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Lox10, a member of the *NK-2* homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella*

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SUMMARY

A novel leech homeobox gene, *Lox10*, is shown to encode a homeodomain sequence characteristic of a phyletically widespread *NK-2* homeobox gene class. *Lox10* expression was examined in leech embryos of various ages by in situ hybridization. In the unsegmented cephalic region, *Lox10* RNA is expressed in a subset of the cells descended from the a′ and b′ micromeres, including a small cluster of cells, believed to be postmitotic neurons, within the supraesophageal ganglion of the central nervous system. Hybridization signal was not detected in either the mesoderm or ectoderm of the trunk segments, and the apparent restriction of *Lox10* ectodermal expression to the nonsegmented cephalic domain resembles the restricted forebrain expression pattern of its mammalian homologues. *Lox10* is also expressed within the endodermal tissues of the leech midgut, which arises by cellularization from a polynucleate syncytium. Endodermal expression is organized into a pattern of transverse stripes and spots which are aligned with the intersegmental septa, and which prefigure the pattern of gut wall constrictions observed at later stages of development. *Lox10* is the first molecular marker of segmentally periodic endoderm differentiation reported for any animal species.

Key words: leech, homeobox gene, *NK-2* gene class, endoderm, segmentation

INTRODUCTION

Homeobox genes have been implicated in a number of regulatory processes associated with the embryonic patterning and cell differentiation of eukaryotes (McGinnis and Krumlauf, 1992). The homeobox is a 180 bp DNA sequence which encodes a 60 amino acid protein motif, the homeodomain, that has been shown to serve as the DNA binding site for a phyletically and functionally diverse class of transcription factors (Gehring et al., 1990). The amino acid sequence and three-dimensional conformation of the homeodomain are highly conserved, but many distinct classes of homeobox gene have been defined on the basis of sequence, genomic organization, expression pattern and/or function (Scott et al., 1989; McGinnis and Krumlauf, 1992).

In this paper, we describe a novel homeobox gene, *Lox10*, isolated from the glossiphoniid leech *Helobdella triserialis*. *Lox10* is characteristic of a recently discovered group of *NK-2*-like homeobox genes whose evolutionary history and functional significance are unclear. The best characterized of these genes encodes thyroid transcription factor 1 (TTF-1), which activates the thyroid-specific transcription of thyroglobulin and thyroperoxidase genes in rat (Guazzi et al., 1990; Civitareale et al., 1989). The *TTF-1* gene has since been cloned in mouse (designated *Nkx-2.1*), and shown to be one of four members of the murine *Nkx-2* gene family (Price et al., 1992). Expression studies reveal that *TTF-1* RNA is restricted to the thyroid, lung and a discrete region of the fetal forebrain (Lazzaro et al., 1991). The closely related *Nkx-2.2* gene is expressed in a distinct region of the murine forebrain, and it has been proposed that the *Nkx-2* gene family plays a developmental role in forebrain regionalization (Price et al., 1992).

Several related genes have been described in invertebrate organisms, but their expression patterns are largely unknown. The reported sites of *TTF-1* and *Nkx-2.2* expression - thyroid, lung and forebrain - are organs specific to the vertebrate lineage (Beklemishev, 1969; Gans and Northcutt, 1983), and it is of considerable interest to learn how the homologous genes are utilized in invertebrates. The murine *Nkx-2* gene family was named for its similarity to the *Drosophila* gene *NK-2*, which was isolated by DNA hybridization (Kim and Nirenberg, 1989). Additional *NK-2*-like homeobox genes have since been found in planaria (Garcia-Fernàndez et al., 1991) and tapeworm (Oliver et al., 1992). To gain further insight into the function and phyletic

conservation of the *NK-2*-like homeobox genes, we have here used in situ hybridization to describe the cellular expression of the *Lox10* gene during the embryonic development of the leech.

Leeches are annelids, and their embryonic development is characterized by an invariant pattern of cell lineage (Stent et al., 1982; Sandig and Dohle, 1988). The fertilized egg undergoes two rounds of asymmetric meridional cleavage to form four positionally identifiable macromeres (Fig. 1). The A, B, and C macromeres give rise to the endoderm, and the D macromere gives rise to the segmented mesoderm and ectoderm of the body trunk. At the third round of division, the macromeres undergo a clockwise spiral cleavage to produce the 'first quartet' micromeres (cells a′, b′, c′ and d′). Additional micromeres are produced at later cleavages (Bissen and Weisblat, 1989), but the first quartet gives rise to the preponderance of ectodermal tissues within the unsegmented cephalic region, or prostomium, which originates at the embryo's animal pole (Weisblat et al., 1984). The micromere cell lineages also give rise to the foregut and the epidermal layer of the provisional integument, a transient extraembryonic membrane which is replaced by the defini-

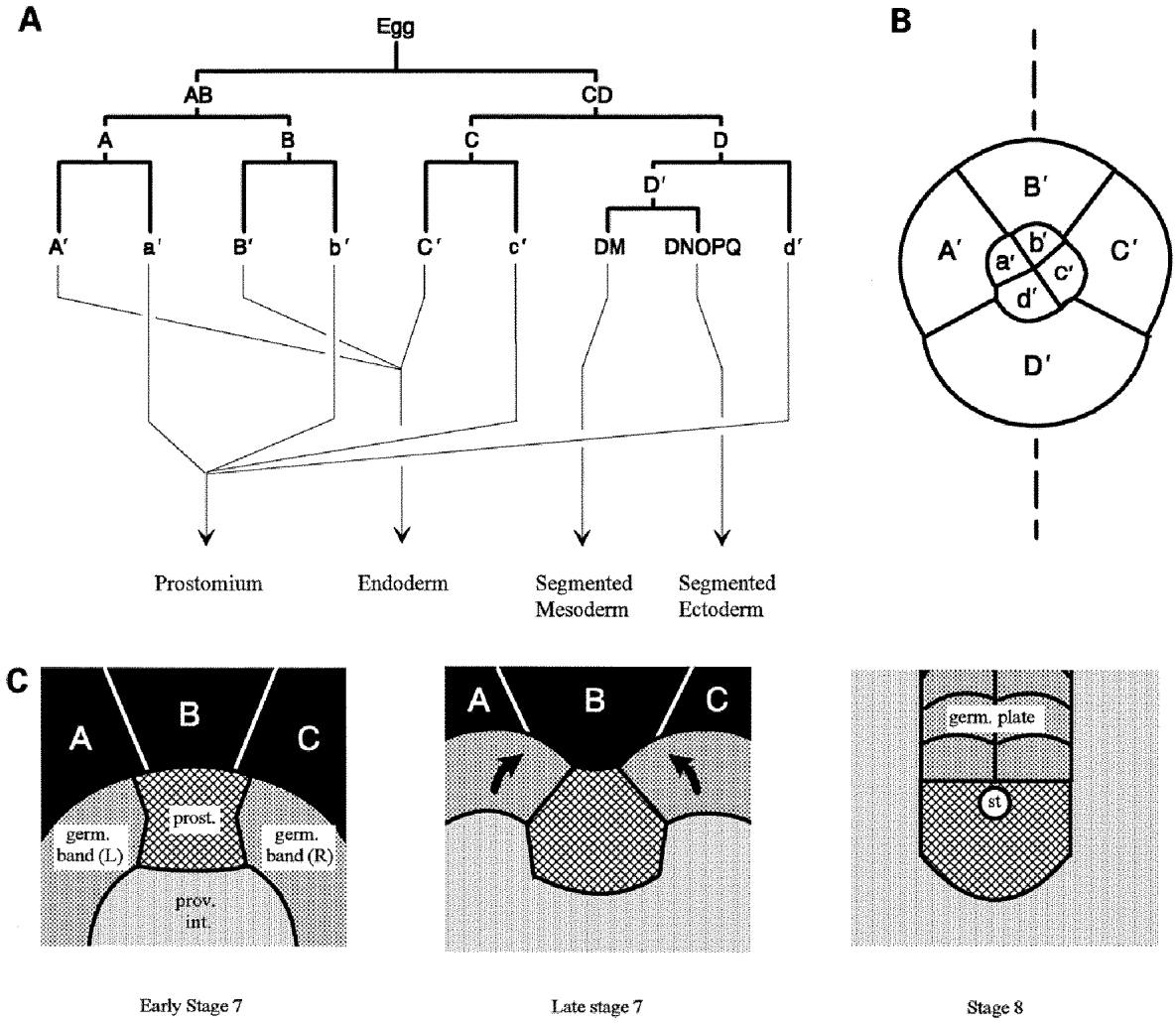

Fig. 1. Overview of early development in the leech *Helobdella*. (A) Cell genealogy of the first 8 cleavages, and the contribution of those blastomeres to the definitive tissues of the adult leech. The three macromeres (A′, B′ and C′) give rise to endoderm; the first quartet micromeres (a′, b′, c′, and d′) give rise to the cephalic domain or prostomium; the DM blastomere gives rise to segmented mesoderm; and the DNOPQ blastomere gives rise to segmented ectoderm. Additional micromeres are produced at later cleavages, and it should be noted that all of the cells shown here make an additional contribution to the extraembryonic tissues of the provisional integument (Weisblat et al., 1984; Ho and Weisblat, 1987). (B) Eight-cell embryo viewed from the animal pole, showing the clockwise arrangement of the first quartet micromeres. The future plane of bilateral symmetry (dashed line) bisects the B and D macromeres. (C) Animal pole view of the developing embryo at various stages of gastrulation. The right and left germinal bands (derived from the D′ macromere) sweep circumferentially over the surface of the embryo from the D quadrant to the B quadrant (arrows), where they fuse to form a segmented germinal plate. During this process the prostomial tissues remain at the animal pole, such that the future anterior end of the embryo points from the animal pole towards the D quadrant. The stomadeum (st) or embryonic mouth develops at the boundary of the prostomium and the germinal plate. The bands are interconnected by a provisional integument, and their circumferential movement thus envelops and internalizes the A, B and C macromeres (prospective endoderm).

tive body wall during the final stages of embryonic development (Weisblat et al., 1984; Ho and Weisblat, 1987).

