ORIGINAL ARTICLE

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Dorsal and Snail homologs in leech development

Abstract As part of an examination of how developmental mechanisms such as axis specification, cell fate specification, and segmentation have evolved, we have cloned homologs of the *Drosophila melanogaster* genes *dorsal* and *snail* from the glossiphoniid leech *Helobdella robusta*. Sequences from one *dorsal*-class gene (*Hro-dl*) and two *snail*-class genes (*Hro-sna1* and *Hro-sna2*) were identified. Polyclonal antibodies were raised against the most conserved domains of HRO-DL and HRO-SNA1. Nuclear staining appeared for both proteins in mid-embryogenesis, in mesodermal and ectodermal precursors. During segmentation, segmentally iterated stripes of cells with strong HRO-DL staining appeared. The stripes of HRO-DL staining were first concentrated in the cytoplasm of cells, and later in the nuclei. Around this time, HRO-SNA levels also appeared in nuclei in segmentally iterated stripes. The localization of HRO-DL and HRO-SNA proteins raise the possibility that these genes are part of a conserved genetic pathway that, instead of specifying the dorsoventral axis and the mesoderm as in flies, might play a role in the diversification of cell types within segment primordia during leech development.

Keywords Leech · Dorsal · Snail · Mesoderm · Segmentation

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Introduction

Understanding how morphological diversity evolves is one of the great challenges of modern biology. The evolution of morphological diversity must entail the modification of pre-existing developmental programs, but whether general rules exist by which alterations to developmental programs produce morphological diversity (or what any such rules might be) remains to be determined. This issue must be approached by analyzing the developmental mechanisms of diverse taxa and comparing them in light of the relevant phylogenetic relationships.

In making such comparisons, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* are prime candidates for reference species, since these are the organisms for which the molecular mechanisms of development are known in most detail. Both these species fall within the Ecdysozoa, and thus represent just one of the three clades of bilaterian animals defined by recent molecular phylogenies (Halanych et al. 1995; Aguinaldo et al. 1997; de Rosa et al. 1999). The main goal of this work is to determine if homologs of genes involved in dorsoventral axis specification and mesoderm specification in *Drosophila* (such as *dorsal* and *snail*) play similar roles in spiralian animals, which belong largely to the Lophotrochozoa (the other protostome clade). The spiralians represent a large group of diverse phyla that have comparable embryonic fate maps but exhibit broad variation in how the mesoderm and the dorsoventral axis are specified (Henry and Martindale 1999). Identifying the molecular players in these events may therefore open a fruitful avenue for examining how development evolves. We also hope to contribute to resolving the question of whether segmentation arose independently within the two great clades of protostomes, by examining the roles of homologs of key fly developmental genes in segmented lophotrochozoans. For these purposes, we use embryos of the glossiphoniid leech *Helobdella robusta* (phylum Annelida), a segmented, spirally cleaving lophotrochozoan.

