for the putative leech segment identity gene Lox2, and use in situ hybridization to characterize the cellular distribution of Lox2 RNA at various stages of segment formation. Our findings indicate that Lox2 is homologous to the fruitfly HOM/HOX genes Ubx and/or abdA, and its segmentally restricted pattern of expression is consistent with an involvement in the process of segmental diversification. The regulation and developmental significance of the Lox2 gene are discussed in the context of previous experimental studies on the establishment of segment identity in this organism.

Comparison of Lox2 in the leeches Helobdella and Hirudo

Our findings confirm and extend the previous observations of Wysocka-Diller et al. (1989), who characterized a genomic fragment of the Lox2 gene (herein designated Lox2-Hme) in the distantly related leech Hirudo. Lox2-Hro and Lox2-Hme encode identical amino-acid sequences in the homeodomain and immediately flanking regions, although diverging dramatically near the predicted C terminus. A comparable pattern of homeodomain conservation and C-terminal divergence has been reported for the abdA gene of three distantly related insect species (Tear et al., 1990; Nagy et al., 1991).

The spatiotemporal pattern of Lox2 RNA expression is also similar in the two leech species. Wysocka-Diller et al. (1989) performed in situ hybridization with radioactive probes on flattened whole mounts of the Hirudo germinal plate, and reported a sharp anterior boundary of expression in segment M6, with the most intense hybridization in the ganglia of the CNS. Whole-mount radioactive labeling does not afford the cellular resolution obtained with the digoxigenin-labeled probes used here, but it was nonetheless apparent that Lox2 expression in Hirudo is restricted to a bilateral pair of posteriorly situated neuron clusters in ganglion M6, and increases in a stepwise fashion in ganglia M7 and M8 (see Fig. 2D in Wysocka-Diller et al., 1989). These striking similarities in HOM/HOX gene expression are consistent with the extensive conservation of identifiable neuron phenotypes between glossiphoniid and hirudinid leeches (Kramer and Goldman, 1981).

There are two noteworthy differences in the observed patterns of Lox2 hybridization. First, we find a sharp posterior boundary of Lox2 expression at the border of the posterior midbody and caudal segments in Helobdella, whereas Wysocka-Diller et al. (1989) were able to detect a low level of Lox2 expression in the terminal ganglion during late stages of Hirudo embryogenesis. Second, we saw intense Lox2 hybridization in the posterior portion of the differentiating genital primordium, but only a low level of diffuse hybridization was observed in the periphery of the Hirudo embryo. This latter distinction could reflect technical differences in the cellular resolution of the two techniques; however, it should be noted that there are pronounced anatomical differences in the genitalia of glossiphoniid and hirudinid leeches (Sawyer, 1986) which might result from a differential utilization of developmental regulatory genes.

The similarity of Lox2 hybridization in these two leech species provides strong support for the specificity of the technique. The Lox2-Hro and Lox2-Hme probes are considerably divergent in nucleotide sequence, and would be very unlikely to give identical patterns of cross-reactivity to other RNA sequences. Moreover, the two probes are entirely (Lox2-Hme) or largely (Lox2-Hro) composed of the homeodomain exon, and should show relatively little discrimination between alternatively spliced Lox2 transcripts.

Comparison of Lox2 with Ubx and abdA

Both sequence analysis and the spatial pattern of expression suggest a close evolutionary relationship between the leech gene Lox2 and the two fruitfly HOM/HOX genes, Ubx and abdA. As is typical of many HOM/HOX genes, these three genes encode highly conserved homeodomains (> 85% amino-acid identity) and also share the upstream amino-acid sequence YPWM (Wilde and Akam, 1987). Lox2 also resembles both Ubx and abdA in homeodomain flanking sequences (Fig. 5). Ubx and abdA are the only pair of fruitfly HOM/HOX genes that display any significant sequence similarity in the downstream flanking region, providing strong evidence that Lox2 is the specific leech homologue of one or both fruitfly genes. Akam et al. (1988) have proposed that Ubx and abdA arose by gene duplication relatively late in arthropod evolution, which would suggest in turn that Lox2 arose from an as yet unduplicated precursor gene present in the last common ancestor of annelids and arthropods. This scenario is consistent with the roughly comparable degree of similarity between Lox2 and each of these two fruitfly sequences; however, we cannot exclude the existence of other, as yet unidentified Ubx/abdA homologues in the leech.