The glossiphoniid leeches have large embryos which are amenable to the intracellular injection of histological tracers, such that particular cell lineages can be labeled and traced for extended periods of time during development (Weisblat et al., 1978; Gimlich and Braun, 1985). To date, lineage tracer studies have largely focused on the segmented ectodermal and mesodermal cell lineages produced by the D macromere (Stent et al., 1982; Weisblat and Shankland, 1985). The D macromere cleaves to form a set of five bilaterally paired stem cells, called teloblasts, each of which generates a chain of segmentally iterated blast cells. These blast cell chains initially form into right and left germinal bands which are located in the D quadrant of the embryo (Fig. 1C). However, the elongating germinal bands undergo epiboly, circling over the surface of the embryo to the B quadrant, where they meet and fuse along the future ventral midline to form the germinal plate. During this epiboly movement the germinal bands remain connected to the prostomium at their anterior ends and by the provisional integument dorsally (Fig. 1C). Thus, the epiboly and fusion of the germinal bands envelops and internalizes the endodermal blastomeres (A, B, and C) as part of gastrulation.

The body axes of the developing leech undergo extensive convolutions due to the movements of germinal bands and their derivatives over the surface of the endodermal macromeres. The germinal bands and germinal plate define a sagittal plane which bisects the so-called D and B quadrants of the embryo. The anterior end of each germinal band initially contacts the prostomium on the D quadrant side (Sandig and Dohle, 1988), but circles around to the B quadrant as epiboly proceeds (Fig. 1C). Thus, the final anterior edge of the prostomium is directed towards the D quadrant, with its ventral surface (the future oral sucker) facing outward from the animal pole.

In this study we show that *Lox10* RNA is expressed in specific portions of the prostomium and in the endodermal tissues of the midgut. There has been limited analysis of the cell lineages that give rise to these organs, and we have therefore used intracellularly injected lineage tracers to characterize the pertinent aspects of morphogenesis, and to identify the developmental provenance of cells that express the *Lox10* gene. In the endoderm, *Lox10* is expressed as a repeating array of transverse stripes and spots, which are in register with the segmentation of the overlying mesoderm. This represents the first reported instance of segmentally periodic gene expression within the endoderm of any animal species.

MATERIALS AND METHODS

Leech embryos

Embryos were obtained from a laboratory breeding colony of *H. triserialis* handled as previously described (Weisblat et al., 1980). Embryonic stages and blastomere nomenclature are taken from Stent et al. (1982).

Isolation of *Lox10* genomic clone

The *Lox10* gene was isolated while probing 8×10^4 recombinant clones of an *H. triserialis* genomic library with a 110 bp gene fragment from the homeobox region of the gene *Lox2* from the closely related species *H. robusta* (Shankland et al., 1991). The library was constructed in λ EMBL3a, and kindly provided by C. J. Wedeen and D. J. Price. The probe sequence corresponded to nt 692-801 of the *Lox2* cDNA sequence reported by Nardelli-Haefliger and Shankland (1992). Phage plating and duplicate filter lifts were carried out according to standard procedures (Sambrook et al., 1989). Prehybridization was carried out in 0.375 M Na_2HPO_4 (pH 7.2), 1% SDS at 60°C; hybridization was performed in the same buffer containing 30 ng of α-^{32}P-labeled *Lox2* fragment (10^9 cts/minute per mg). Filters were given three 1-hour washes in 0.15 M Na_2HPO_4 (pH7.2), 1% SDS at 60°C. Two positive clones were plaque purified, and the phage DNA prepared from a single plaque. Phage DNA was digested with a panel of restriction enzymes, separated on an agarose gel, and transferred to a nylon membrane (Amersham), then hybridized with the *Lox2* probe as described above. The two positive clones appeared to be identical, and a hybridizing 1.4 kb *Sma*I restriction fragment was subcloned into the Bluescript KS^+ vector (Stratagene). This fragment was partially sequenced by the dideoxy method (U.S. Biochemical Sequenase kit).

In situ hybridization

In situ hybridization was performed with a digoxigenin-labeled RNA probe as described previously (Nardelli-Haefliger and Shankland, 1992). A probe complementary to the *Lox10* coding strand was obtained by in vitro transcription from the cloned 1.4 kb *Sma*I fragment using T3 polymerase (Boehringer-Mannheim), and subsequently hydrolyzed into shorter fragments. In short, embryos of various ages were fixed in formaldehyde and permeabilized by Pronase E digestion. Stage 6-8 embryos were digested for 20-25 minutes; stage 9-11 embryos for 40-45 minutes. Digested embryos were acetylated and post-fixed with formaldehyde, then hybridized overnight at 59°C in 50% formamide with approximately 1 ng/μl digoxigenin-labeled RNA. Washed embryos were treated with RNAase A (Sigma) to degrade unhybridized probe. Hybridized probe was visualized immunologically with an alkaline phosphatase (AP)-conjugated anti-digoxigenin (Boehringer-Mannheim) reacted for periods ranging from 15 hours to 3 days, using NBT and X-phosphate color reagents. Previous studies using this protocol on leech embryos indicate that the staining pattern obtained here is specific to the *Lox10* probe (Nardelli-Haefliger and Shankland, 1992).

Intact embryos were cleared either in 80% glycerol, or by dehydration in ethanol and propylene oxide followed by infiltration with Polybed plastic embedding medium (Polysciences). Some of the embedded specimens were cured, cut into sections of 0.1 mm thickness using a hand-held razor blade, mounted under a coverslip in Fluoromount (Biomedical Specialities; Santa Monica, CA), and photographed with Nomarski optics.

Fluorescent lineage tracers

To ascertain the lineage history of cells that express *Lox10* transcripts, identified embryonic blastomeres were pressure injected with 50 mg/ml fluorescent dextran in 0.1 M KCl and 2% fast green FCF. The dextrans used in this study were tetramethylrhodamine-dextran-amine (RDA; obtained from Molecular Probes, Eugene, OR, catalog no. D-1817) and fluorescein-dextran-amine (FDA; calatog no. D-1820). 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 *Lox10* 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.

RESULTS

Isolation and sequencing of *Lox10*

An *H. triserialis* genomic library was screened at moderate stringency with a homeobox sequence (*Lox2*; see Shankland et al., 1991) from the closely related species *H. robusta*, leading to the isolation and subcloning of a hybridizing 1.4 kb *Sma*I fragment. DNA sequencing revealed a 552 bp open reading frame (not shown) which is terminated at the 3′ end by a *SmaI* cleavage site. This reading frame encodes a novel homeodomain (Fig. 2) which is only 42% identical to *Lox2* at the amino acid level, although it contains a stretch of 19 identical nucleotides (homeobox nt 134-152) which presumably accounts for the observed hybridization. In keeping with the nomenclature established by Wysocka-Diller et al. (1989), we have named this novel leech homeobox gene *Lox10*. The *Lox10* homeobox sequence has been deposited in the EMBL Library Database under accession number Z22635.

Homeodomain comparison

The deduced *Lox10* homeodomain amino acid sequence is 88% identical to the planarian gene *Dth-2* (Garcia-Fernàndez et al., 1991), one of a phyletically widespread group of homeodomains whose first described member was the *Drosophila* gene *NK-2* (Kim and Nirenberg, 1989). In Fig. 2B, the *Lox2* homeodomain sequence is compared with eight other *NK-2*-like genes, collectively defined here as the *NK-2* homeobox gene class. Pairwise comparison of *Lox10* and these other eight homeodomains reveals sequence identities in the range 63-88%.

Fig. 2A presents a consensus sequence of amino acid residues conserved among at least six of the nine *NK-2* class genes. Some residues are shared by nearly all homeodomains (Gehring et al., 1990), but there are nine conserved residues which seem to be relatively unusual in that they were observed in no more than 1 of the 85 homeobox genes surveyed by Scott et al. (1989). This is the case for the leucine at position 7 (Leu-7), Tyr-14, Arg-19, Ala-28, His-33, Ile-38, Pro-42, His-52 and Tyr-54. Six of the *NK-2* class gene products share all nine of the unusual residues, while the planarian gene *Dth-1* and tapeworm gene *EgHbx3* share seven, and the mammalian gene *Nkx-2.3* shares five.

The large number of unusual residues in this consensus sequence supports the idea that the *NK-2*-like homeobox genes should be defined as a discrete gene class. Similarities have previously been noted between *NK-2* and the other three *Drosophila NK* genes (Kim and Nirenberg, 1989), as well as members of the phyletically widespread *Distal-less* gene class (Cohen et al., 1989; Price et al., 1991). However, none of the latter genes shares more than three of the unusual amino acids found in our *NK-2* class consensus sequence, and they also exhibit a lower percentage of overall identity (Fig. 2C). Six of the unusual amino acids (Tyr-14, Ala-28, His-33, Ile-38, Pro-42, and His-52) are not shared by any of these other genes. However, two of the other three *NK* genes and all known members of the *Distal-less* gene class share the Arg-19 residue, suggesting that they may be more

Fig. 2. Alignment of *Lox10* homeodomain amino acid sequence with similar homedomains from other organisms. (A) Consensus sequence for the proposed *NK-2* gene class. Listed residues are shared by at least 6 of the 9 genes in Part B. The 9 underlined residues are deemed to be unusual, in that they were found in no more than 1 of the 85 homeobox genes surveyed by Scott et al. (1989).
(B) Comparison of the *Lox10* homeodomain with the other *NK-2* class genes. Dashed residues are identical to *Lox10*. Overall percentage of identity to *Lox10* is shown to the right. Sequence data: *NK-2*, *TTF-1* and *Nkx*-2 genes (Price et al., 1992); *Dth* genes (Garcia-Fernàndez et al., 1991); *EgHbx3* (Oliver et al., 1992). (C) Comparison of *Lox10* with the other three *NK* genes and members of the *Distal-less* gene class. Sequence data: *NK* genes (Kim and Nirenberg, 1989); *Dll*, *Dlx-1* and *Xdll* (Asano et al., 1992); *Dlx-2* (Porteus et al., 1991).

closely related to the *NK-2* class than to other classes of homeobox. In any case, the phylogenetic diversity of the *NK-2*-like genes argues that the ancestral gene must have diverged from other homeobox gene classes prior to the radiation of the major animal phyla.

We did not find significant sequence similarities between *Lox10* and the other *NK-2* class genes outside the homeodomain. The *Drosophila NK-2* gene and its mammalian homologues share a highly conserved stretch of 17 amino acids located a short distance downstream from the homeodomain (Price et al., 1992). We did not observe a comparable peptide sequence within the 49 amino acids separating the *Lox10* homeodomain from the 3′ end of the cloned fragment.

Lox10 expression in prostomial development

In situ hybridization was performed on fixed, permeabilized *Helobdella* embryos using a digoxigenin-labeled RNA probe complementary to the *Lox10* coding strand (Fig. 3). Hybridized *Lox10* probe was revealed with AP-conjugated anti-digoxigenin. This procedure gave a discrete pattern of staining distinct from that seen with plasmid sequences, or probes directed to other leech homeobox genes (Nardelli-Haefliger and Shankland, 1992, and unpublished results).

Lox10 hybridization was first apparent at the beginning of stage 7, and was localized to a bilateral pair of cells situated at the anterior end of the prostomium (Fig. 4A). The hybridization reaction product was relatively uniform within the cell, occupying both nucleus and cytoplasm. Over the next 2 days of development each cell is replaced by a cluster of smaller cells, possibly their progeny, which show predominantly cytoplasmic hybridization (Fig. 3A). During stage 8, each cluster consists of one or two intensely labeled cells lying at the boundary between the prostomium and the provisional integument, and several more faintly labeled cells extending posteriorly (Fig. 3A). In some embryos the two cell groups appear bilaterally paired, but in other embryos they have merged at the midline (Fig. 4C). *Lox10* probe ceases to hybridize to cells in this region of the embryo by the end of stage 8.

The ectodermal tissues of the prostomium are derived largely from the first-quartet micromeres, cells a′-d′ (Weisblat et al., 1984). To ascertain the lineal origin of the cells that express *Lox10*, we injected individual micromeres with tetramethylrhodamine-dextran-amine (RDA) at embryonic stage 4, examining the injected embryos immediately thereafter with low light-level fluorescence to verify the identity of the labeled cell. At stages 7 or 8, the injected embryos were fixed and hybridized with a *Lox10* probe, and the prostomial tissues examined for colocalization of RDA

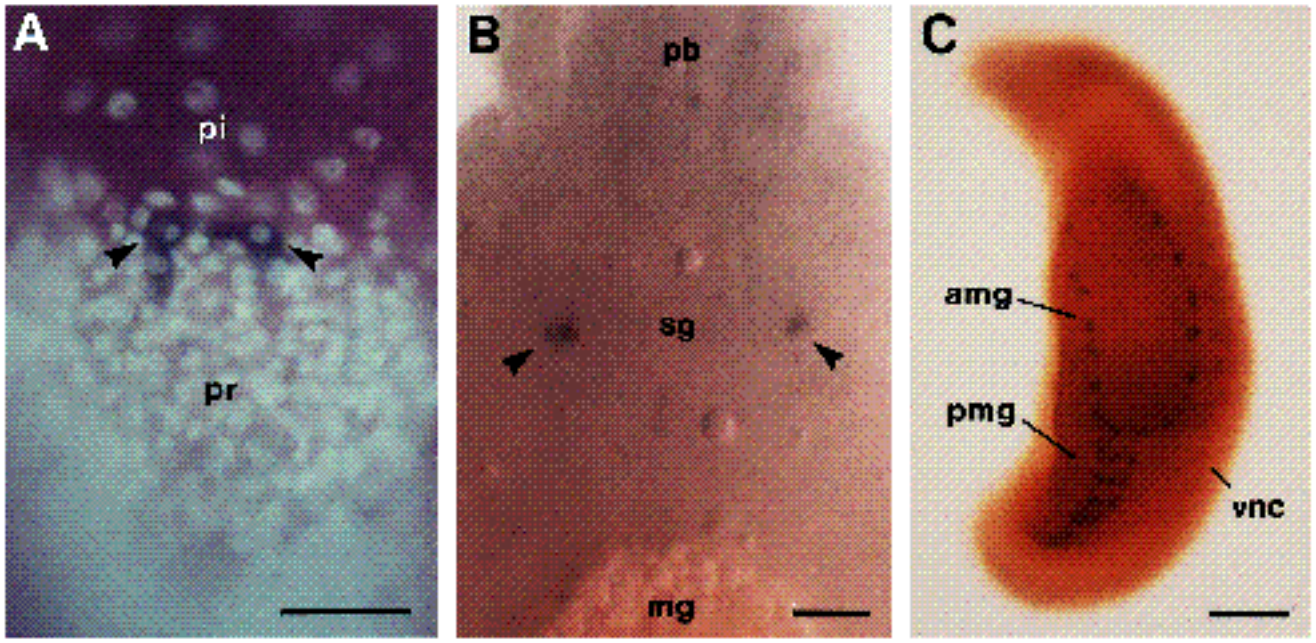

Fig. 3. Expression of *Lox10* RNA in leech embryos of various ages as revealed by in situ hybridization. Anterior is to the top. (A) During embryonic stage 8, *Lox10* RNA is seen in a bilaterally paired group of cells (arrowheads) situated at the anterior edge of the prostomium (pr), at its boundary with the provisional integument (pi). Nuclei have been counterstained with Hoechst 33258, and fluoresce blue-white. Hybridization reaction product is seen as dark blue cytoplasmic staining. Bar, 50 μm. (B) At stage 10, Lox10 RNA is observed in a bilaterally paired cluster of cell bodies (arrowheads) situated in right and left lobes of the supraesophageal ganglion (sg). This ganglion encircles the foregut between the proboscis (pb) and the yolk-filled midgut (mg). Bar, 20 μm. (C) At stage 10, Lox10 RNA is also expressed in a segmentally repeated pattern within the developing midgut. This embryo is shown in an oblique lateral view, with the unstained ventral nerve cord (vnc) to the right. In the posterior midgut (pmg), hybridization reaction product is arrayed in transverse stripes. Each stripe extends from the lateral edge of the ventral nerve cord to the dorsal edge of the expanding germinal plate. In the anterior midgut (amg), hybridization reaction product is arrayed as dorsal and ventral spots whose positions approximate the ends of the stripes seen in more posterior segments. Note the incipient swelling of the anterior midgut, which will give rise to the crop of the mature leech. Bar, 50 μm.

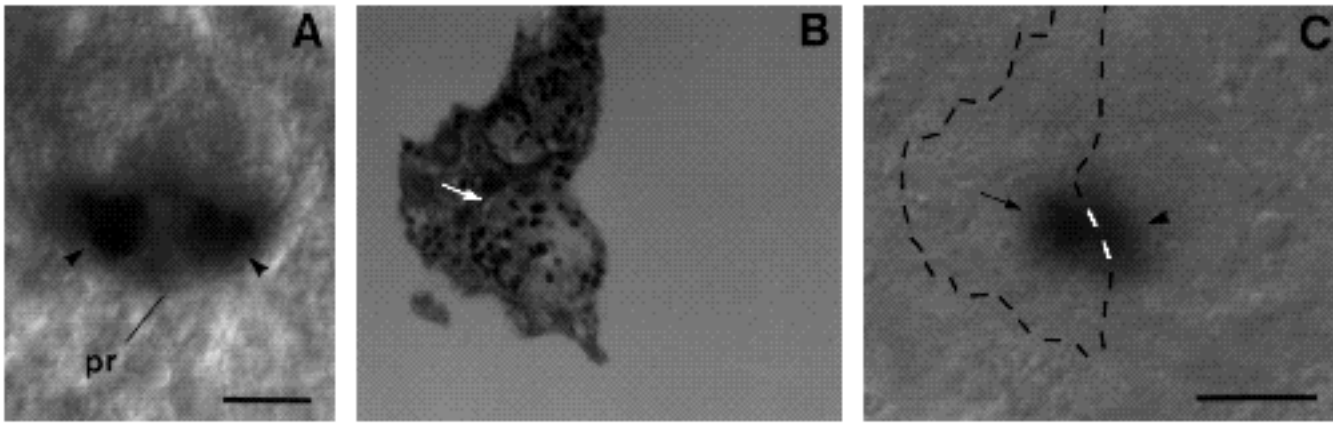

Fig. 4. Expression of *Lox10* RNA in the developing prostomium of late stage 7 embryos as revealed by in situ hybridization. Embryos are viewed from the animal pole, with the future anterior end of the prostomium oriented towards the bottom. (A) Lox10 hybridizes to a bilateral pair of spots situated near the prostomium's anterior edge (pr). Within each spot, hybridization reaction product is largely restricted to a single spherical cell (arrowheads). Bar, 20 μm. (B,C) The prostomial cells that express Lox10 RNA are descendants of the first quartet micromeres. B is an optical section taken with a confocal microscope showing a clone of RDA-labeled cells descended from an injected a′ micromere. Rhodamine fluorescence is shown as black. The prostomial midline is demarcated by the right-hand clonal boundary. Part C is the same specimen viewed with Nomarski optics to reveal the distribution of Lox10 hybridization. The boundaries of the RDA-labeled clone have been outlined. In this embryo there is a patch of reaction product straddling the midline, with colocalization of RDA fluorescence in a cell on the left (arrows), indicating that it is part of the a′ clone. Hybridization is also observed to the right of the midline in an unlabeled cell (arrowhead) descended from the b′ micromere. Bar, 20 μm.

and hybridization reaction product. This analysis revealed that the a′ micromere gives rise to a clone of descendants which lies along the left side of the prostomial midline, including the cell(s) that hybridize to the Lox10 probe (Fig. 4). The b′ micromere gives rise to a symmetrical clone which includes the hybridizing cell(s) on the right.

We encountered a few stage 7 embryos in which hybridization reaction product was apparent within scattered nuclei of the provisional integument, but this staining pattern was neither as intense nor as reliable as the prostomial expression. Lengthy color reactions typically gave staining within the germinal bands, but this phenomenon has been seen with a variety of RNA probes, and most likely represents a nonspecific background staining.

Lox10 expression in the supraesophageal ganglion

During embryonic stage 9, *Lox10* expression appears in a restricted portion of the supraesophageal ganglion, the prostomial component of the leech's otherwise segmented central nervous system (CNS). Ganglionic expression is localized to a small cluster of cells which are likely to be postmitotic neurons. In the best preparations, this cluster was seen to include three adjacent cell bodies situated on either side partway around the ganglion's circumference (Fig. 5A). Neural hybridization persists through the end of embryonic stage 10, but disappears at later stages, probably due to the reduced permeability of the maturing body wall (Nardelli-Haefliger and Shankland, 1992). Lengthy color reactions gave a faint, uniform staining of the CNS, but this latter phenomenon has been seen with various RNA probes and is thought to represent non-specific background staining.

The supraesophageal ganglion is largely if not entirely derived from progeny of the first quartet micromeres (Weisblat et al., 1984), and we therefore examined the lineal origin of the *Lox10*-expressing neurons by injection of the tracer RDA. Injection of each micromere labeled a specific quadrant of the supraesophageal ganglion, with the a′ micromere giving rise to an anterior portion of the ganglion on the left, and the b′ micromere giving rise to an anterior portion of the ganglion on the right. The c′ micromere gives rise to a somewhat larger posterior portion of the ganglion on the left, and the d′ micromere gives rise to a similar clone on the right. (Anterior and posterior are here defined with respect to stage of ganglion formation (Fig. 6C), before this ganglion adopts its retroflexed adult configuration; Weisblat et al., 1984.) Colocalization of RDA lineage tracer and *Lox10* hybridization reaction product was observed on the left side of the supraesophageal ganglion in 3 embryos following injection of the a′ micromere (Fig. 5B,C), and on the right side of the supraesophageal ganglion in 1 embryo following injection of the b′ micromere. Colocalization of lineage tracer and *Lox10* hybridization was never observed following injection of the c′ or d′ micromeres (4 embryos).