The segmental pattern of Lox2 expression further supports its homology to Ubx and abdA. In the fruitfly, Ubx and abdA gene products are restricted to distinct but extensively overlapping segmental domains which span either the posterior two-thirds (Ubx) or one-half (abdA) of the germinal band, with neither extending into the terminal abdominal segment (Beachy et al., 1985; Karch et al., 1990). In Helobdella, Lox2 expression is restricted to a domain of 16 contiguous segments which constitutes the posterior two-thirds of the midbody, but likewise does not seem to extend into the terminal segments. Moreover, all three genes show distinct patterns of neuronal expression within different segments of their expression domain, with reduced expression near the boundaries of the domain (Doe and Scott, 1988).
In the leech, the segmented tissues of the germinal plate are composed of an iterated array of blast cell clones, and we have shown that blast cell clones in the boundary segments of the *Lox2* expression domain display a mosaic pattern of *Lox2* hybridization (Fig. 8B). In the fruitfly, the differential expression of HOM/HOX gene products within successive segments is thought to arise both through the segment-specific utilization of *cis*-regulatory elements, as well as cross-regulatory interactions between HOM/HOX genes with overlapping expression domains (Peifer et al., 1987; Akam et al., 1988).

Akam et al. (1988) propose that the various body regions or tagmata of the modern insect (e.g. thorax; abdomen) were originally established as multisegmental domains of equivalent HOM/HOX gene expression. In this context, it is interesting to note that the segmental domain of *Lox2* expression in the *Helobdella* embryo demarcates the posterior midbody region, M7-M21. Anterior to this region are the two reproductive segments, M5 and M6, which are distinguished by the presence of genitalia and of specialized postembryonic neuroblasts (Baptista and Macagno, 1988), and — in *Helobdella* — by the absence of nephridia (Weisblat and Shankland, 1985). (In *Helobdella*, the genital primordia of segments M5 and M6 arise from the two mesodermal blast cell clones which fail to generate nephridia (M.S., unpublished). However, the nephridium becomes posteriorly displaced with respect to the remainder of the blast cell clone, and thus the gap corresponds to segments M6 and M7 (Weisblat and Shankland, 1985).) Posteriorly, the observed boundary of *Lox2* expression demarcates the border between the midbody segments and the caudal sucker.

**Role of *Lox2* in segmental specification**

In the leech embryo, segmentally homologous blast cells arise through the iterated divisions of a teloblast stem cell, and normally contribute their descendant clones to particular body segments in accordance with their birth rank in the stem cell lineage. Blast cells that have been relocated along the anteroposterior body axis generate descendant clones composed of cellular phenotypes appropriate for their normal segment of destiny (Martindale and Shankland, 1990b; Shankland and Martindale, 1992; Gleizer and Stent, 1992). Thus, it would appear that the newly born blast cell acquires a segment identity in accordance with its birth rank, and these ordinal identities equate to segment identities because of the orderly assembly of blast cell clones during the formation of the germinal plate.

In both *Helobdella* and *Hirudo*, *Lox2* RNA has only been identified in cells that are actively undergoing terminal differentiation, *i.e.* distant descendants of the primary blast cells. Studies of neurogenesis and neuronal differentiation (Stewart et al., 1986; Stuart et al., 1987) indicate that most or all of the *Lox2*-expressing cells observed in the CNS are postmitotic neurons, and muscle differentiation is well advanced (Torrence and Stuart, 1986) at the stage of the earliest mesodermal expression. We cannot exclude the possibility that teloblasts and/or primary blast cells express *Lox2* gene products that are functionally significant but below our current level of detection, although it must be noted that we can visualize robust RNA expression from another homeobox gene, *Lox5* (Shankland et al., 1991), in teloblasts and blast cells using this same hybridization technique (M.S. and D.N.H., unpublished).

Taken at face value, these findings argue that *Lox2* gene products are unlikely to be involved in developmental commitments occurring at the level of the teloblast or primary blast cell, *i.e.* the time when segment identities are first being established. This conclusion would imply that leech HOM/HOX genes, at least as typified by *Lox2*, are divorced from the initial specification of segment identity. However, the relatively late expression of *Lox2* gene products does not preclude a role in segmental diversification. In the CNS, the embryonic cell lineages give rise to a similar number of ganglionic neurons in different body segments (Stewart et al., 1986), and many segmental differences are manifested through the differential survival or phenotypic modification of segmentally iterated neurons (Loer et al., 1987; Martindale and Shankland, 1988), as well as segment-specific patterns of postembryonic neurogenesis (Baptista and Macagno, 1988). Such events occur after the onset of *Lox2* RNA accumulation, and the protein product of this gene could serve as a segment-specific regulator of neuronal differentiation.