Rearrangement of the micromere clones

The relative orientation of the a′-d′ micromere clones in the supraesophageal ganglion is inverted with respect to the stage of micromere formation (Fig. 6). To understand better the rearrangement of these clones during prostomial morphogenesis, we performed experiments in which one micromere was injected with RDA and a second micromere with fluorescein-dextran-amine (FDA). The distribution of the two lineage tracers was then examined at later stages in development (Fig. 7).

The first quartet micromeres are initially arrayed in clockwise order around the embryo's animal pole (Fig. 1B). Lineage tracer injections revealed that the developmental fate of their descendant clones is stereotyped and symmet-

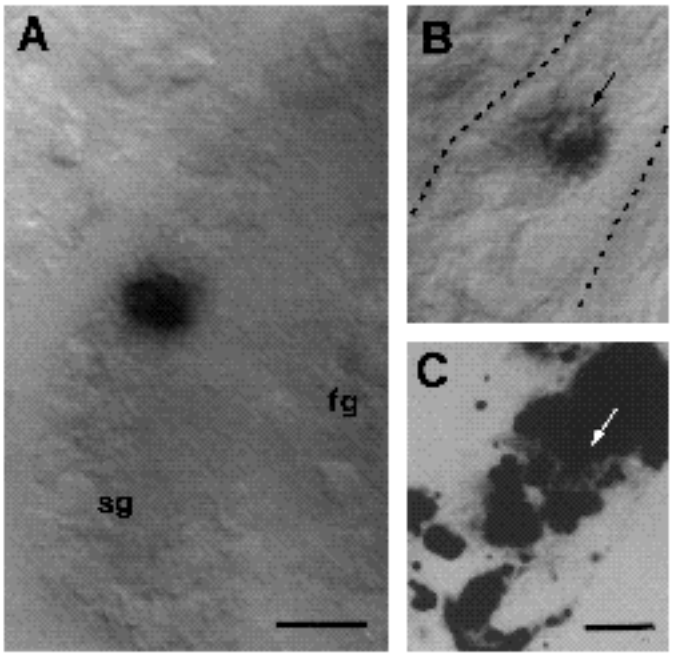

Fig. 5. Expression of *Lox10* RNA in the supraesophageal ganglion of stage 10 embryos as revealed by in situ hybridization. Photographs are taken from thick sections in the plane of the ganglion, with the anterior end of the ganglion oriented toward the top. (A) The *Lox10* probe hybridizes to a cluster of three cell bodies, presumed to be neurons, here shown on the left side of the supraesophageal ganglion (sg) as it encircles the foregut (fg). Bar, 10 μm. (B,C) Lineage tracer injections reveal that the cells that express *Lox10* are derived from the a′ micromere on the left and the b′ micromere on the right. B shows hybridization reaction product within a single cell body (arrow) on the left side of the ganglion. Dotted lines mark the ganglion's edge. C is an optical section of the same specimen taken with a confocal microscope, and shows the distribution of RDA-labeled cells (black) descended from an injected a′ micromere, including the cell labeled in B (arrow). Bar, 5 μm.

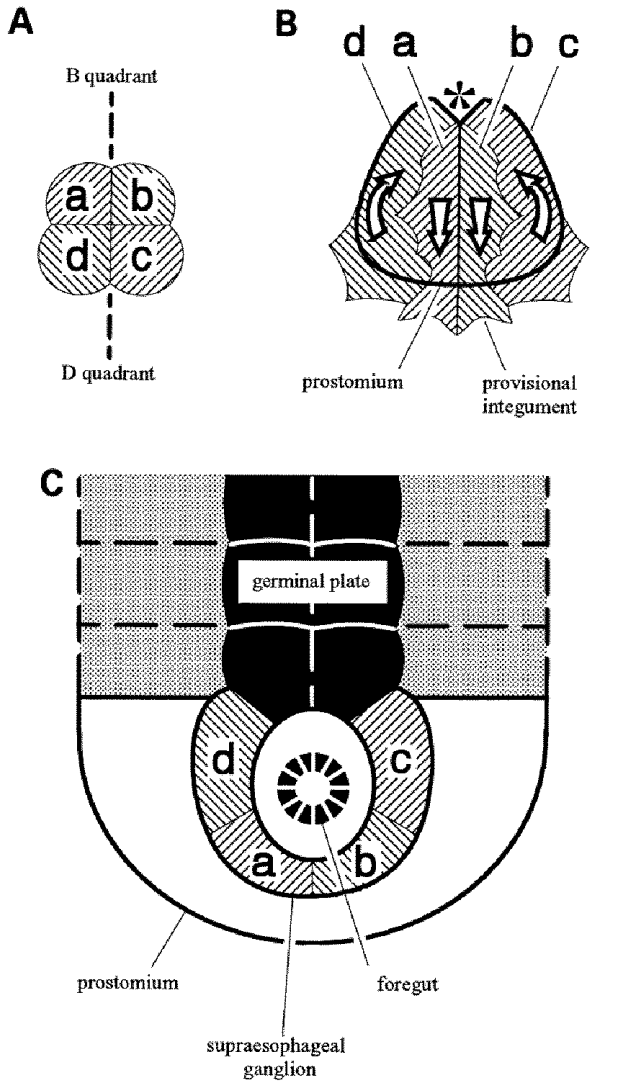

Fig. 6. Inversion of the micromere clones about the animal pole during prostomial morphogenesis. (A) The first quartet micromeres are initially arrayed in clockwise order, with future prostomial midline represented by a plane of symmetry separating the a′ and d′ micromeres on the left from the b′ and c′ micromeres on the right. The plane of micromere symmetry becomes aligned with the B and D quadrants of the embryo as a whole, and all three panels reflect this final orientation. (B) In embryonic stage 7, the a′/b′ clones elongate towards the D quadrant at the midline, while the d′/c′ clones separate laterally and rotate towards the B quadrant. During this time cells emigrate from the prostomial primordium (outlined) into the provisional integument (Weisblat et al., 1984). These provisional tissues become separated from the prostomium during subsequent development. An asterisk marks the future site of stomadeal invagination. (C) By stage 8, rotation is complete and the micromere clones now exhibit an anticlockwise arrangement as viewed from the animal pole of the egg, i.e. the future ventral surface of the prostomium. The anterior edge of the prostomium is oriented towards the bottom, and the posterior edge is joined to the segmental tissues of the germinal plate. At this stage the micromere clones are distributed across several tissue layers (see Fig. 7C,D), but only their contribution to the supraesophageal ganglion has been shown. This ganglion encircles the foregut to connect with the segmented subesophageal ganglion (black).

rical with respect to a meridional plane separating the a′ and d′ clones on the left side of the embryo from the b′ and c′ clones on the right (Fig. 6). In contrast to many other leeches and the vast majority of spiralian embryos (Anderson, 1975), it should be noted that the plane of micromere symmetry in the *Helobdella* embryo is not coincident with the future sagittal plane at the time of micromere formation (Fig. 1B), but rather becomes aligned during the course of subsequent morphogenesis.

The first quartet micromeres begin subsidiary divisions shortly after they are born (Sandig and Dohle, 1988), and in embryonic stage 7 their descendant clones undertake a series of morphogenetic movements which alter the arrangement of those clones about the animal pole (Figs 6, 7). This rearrangement begins with the lengthening of the four micromere clones along the future anteroposterior body axis (Fig. 7B). During this time the d′/c′ clones become separated by the a′/b′ clones, and are thus displaced laterally from the prostomial midline. The relative displacement of these clones continues into the first half of embryonic stage 8, with the d′/c′ clones symmetrically circumnavigating the a′/b′

clones so that they come to lie between a′/b′ clones and the first trunk segment of the germinal plate (Fig. 6C). The result of these movements is that the anteroposterior position of the micromere clones is inverted on either side of the midline (Fig. 7C,D), and hence the clones assume an anticlockwise arrangement as viewed from the animal pole, i.e. the future ventral surface of the prostomium (Fig. 6C).

During embryonic stage 8 the prostomium undergoes organogenesis, with the a′-d′ clones separating into an outer epidermal cell layer (Fig. 7C) and an internal ring of neural tissue - the supraesophageal ganglion - surrounding the embryonic mouth or stomadeum (Fig. 7D). This separation does not significantly alter the relative positions of a′-d′ clones, and hence the contribution of these micromeres to the supraesophageal ganglion (seen in ventral view) reflects their final anticlockwise disposition as seen externally from the animal pole.

Segmental expression of *Lox10* in the midgut

Beginning at stage 9 of *Helobdella* embryogenesis, the *Lox10* probe also begins to hybridize to midgut tissues in the segmented midbody region. This hybridization consists

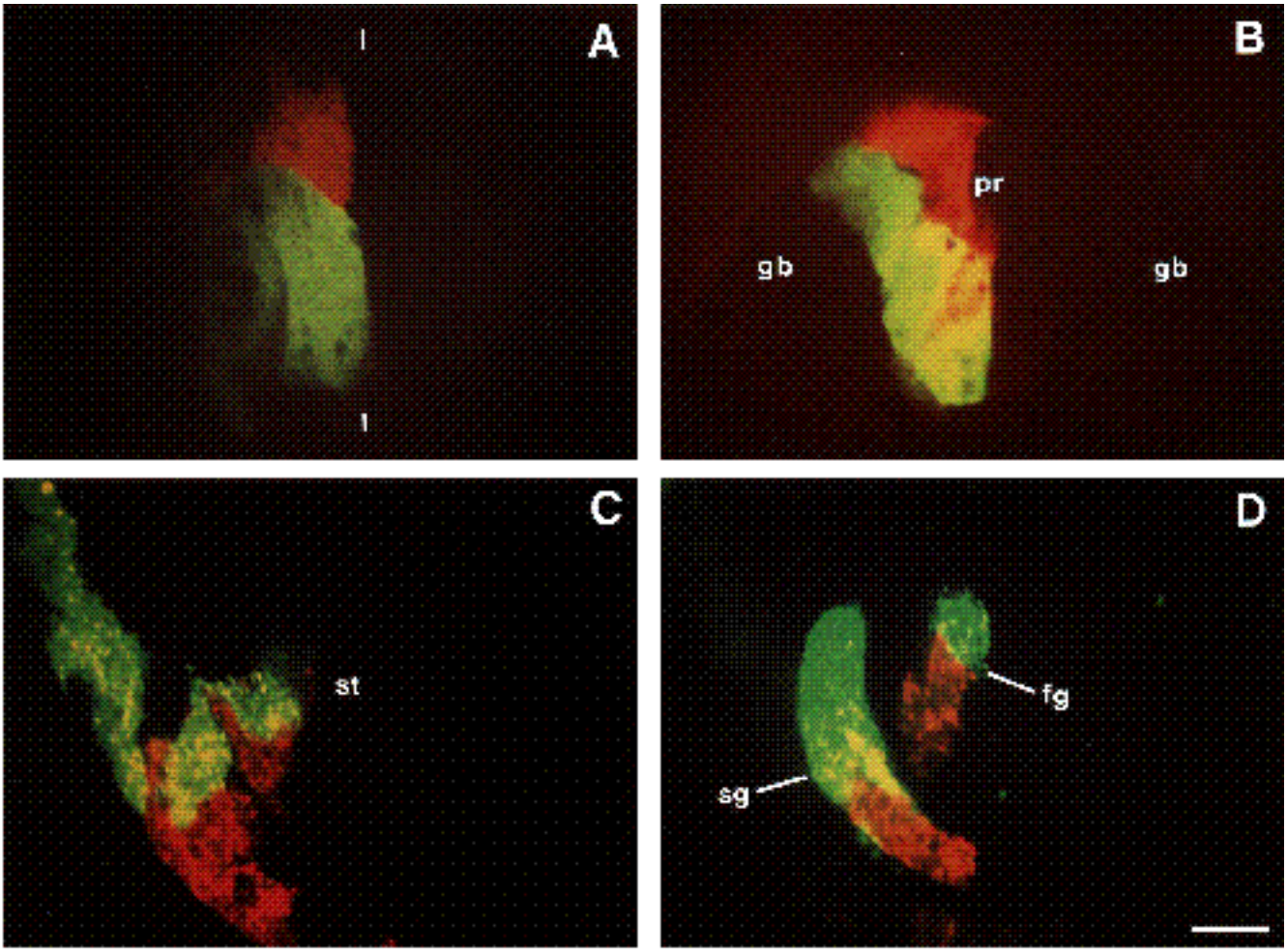

Fig. 7. Morphogenesis of the prostomium examined by differential labeling of the a′ and d′ micromere clones. In these embryos, the a′ micromere was injected with the fluorescent lineage tracer RDA (red) and the d′ micromere was injected with the fluorescent lineage tracer FDA (green/yellow). Each panel is a confocal microscope optical section, and represents the merger of images taken from rhodamine and fluorescein channels. All embryos are viewed from the animal pole, with the future anterior end of the prostomium oriented towards the bottom. (A) At embryonic stage 6, the first quartet micromeres have divided, but their descendant clones have not intermingled and retain the initial arrangement about the pole. The a′ and d′ clones lie to the left of the future midline (white dashes); the unlabeled b′ and c′ clones are located to the right. Note that the labeled clones do not violate the midline during subsequent morphogenetic movements. (B) In stage 7 the micromere clones elongate along the anteroposterior axis, with the d′ clone extending along the lateral side of the a′ clone. During this stage the embryo elaborates right and left germinal bands (gb) whose future anterior ends embrace the prostomium (pr). Note that the lateral elongation of the d′ clone allows it to remain in contact with the end of the left germinal band as the band circles the embryo from the D quadrant (bottom) to the B quadrant (top). (C,D) By stage 8 the d′ clone has been completely translocated to the posterior side of the a′ clone. C is an optical section at the level of the prostomial epidermis. Both a′ and d′ clones extend into the invaginating stomadeum (st) located at the midline. D is an optical section in the plane of the supraesophageal ganglion (sg). Note that the d′ clone gives rise to posterior components of both the ganglion and the differentiating foregut (fg), which is continuous externally with the stomadeum. Bar, 20 μm.

of a longitudinally iterated pattern of cells associated with the region of contact between the midgut primordium and the overtly segmented tissues of the germinal plate (Figs 3C, 9). There was no appreciable staining within the depths of the midgut primordium, nor in the germinal plate itself.

The development of the leech midgut is depicted in Fig. 8. The midgut primordium arises from the fusion of the A, B and C macromeres, which cease to cleave during embryonic stage 4 but continue karyokinesis and fuse to form a polynucleate syncytium (Whitman, 1878). This syncytial primordium is roughly spherical through the end of stage 8, and is encircled along the future ventral midline by a ribbon-like germinal plate whose anterior end is joined to the prostomium and foregut at the animal pole (Fig. 8A). During stages 9-10, the embryo elongates and the germinal plate widens and spreads dorsally to engulf the midgut primordium within the definitive body wall (Fig. 8B,C).

As the midgut primordium is engulfed by the expanding germinal plate, it develops a pattern of regional differentiation characteristic of the adult digestive tract. This regional differentiation is first apparent at the beginning of stage 10, when anterior and posterior subdivisions of the midgut begin to exhibit distinct patterns of morphogenesis (Fig. 8B). The posterior midgut narrows, expelling the yolky contents of its lumen into the anterior midgut, and differentiates to form the intestine and rectum (Fig. 8D). The maturing intestine forms lateral bulges or caeca in segments M13-M16, but the rectum does not develop any overt segmentation. In both of these organs, *Lox10* expression is seen in a segmentally repeated array of bilaterally paired transverse stripes, which extend ventrally to the lateral edge of the CNS and dorsally to the edge of the expanding germinal plate. The hybridizing cells lie in the inner, endodermal layer of the gut wall, immediately beneath the junction of the gut wall with the segmentally arrayed mesodermal septa (Fig. 10A-C). In the intestine, the stripes of *Lox10* expression are situated at the constrictions which separate successive caeca.

The anterior midgut shows a distinct pattern of *Lox10* hybridization from the earliest stages of expression. With the narrowing of the posterior midgut, the anterior midgut swells to form large lateral caeca in segments M7-M12 and differentiates into the crop (Fig. 8D). (The vestigial M7 caecum is resorbed shortly after its formation in *H. triseri - alis* taken from our laboratory breeding colony (Fig. 8D), although adults collected in the wild (Kutschera, 1987) are reported to have 6 crop caeca.) In the anterior midgut, *Lox10* hybridization is initially restricted in each hemilateral segment to a pair of roughly circular spots which are situated immediately lateral to the segmental ganglion and at the dorsal edge of the germinal plate, i.e. at approximately the same position as the two ends of the *Lox10* stripes seen in the posterior midgut (Figs 3C, 9). Each spot consists of a cluster of three or more neighboring, but not necessarily adjacent, nuclei surrounded by hybridization reaction product (Fig. 9B). In stage 9 embryos the *Lox10* probe occasionally hybridizes to a few scattered cells lying between the dorsal and ventral spots, but this intermediate staining disappears by stage 10. The transition between striped and spotted patterns consistently falls at the M12/M13 segment boundary (the future crop/intestine junction) even before these organs exhibit any obvious morphological distinction.

Description of the segmental expression pattern in the anterior midgut is complicated by developmental changes in the overall morphology of the digestive tract. The yolk-filled midgut initially extends up to segment M2, but withdraws posteriorly during stage 10 such that the front end comes to lie at the M6/M7 segment boundary. Prior to this compaction, we typically found 11 ventral spots of *Lox10* expression lying in close, but not perfect, correspondence with the mesodermal septa and ganglia of segments M2-M12 (Fig. 9A). However, the number of distinct spots decreased with the onset of compaction, and only 6 ventral spots of *Lox10* expression (segments M7-M12) were evident once the process was complete. This sequence of events clearly requires some degree of plasticity in *Lox10* expression, e.g. a selective loss of anterior spots as the midgut withdraws from the front-most segments, or cellular rearrangements which bring about a fusion of *Lox10* spots that were originally situated in adjacent segments.

At the end of midgut compaction (late stage 10), the dorsal and ventral spots of *Lox10* expression are situated at the two ends of each nascent intercaecal constriction. These constrictions arise because germinal plate expansion pinches the midgut between the right and left dorsoventral muscles, which arise from the medial edges of the intersegmental septa (Sawyer, 1986). The constrictions are first seen as oblique furrows, and shortly after their appearance aligned rows of cells begin to display *Lox10* expression along the furrow's length (Fig. 9C). It may be that recruitment of additional *Lox10* expressing cells within the furrow eventually leads to the formation of a continuous intercaecal stripe comparable to that seen in posterior segments, but due to technical limitations we were unable to trace the maturation of this staining pattern past the beginning of stage 11 (Nardelli-Haefliger and Shankland, 1992).

Endoderm formation

The gut wall of the leech is composed of an inner layer of endoderm and an outer layer of visceral mesoderm. The endodermal cell layer is traditionally thought to arise by cellularization of the polynucleate midgut syncytium which originates from the A, B, and C macromeres, with cellularization occurring between the visceral mesoderm externally and the 'residual yolk' (i.e. the yolky remnants of the syncytium) within the gut lumen (Whitman, 1878). We here reexamined this phenomenon using intracellularly injected lineage tracers, showing that *Lox10* RNA is expressed in the inner, endodermal layer, and that this layer is in fact derived from the midgut syncytium.

The A, B, and/or C macromeres were injected with RDA at stage 7 (i.e. after micromere formation is complete), and the labeled embryos fixed and sectioned at stages 9-10. Intense rhodamine fluorescence was observed in the residual yolk, and a lesser amount of labeling was also found in the endoderm of the developing gut wall (Fig. 10A-C), which only covers that ventral portion of the syncytium which is in contact with the germinal plate (Fig. 8). No fluorescent labeling was observed in the thin, external layer of the gut wall or other body tissues following these late macromere injections (Fig. 10B).

It is not entirely clear why the injected dextrans give such faint labeling of the endoderm relative to the residual yolk. We were concerned that a small amount of tracer might have reached the endodermal layer by a means other than lineal inheritance (e.g. phagocytosis of the residual yolk mass), and that the endoderm could therefore be arising from another source. However, this same unequal labeling was observed following injections of the fertilized egg (Fig. 10G), indicating that the inequality is a normal consequence of endoderm formation, and arguing against an alternative route of endoderm derivation. Unequal labeling may arise because the endoderm originates as a small cell population which proliferates rapidly, thereby diluting the tracer, to keep pace with the expansion of the overlying germinal plate (cf. Fig. 8A,B).