It should be noted that expression of the HOM/HOX genes is not thought to be the primary determinant of segment identity in the fruitfly. Analysis of the Bithorax complex has shown that the transcription units are associated with multiple *cis*-regulatory regions, and that each regulatory region is utilized in only a subset of the iterated cellular domains (‘parasegments’) that constitute the fruitfly embryo (Peifer et al., 1987; Duncan, 1987). Peifer et al. (1987) propose that an early developmental decision distinguishes the individual parasegments by determining which of the HOM/HOX gene regulatory regions can be used for transcriptional regulation, and the accessibility or inaccessibility of a given regulatory region is then determined during subsequent development through secondary changes in chromatin structure (Paro, 1990; Pearce et al., 1992). In this model, the primary molecular address of the parasegment is determined by the accessibility of HOM/HOX gene regulatory regions, and not HOM/HOX gene transcription per se. By analogy, one could envision that the leech blast cell establishes its segment identity by specifying heritable restrictions on the regulatory potential of HOM/HOX genes such as *Lox2* in its descendant clone, even though these genes may not be transcribed nor their products utilized until several days later in clonal development.

**Segmental specification of *Lox2* expression in different teloblast lineages**

If segment identity specification is functionally independent in the various teloblast lineages, there is no a priori reason to assume that different lineages should adopt the same segmental patterns of HOM/HOX gene expression. However, intracellularly injected lineage tracers have shown that the teloblast lineages have almost exactly the same anterior boundary of *Lox2* expression. Hybridization reaction product could be localized to identifiable progeny of four of the five embryonic teloblasts and, in every case, the most anterior cells to exhibit detectable expression were situated...
within 1/2 segment of the M6/M7 segment border. The posterior boundary of *Lox2* expression was not examined in as much detail, but it is clear that expression in both the CNS and the peripheral mesoderm extends posteriorly to the caudal sucker. These findings indicate that the different teloblast lineages utilize at least one putative segment identity gene in common, and express the products of this gene in a similar spatiotemporal pattern.

There are two scenarios which might explain the coordinate disposition of *Lox2* expression in the different teloblast lineages. First, one could envision that *Lox2* expression is evoked by positional information after the teloblast progeny have fused together to form a segmentally organized germinal plate. Such a model would be consistent with what is known of HOM/HOX gene expression in insects and vertebrates, but is difficult to reconcile with previous studies demonstrating that leech blast cells have intrinsic segment identities several days before they come into segmental register within the germinal plate (Martin-dale and Shankland, 1990b; Shankland and Martin-dale, 1992; Gleizer and Stent, 1992). Moreover, experimental displacements of the N lineage indicate that *Lox2* RNA is expressed in a domain determined by the blast cell’s lineage, regardless of the identity of the host segment (M.S. and D.N.H., unpublished data).

If the various teloblast lineages do establish their segment identities autonomously, then their coordinate expression of *Lox2* gene products places some constraints on the mechanism of gene regulation. The fact that both the N and Q lineages — which generate two blast cells/segment — and the M and O lineages — which generate one blast cell/segment — show the same segmental domain of expression indicates that *Lox2* expression is not determined by a particular number of stem cell divisions common to all teloblasts. If this were the case, then the N and Q lineages would show a more anterior domain of expression (cf. Fig. 3B). The same argument rules out a temporal signal acting globally during a restricted period of blast cell production, since all five teloblasts generate blast cells at the same rate (Wordeman, 1983).

We envision that the mechanism that specifies *Lox2* expression is operating at the level of the teloblastic stem cell divisions, but that the M, O and P lineages advance segment identities at intervals of a single blast cell, while the comparable mechanism in the N and Q lineages advances segment identities at intervals of two blast cells. One possibility is that the teloblasts progressively respecify segment identity through an activity which is expressed in association with the cell cycle, and thus convey different regulatory information to successive blast cell daughters. The respecification activity would be expressed at every cell cycle of the M, O and P teloblasts, but in the N and Q teloblast it would be suppressed at alternate cell cycles — either autonomously or through interaction with the newly formed blast cell (Bissen and Weisblat, 1987). Thus, sequential pairs of n and q blast cells would share the same segment identity, although differing in other aspects of their subsequent differentiation (Zackson, 1984; Weisblat and Shankland, 1985).

The fact that leech embryos utilize the same segmental pattern of HOM/HOX gene expression in several developmentally independent stem cell lineages may have a phylogenetic explanation. Comparative studies indicate that the HOM/HOX gene complex very likely served as a means of specifying anteroposterior patterns of cell identity prior to the evolution of overtly segmented organisms (Akam, 1989; Kenyon and Wang, 1991). With the appearance of teloblastic segmentation in the annelids (and certain arthropods; Dohe and Scholtz, 1988), various cell lineages could have inherited the same primitive pattern of HOM/HOX gene expression, although now placing the construction of that pattern under the control of different programs of iterative cell divisions. In this scenario, the coordinate patterning of *Lox2* expression in different teloblast lineages of the modern day leech would simply represent the coordinate retention of a primitive expression pattern.