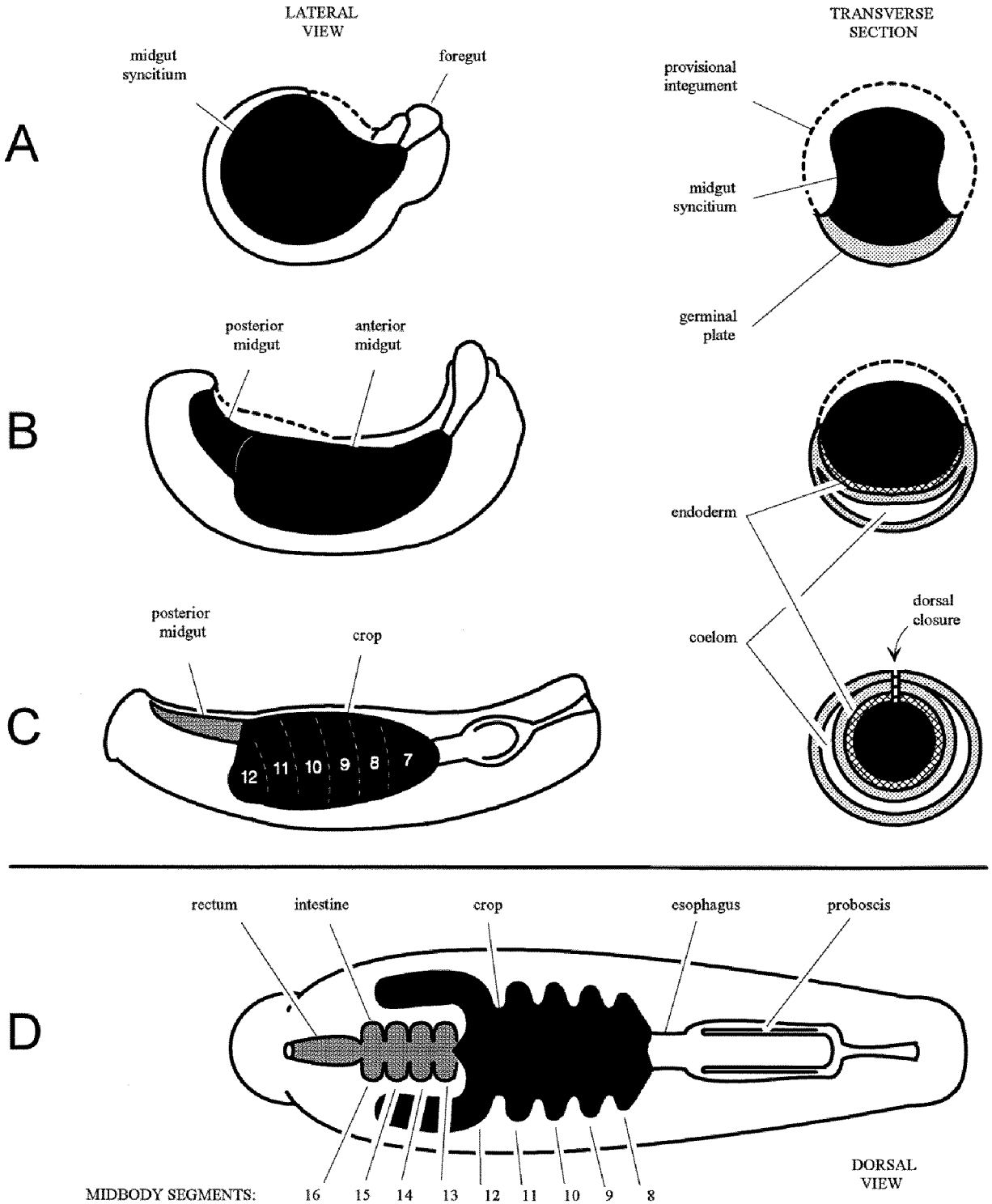

Fig. 8

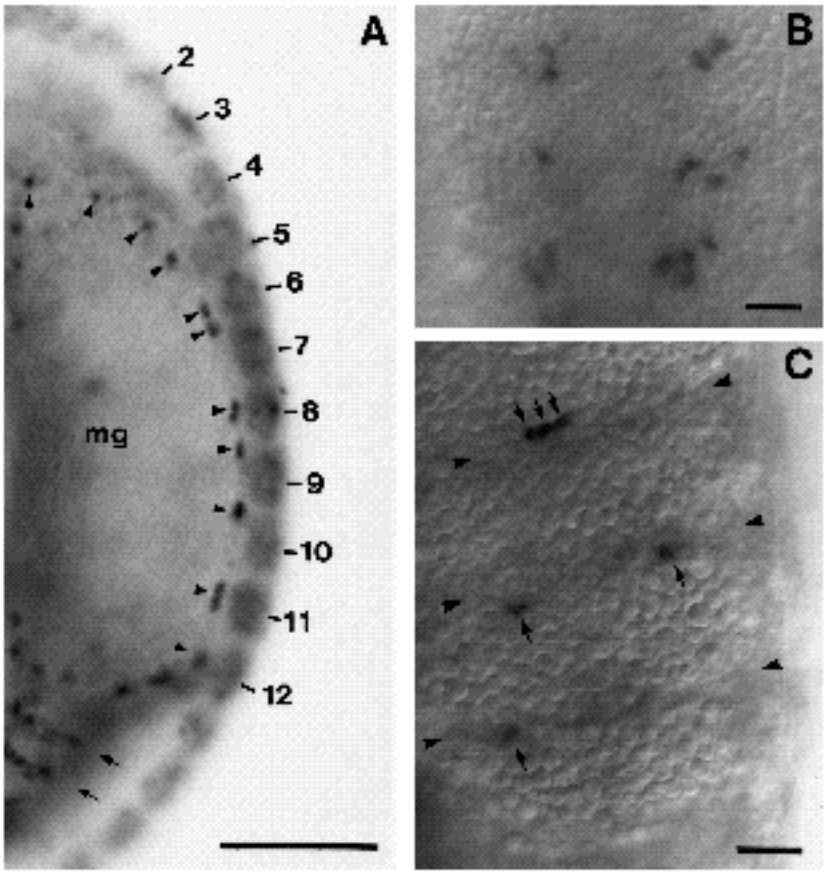

Fig. 9. Expression of *Lox10* RNA in the midgut of stage 10 embryos as revealed by in situ hybridization. Embryos are oriented with anterior to the top. (A) Lateral view of an embryo showing the segmental pattern of hybridization associated with the midgut (mg). Posteriorly, each segment of the midgut has a stripe of hybridizing cells (arrows) along its lateral side. Anteriorly, hybridization is restricted to segmentally arrayed spots at the dorsal and ventral edges of the midgut. Arrowheads mark the ventral spots as well as the unpaired spot located at the anterior end of the midgut. The corresponding midbody ganglia of the ventral nerve cord are numbered to the right. Note the imprecise relationship between the segmental patterning of *Lox10* expression in the midgut and the segmental organization of the other tissues. Bar, 100 μm. (B) Ventral view showing a cluster of cells expressing *Lox10* RNA on either side of three unlabeled segmental ganglia. Note the variable number and array of cells within each cluster. The yolk platelets within the midgut lumen appear as unlabeled spheres. Bar, 20 μm. (C) By the end of stage 10, the midgut begins to develop oblique furrows, which give rise to the intercaecal constrictions, and individual cells begin to express *Lox10* RNA (arrows) within each furrow. Embryo is shown in lateral view with ventral to the right. Arrowheads mark the ends of the three most posterior furrows (segments M9-M12). Bar, 20 μm.

Fig. 8. Overview of gut morphogenesis. Lateral and transverse views are shown with dorsal toward the top; lateral and dorsal views are shown with anterior to the right. The midgut arises from the yolky macromeres, and the yolk-filled portions of the gut are shown in black. (A) At the beginning of stage 9, the head and foregut protrude from the previously spherical embryo. The midgut syncytium is in contact ventrally with the germinal plate, and dorsally with a body cavity enclosed by the provisional integument. (B) By the beginning of stage 10, the embryo has elongated and the midgut has become subdivided into morphologically distinct anterior and posterior regions. During this time the germinal plate separates into somatic and visceral layers enclosing the coelom, and expands dorsally to engulf the midgut syncytium. A cellular layer of definitive endoderm arises between the germinal plate and the syncytium. (C) By the end of stage 10, the residual yolk has been expelled from the posterior midgut into the differentiating crop, which develops lateral furrows separating it into six caeca situated in segments M7-M12. The foregut involutes around this same time. Stage 10 ends with dorsal closure, when the right and left edges of the germinal plate meet and enclose the midgut. (D) The stage 11 embryo displays a regionalized digestive tract reminiscent of the adult. The foregut comprises the internalized proboscis, its sheath, and a short esophagus (Sawyer, 1986). The midgut comprises a crop, which has lateral caeca in segments M8-M12, an intestine, which has lateral caeca in segments M13-M16, and a rectum. The hindgut (not shown) is limited to the region of the anus. At this age the crop has lost its vestigial caecum in segment M7, but still contains embryonic yolk.

Alternatively, dextrans within the syncytium may be partitioned away from the site of cellularization. RDA injected into the midgut lineage is typically seen as large, flocculent clumps interspersed between the yolk platelets (Fig. 10E), and like the platelets it could be excluded from the cortical cytoplasm where cellularization will occur.

We have previously shown that the thin outer layer of the gut wall is composed of the visceral mesoderm (Nardelli-Haefliger and Shankland, 1992), and here reexamined this point by injecting RDA into the M teloblast, the hemilateral mesodermal progenitor. As expected, M teloblast injections gave intense labeling of the visceral mesoderm (Fig. 10D-F), intersegmental septa and the muscular layer of the body wall. Such injections also gave a very faint labeling of the endoderm and residual yolk, presumably because the spent teloblast - which has completed formation of segmental blast cells - fuses with the midgut syncytium prior to midgut cellularization.

Colocalization of injected lineage tracers and hybridization reaction product reveals that *Lox10* RNA is expressed exclusively within the endodermal layer of the gut wall (Fig. 10). Examination of embryos labeled with the DNA stain, Hoechst 33258, indicates that the reaction product is heavily concentrated around particular nuclei, but we do not yet know when cellularization reaches completion, and it is therefore unclear whether we are observing *Lox10* expression within endodermal cells, or if the RNA is simply immobilized around syncytial nuclei from which it has been transcribed (Ralston and Hall, 1992).

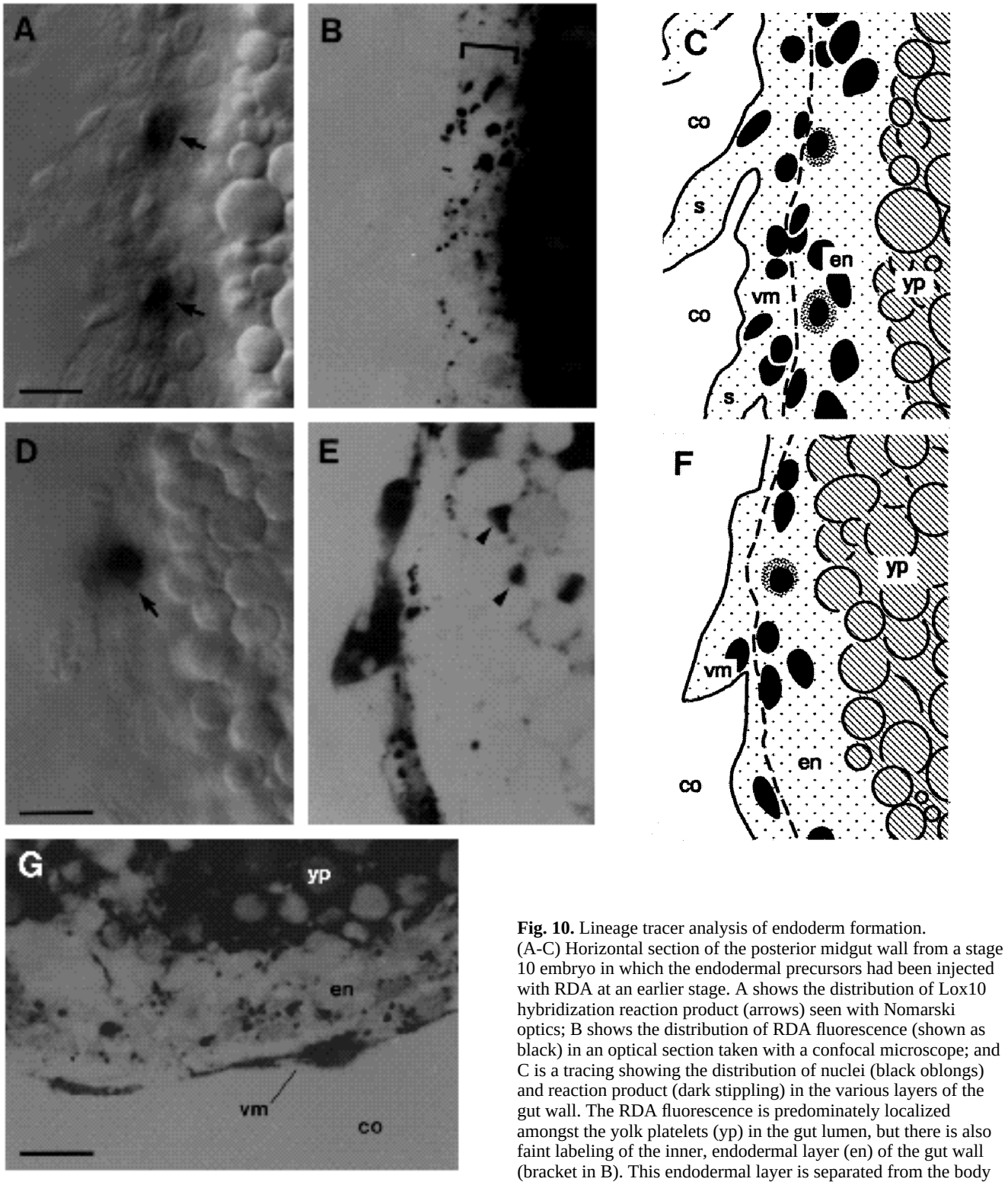

Fig. 10. Lineage tracer analysis of endoderm formation. (A-C) Horizontal section of the posterior midgut wall from a stage 10 embryo in which the endodermal precursors had been injected with RDA at an earlier stage. A shows the distribution of Lox10 hybridization reaction product (arrows) seen with Nomarski optics; B shows the distribution of RDA fluorescence (shown as black) in an optical section taken with a confocal microscope; and C is a tracing showing the distribution of nuclei (black oblongs) and reaction product (dark stippling) in the various layers of the gut wall. The RDA fluorescence is predominately localized amongst the yolk platelets (yp) in the gut lumen, but there is also faint labeling of the inner, endodermal layer (en) of the gut wall (bracket in B). This endodermal layer is separated from the body coelom (co) by an outer layer of unlabeled visceral mesoderm (vm). Note that the hybridizing cells are part of the endoderm, and lie immediately beneath the point where the intersegmental septa (s) join the gut wall. Bar, 10 μm. (D-F) Transverse section of the anterior midgut from a stage 10 embryo in which the mesodermal precursor had been labeled with RDA. Organization of the panels and labeling as in A-C. The visceral mesoderm is heavily labeled, while the endoderm and yolky gut contents only display small clumps of RDA (arrowheads in E). Note that the cell that hybridizes with the *Lox10* probe is situated within the endoderm. Bar, 10 μm. (G) Intracellularly injected RDA is unequally partitioned to the endoderm during normal development. This stage 10 embryo was injected with RDA prior to the onset of embryonic cleavage, and is shown here in a transverse optical section of the anterior midgut. The tracer shows intense labeling in the visceral mesoderm and between the yolk platelets of the gut lumen, but only faint labeling of the endoderm. Bar, 10 μm.

DISCUSSION

Molecular analysis of *Lox10*

The *Lox10* homeobox sequence shows pronounced similarities to a recently discovered class of homeoboxes typified by the *Drosophila* gene *NK-2* (Kim and Nirenberg, 1989). *NK-2* is one of four *Drosophila* genes given the *NK* designation, but the degree of amino acid identity among the four *NK* homeodomains (45%-65%) is only slightly greater than with other homeodomain classes (e.g. 41-49% identity to *Antennapedia*). Homeodomains showing a higher degree of similarity (75-93%) to *NK-2* have since been described in rat (*TTF-1*, Guazzi et al., 1990), mouse (Nkx-2 gene family, Price et al., 1992), planaria (*Dth* genes, Garcia-Fernàndez et al., 1991), tapeworm (*EgHbx3*, Oliver et al., 1992), and now leech (*Lox10*), and we here propose to designate these sequences as a distinct *NK-2* homeodomain class. The phyletic diversity of these *NK-2*-like genes indicates that the founding member of this class must have been established prior to the radiation of the major animal phyla, and comparative analysis can therefore yield insight into the role of this particular gene class in relation to the development and evolution of morphologically divergent animal species.

The *NK-2* class homeodomains share a large number of amino acid residues that are but rarely found at equivalent positions in other homeobox genes (Fig. 2). One of these unusual residues, Ala-28, is located at a position which has recently been implicated in DNA binding specificity for the homeodomain of the *Drosophila* segmentation gene *fushi tarazu* (Furukubo-Tokunaga et al., 1992). The presence of an unusual amino acid residue at this position may help to resolve certain questions regarding the DNA binding specificity of *NK-2* class homeodomain proteins. *TTF-1, Nkx-2.2* and *Nkx2.3* are all identical to *Antennapedia* in the downstream portion of the so-called 'recognition helix' (Hanes and Brent, 1989; Treisman et al., 1989), but do not show the same DNA sequence specificity (Guazzi et al., 1990; Price et al., 1992). Homeodomain residue 43 has also been implicated in binding specificity (Furukubo-Tokunaga et al., 1992), and it is interesting to note that at this position the *NK-2* class consensus sequence includes another amino acid (Thr) that differs from the *Antennapedia* class consensus sequence (Scott et al., 1989).

Lox10 expression and midgut morphogenesis

Lox10 is the first molecular marker shown to display a segmentally periodic distribution in the endoderm of any animal species. In annelids, the definitive endoderm is formed relatively late in embryonic development by the cellularization of a polynucleate midgut syncytium (Whitman, 1878; Anderson, 1975). We have here used intracellularly injected lineage tracers to verify that the endodermal layer of the *Helobdella* gut wall originates from this syncytium, and to show that cells which express *Lox10* RNA are segmentally distributed within the endodermal layer. Cellularization is initiated only in that ventral region of the midgut syncytium which, at early stage 9, is in contact with the germinal plate. The endodermal layer then spreads dorsally in conjunction with the germinal plate to envelop the remnants of the syncytium over a period of several days. However, it is not known whether the accompanying increase in endodermal surface area results from changes in cell shape, mitotic activity, or continued recruitment of cells from the underlying syncytium.

Sequence analysis indicates that the *Lox10* gene encodes a homeodomain transcription factor, and its early expression suggests a role in patterning the gut. For instance, morphogenetic analysis indicates that *Lox10* expression tends to be associated with future sites of midgut constriction. Endodermal expression is first evident at a stage when the midgut primordium is morphologically simple, exhibiting neither segmental organization nor anteroposterior subdivision into the anatomically distinct organs of the adult digestive tract. The spatial transition in *Lox10* expression (spots versus stripes) at the M12/M13 segment boundary prefigures the anteroposterior regionalization of the midgut, in which the posterior region - which expresses *Lox10* over a larger fraction of its surface area - undergoes a dramatic constriction that expels its yolky contents into the swelling anterior midgut. The boundary between these two morphogenetic zones will ultimately correspond to the crop/intestine junction. In addition, there is a close correlation between the positioning of *Lox10*-expressing cells within each segment and the formation of intercaecal constrictions in the intestine and crop.

Morphogenetic events within the midgut could originate from mechanical forces generated either by the endoderm itself or by the overlying mesoderm. In the former case, one could envision that the putative Lox10 protein regulates the expression of downstream gene products (e.g. cytoskeletal proteins or secreted morphogens) which alter the curvature of the endodermal cell layer and thereby shape the gut wall. Alternatively, the correlation of *Lox10* expression with sites of gut constriction could reflect parallel responses to an extrinsic signal derived from the mesoderm. This latter scenario would seem more likely for the delayed expression of *Lox10* in the intercaecal grooves of the developing crop, since these grooves are thought to arise by passive deformation as the expanding germinal plate pulls the dorsoventral muscles against the crop like taut ropes pressing into a balloon (Whitman, 1878).