The authors are deeply indebted to Michelle C. Perkins for her unflagging technical assistance in these studies. We would also like to thank Euan Baxter and David J. Price for the genomic DNA library, David Paul for his many helpful discussions, Richard Harland for sharing unpublished tips on the in situ hybridization protocol, and Welcome Bender for his critical reading of the manuscript. This work was supported by NIH grant ROI-HD21735 and March of Dimes grant 0F92-0900 (to M.S.), and postdoctoral grant 823A-026134 from the Fonds National Suisse de la Recherche Scientifique (to D.N.H.).

**References**


(Accepted 3 July 1992)
**Fig. 2.** Lineage tracer injections demonstrate the differing relationship between cell lineage and segment formation in the M lineage, which has one blast cell clone/segment, and the N lineage, which has two blast cell clones/segment. In each embryo, the left M teloblast was injected with RDA (red) and the right N teloblast injected with FDA (green) at the same time during blast cell formation. Following the appearance of overt segmentation, the embryos were fixed and dissected, and counterstained with the blue-fluorescing nuclear dye Hoechst 33258. The anterior borders of RDA- and FDA-fluorescence demarcate the boundary between anterior unlabeled clones, whose ancestral blast cells were generated prior to injection, and posterior labeled clones, whose ancestral blast cells were generated following injection. A and B show two synchronously developing sibling embryos in which identical injections were performed 12 hours apart. Brackets mark the relative location of the dye boundaries in the two embryos. Comparison reveals that the M teloblast generated 10 additional blast cells (= 10 segments) and the N teloblast 12 additional blast cells (= 6 segments) during the 12 hour interval. Hence, the N teloblast gives rise during this time to progeny which are compressed into a narrower and more anteriorly situated subset of body segments. Scale bar, 50 µm.

**Fig. 6.** Lox2 RNA expression in the leech embryo as revealed by in situ hybridization with digoxigenin-labeled probes, and visualized by alkaline phosphatase immunostaining. Parts A and B show whole, cleared embryos; parts C, E and F are Nomarski images of embryos dissected following hybridization; and part D is a Nomarski image of a transverse section. (A) Lateral view of an early stage-10 embryo, with anterior toward the top and ventral to the right. The midbody segments are demarcated by brackets. Intense hybridization is evident in the ganglia of the ventral nerve cord, with an anterior boundary associated with midbody ganglia 6-8. At this stage, there is a gradual decrease in hybridization intensity in the more posterior midbody segments. Intense peripheral staining is evident in the genital primordium (gp), and in fainter posterior stripes corresponding to the dorsoventral muscles. Scale bar, 100 µm. (B) Ventral view of the anterior boundary of the Lox2 expression domain, with anterior toward the top. Brackets demarcate midbody ganglia 6, 7 and 8. Bilaterally paired clusters of hybridizing neurons are evident in the posterior of midbody ganglion 6 (arrowhead), and throughout midbody ganglion 7. The more posterior ganglia show an essentially uniform pattern of hybridization. Hybridization is also evident in the dorsoventral muscles (dv), which project laterally and anteriorly from the interganglionic borders. The most anteriorly labeled dorsoventral muscle is associated with the intensely hybridizing genital primordium (gp). Scale bar, 50 µm. (C) Anterior boundary of the Lox2 domain at the onset of expression, seen in ventral view of a stage-9 embryo. Brackets demarcate borders between segments, and extend to the lateral edges of the segmental ganglia. There is a laterally situated pair of bilaterally hybridizing neurons specific to midbody ganglion 7 (arrowheads), as well as clusters of hybridizing neurons near the midline of this and more posterior ganglia. Scale bar, 20 µm. (D) Transverse section of a posterior midbody ganglion (g), with dorsal towards the top. Hybridization reaction product is restricted to the cell body cortex, and is not evident in the dorsally situated neuropil. Hybridization is not evident in the ventral body wall (bw). The midgut is situated more dorsally, and is packed with yolk platelets (yp) at this stage. Scale bar, 20 µm. (E) Anterior boundary of Lox2 domain in the CNS of a stage-10 embryo, shown in dorsal view. Midbody ganglia 5 - 8 are labeled in their upper right corners. Note the progressive increase in the number of hybridizing cells in successive ganglia. Scale bar, 20 µm. (F) Posterior boundary of Lox2 domain in a late stage-10 embryo, shown in dorsal view. Lines demarcate the boundary between the midbody segments (mb) and the caudal sucker (cs), and extend to the lateral edges of the CNS. There is a progressive decrease in the number of hybridizing cells in midbody ganglia 19 - 21, and no apparent hybridization in neuromeres of the caudal sucker. Scale bar, 20 µm.