The segmented ectoderm and mesoderm of the leech embryo arise from embryonic stem cells via iterated cell lineages that establish the longitudinal periodicity of their descendant tissues (Shankland, 1991). However, iterated patterns of cell division could not account for the segmental character of endodermal tissues which have arisen by cellularization of a polynucleate syncytium. One possibility is that the overlying mesoderm imprints its segmental organization onto the endoderm through inductive cell interactions. Such an induction is supported by experimental manipulations: photoablation of the leech mesoderm over a short stretch of contiguous hemisegments leads to a localized loss of *Lox10* expression and gut morphogenesis (D. N. H. and M. S., unpublished results). Moreover, the leech mesoderm has an intrinsic segmental identity from an early developmental stage (Gleizer and Stent, 1993), and could provide the anterior and posterior midgut with differing signals that might account for their distinct patterns of *Lox10* expression. Ablation of the mesoderm has been shown to disrupt the regionalization of gut differentiation in

another annelid, *Eisenia* (Devriès, 1974), and mesoderm/endoderm interactions are likewise implicated in the regionalization of the vertebrate digestive tract (Wessels, 1974).

Both homeodomain transcription factors and mesoderm-endoderm inductions play an important role in the gut development of *Drosophila*. The segment identity genes, which govern the fly's ectodermal differentiation, are also expressed in anteroposterior order in the visceral mesoderm of the midgut (Tremml and Bienz, 1989; Reuter et al., 1990). Mutational analysis indicates that the expression of these segment identity genes is essential for regionalized gut morphogenesis, although there is as yet no evidence of a segmental periodicity such as that shown by *Lox10* in the leech endoderm. In the best studied example, the *Ultra-bithorax* (*Ubx*) gene is expressed within particular regions of the fly's visceral mesoderm, where it activates synthesis of the decapentaplegic (dpp) growth factor. Spatially restricted dpp secretion within the mesoderm leads in turn to a localized expression of the regulatory gene *labial* in the underlying endoderm (Reuter et al., 1990; Panganiban et al., 1990). A leech *Ubx* homologue, *Lox2* (Wysocka-Diller et al., 1989), has recently been cloned in *Helobdella* (Nardelli-Haefliger and Shankland, 1992), and in situ hybridization reveals that *Lox2* is expressed in the dorsoventral muscles, which arise from the septal mesoderm and lie in the groove of the intercaecal constrictions in close association with the endodermal cells that express *Lox10*. In addition, the rostral limit of *Lox2* expression corresponds to the anterior boundary of the crop and therefore to the final anterior boundary of *Lox10* midgut expression. One could speculate that *Lox2* plays a role in the midgut regionalization of the leech comparable to that of *Ubx* in *Drosophila*, and that *Lox10* could be one of the downstream genes whose endodermal expression it controls. *NK-2*, the *Drosophila* homologue of *Lox10*, is also expressed in the midgut (K. Nakayama and M. Nirenberg, personal communication), but its function and the details of its midgut expression pattern are as yet unknown.

In the distantly related leech *Hirudo*, the homeobox genes of the *Lox3* complex are likewise expressed in the developing gut in segmentally repeated transverse stripes, although it is not known whether their expression is restricted to the endoderm or the visceral mesoderm (J. Wysocka-Diller and E.R. Macagno, personal communication). The *Lox3* genes belong to a recently characterized homeobox gene family that includes only two other identified members: the *Helobdella* gene *Htr-A2* (Wedeen et al., 1990); and *XlHbox8*, which is expressed in a narrow band of endoderm in the *Xenopus* embryo (Wright et al., 1988). Thus, several distinct classes of homeodomain transcription factor seem to play a role in the gut differentiation and regionalization of a wide variety of animal species.

Lox10 expression in the prostomium

In situ hybridization also reveals *Lox10* expression within a subset of the micromere lineages that give rise to the prostomium, the unsegmented cephalic region of the leech body. The first quartet micromeres arise from radially symmetric cell divisions, and in support of previous cell lineage studies (Weisblat et al., 1984) we have found that their descendant clones make radially arrayed components of the supraesophageal ganglion and prostomial epidermis. Thus, the micromere lineages differ from the iterated cell lineages that generate the mesoderm and ectoderm of the segmented body trunk, arguing against traditional views which hold that the prostomium represents the two anteriormost body segments (Mann, 1962; Sawyer, 1986). Further evidence against the segmentation of the prostomial ectoderm is the absence of longitudinally iterated neuronal phenotypes, e.g. the single cluster of supraesophageal *Lox10* neurons. In our view, the prostomium represents a non-segmental anterior component of the leech's body plan, at least formally comparable to the non-segmental tissues found at the anterior end of the insect (Finkelstein and Perrimon, 1991) and of the vertebrate CNS (Gans and Northcutt, 1983).

The apparent restriction of *Lox10* expression to progeny of the a′/b′ micromeres at both early and late stages of embryonic life raises the possibility that the prostomial cells that hybridize at embryonic stages 7/8, or their progeny, migrate into the supraesophageal ganglion and give rise to the hybridizing neurons seen at stages 9-10. Such migration seems unlikely, in that the early expression is located at the extreme anterior end of the a′/b′ clones (even after the stage of micromere inversion), while the neuronal expression seen at later stages is situated at the posterior border of these same clones. Moreover, we did not encounter hybridizing cells at any intermediate position, although there is a period of time around the stage 7/8 transition when in situ hybridization repeatedly failed to reveal prostomial *Lox10* expression. Thus, we cannot rule out the possibility that there is a transient loss of *Lox10* transcripts while the cells in question are undergoing migration.

Lineage tracer analysis of the first quartet micromere clones reveals a previously undescribed rearrangement of these clones during prostomial morphogenesis. The d′/c′ clones switch positions with the a′/b′ clones along the future anteroposterior body axis, thereby transforming the initial clockwise arrangement of these micromeres about the animal pole of the egg to the anticlockwise arrangement of their descendant clones about the animal's mouth (Fig. 6).

It appears that the a′/b′ clones remain stationary throughout this rearrangement, and that it is the c′/d′ clones that are (either actively or passively) displaced relative to the underlying macromeres that form the bulk of the embryo. The a′/b′ clones remain apposed to the future anterior side of developing foregut (Anderson, 1975), while the d′/c′ micromeres rotate from the future anterior (D quadrant) to the future posterior (B quadrant) side of the foregut as the rearrangement occurs. This micromere rotation mirrors the epiboly movement of the left and right germinal bands (Fig. 1C), which are in contact with the d′ and c′ clones from the onset of blast cell formation (Sandig and Dohle, 1988). Thus, the d′ and c′ clones may respectively join the anterior ends of the left and right germinal bands to the a′ and b′ clones, and their posteriad displacement would therefore be linked to the symmetric rotation of the germinal bands about the animal pole. Further support for this idea comes from labeling of the nopq″ micromeres (cells 't^{II}'; see Sandig and Dohle, 1988), which are formed bilaterally between the d′/c′ micromeres and the anterior ends of the left and right germinal bands, and which give rise to neuronal progeny situated at the junction of the supraesophageal ganglion and

the first segmental neuromere (S. R. Mase and D. K. Stuart, personal communication).

Evolutionary history of the *NK-2* gene class

Our present analysis of the leech *Lox10* gene represents the first detailed characterization of *NK-2* class homeobox gene expression in an invertebrate organism, and reveals several marked anatomical correlations with the utilization of the *TTF-1* and *Nkx-2* genes in mammals. First, *Lox10* is expressed in the leech endoderm, and two major sites of *TTF-1* expression (thyroid and lung) are organs of endodermal derivation. Second, *Lox10* is expressed in a lineally distinct anterior region of the leech embryo, particularly in the anteriormost component of its CNS. Both *TTF-1* and *Nkx-2.2* are expressed in the mammalian forebrain (Lazzaro et al., 1991; Price et al., 1992), anterior to the segmented rhombomeres of the hindbrain (McGinnis and Krumlauf, 1992). Preliminary data on the *Drosophila NK-2* gene indicates that it is also expressed in the midgut and CNS, although its neural expression is more widely distributed along the body axis (K. Nakayama and M. Nirenberg, personal communication). Caution must be applied when drawing homologies between phyletically diverse organisms; nonetheless, our findings suggest that some aspects of gene utilization within the *NK-2* class may be conserved features retained from a common ancestor.

Restriction of the neural expression of *Lox10* to the supraesophageal ganglion of the leech and of the *TTF-1/Nkx-2* gene family to the forebrain of mammals is intriguing in light of other work on genetic similarities between the cephalic region of mammals and insects. Expression of the *empty spiracles* (*ems*) and *orthodenticle* (*otd*) genes is restricted to the cephalic segments of the *Drosophila* embryo, and their mammalian homologues *emx1* and *emx2* (Simeone et al., 1992a) and *otx1* and *otx2* (Simeone et al., 1992b) are expressed in overlapping subregions of the forebrain. Thus, in both organisms the expression of these genes is restricted to an extreme anterior body region which does not overlap with a much more extensive axial domain that is characterized by HOM/*Hox* gene expression (Finkelstein and Perrimon, 1991; McGinnis and Krumlauf, 1992). One interpretation of these findings is that *ems* and *otd* expression may help to define a cephalic domain whose genetic distinction predated the divergence of vertebrates and protostome invertebrates (Holland, 1992; Holland et al., 1992). Annelids such as the leech are also members of the protostome lineage, and the restriction of *NK-2* class gene expression to the extreme anterior ectoderm of leeches and mammals is consonant with the idea of a homologous - and thus phyletically primitive - cephalic domain. This view would require that the widespread expression of *NK-2* in the *Drosophila* ectoderm is a derived character, and clearly expression data must be compiled from *NK-2* class genes of additional organisms before any consensus can be drawn.

Traditional views of phylogeny are framed around an early split in the protostome and deuterostome lineages, and hold that these lineages originated from a coelenterate-like organism that was organized along an oral-aboral axis, with the aboral pole giving rise to the anterior end of both protostome and deuterostome body plans (Beklemishev, 1969). Many protostomes retain this organization in their larval forms, with the aboral and oral hemispheres giving rise respectively to the cephalic and trunk tissues of the adult. In these organisms the homologues of the leech first quartet micromeres generate an aboral sense organ that transforms into the cephalic ganglion of the mature CNS. Some evolutionary theories hold that the aboral sense organ of the ancestral deuterostome made no contribution to the vertebrate CNS (Beklemishev, 1969), but others allow for incorporation of this sensory structure at the anterior end of the neural tube (Crowther and Whittaker, 1992; Garstang, 1928). In any case, the growing number of shared genetic distinctions between the cephalic and trunk domains of the protostome invertebrates (e.g. flies, leeches) and the vertebrate CNS makes it seem likely that these domains reflect a phyletically ancient subdivision of the primary body axis.

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