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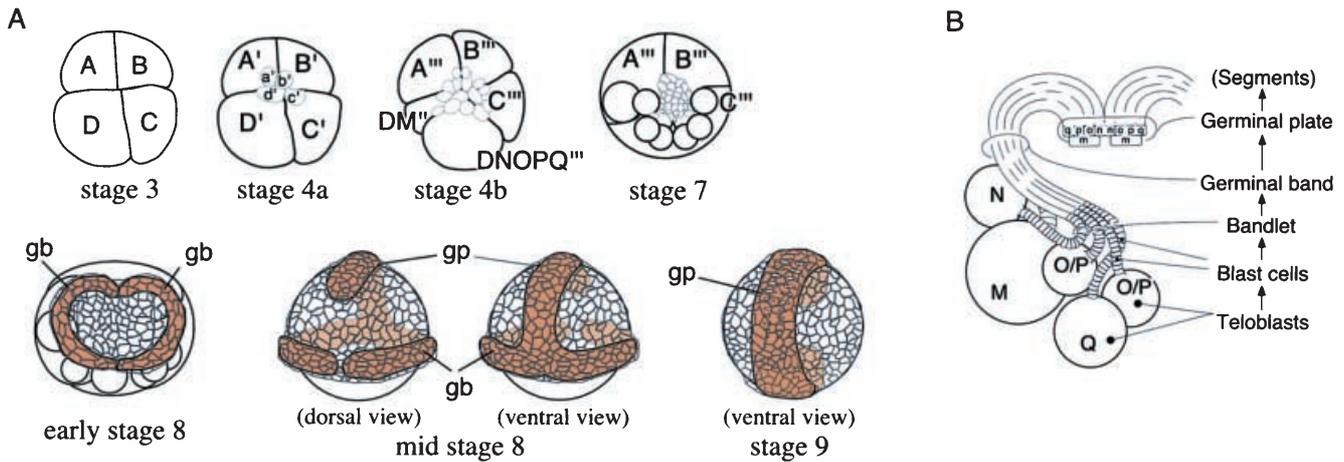
Fig. 1A, B Schematic summary of development of the leech *Helobdella robusta*. **A** Selected developmental stages. *Orange* Germinal bands (*gb*) and germinal plate (*gp*), *gray* micromere cap. Segmentally iterated coelomic spaces are shown in the stage 9 embryo. **B** Diagram of segment formation from teloblasts in an early stage 8 embryo

The first two cleavages in *Helobdella* produce four cells called A, B, C and D (Fig. 1). Cell D is larger than the other three cells, inherits most of the yolk-deficient cytoplasm (teloplasm) and is the precursor of all segmental mesoderm and ectoderm. The D cell undergoes an unequal division, producing a micromere and a macromere, and the macromere next divides to form the mesodermal precursor DM and the ectodermal precursor DNOPQ. Thus, we might expect any putative segregated determinants that specify dorsal and/or mesodermal fates to be localized exclusively to the D cell, and a putative segregated determinant for mesoderm to be further segregated to DM. A determinant specifying other fates might be localized in a complementary pattern. Embryological experiments in other spiralians, such as *Ilyanassa obsoleta* and *Bithynia tentaculata*, suggest additional predictions for where such determinants should be localized in such organisms (Clement 1952; van Dam et al. 1982). To date, no molecules with these predicted patterns of localization are known in any spiralian.

Flies develop segments by making use of gradients of proteins, such as transcription factors and translational regulators, within a multinucleate syncytium, to subdivide a common cytoplasm into unique regions. Unlike flies, and like most other animals, *Helobdella* embryos undergo holoblastic cleavages during early development. Segmental tissues arise in *Helobdella* from anteroposteriorly oriented columns of segmental founder cells (blast cells). The blast cells arise in an anteroposterior progression from five bilateral pairs of stem cells (M, N, O/P, O/P, and Q teloblasts) produced during late cleavage from DM and DNOPQ, the mesodermal and ectodermal precursors discussed above.

In *Drosophila*, *dorsal* and *snail* are key regulators of dorsoventral axis specification and mesoderm specification; *dorsal* encodes a *rel*-domain transcription factor

that is present throughout the embryo but which only enters nuclei on the ventral side of the embryo, under the control of the *Toll* signaling pathway (Belvin and Anderson 1996). In ventral nuclei, Dorsal protein binds to the promoter of *snail*, activating *snail* expression (Rusch and Levine 1996). Snail is a zinc finger transcription factor; it plays a role in ensuring that a mesoderm-specific pattern of gene expression arises in ventral cells (Leptin 1991). Similarities in the expression patterns between fly and vertebrate versions of these genes (Nieto et al. 1992; Smith et al. 1992; Essex et al. 1993; Hammerschmidt and Nusslein-Vollhard 1993; Thisse et al. 1993; Bearer 1994) suggest that the common ancestors of flies and vertebrates might have used these genes similarly, at least to some degree. We therefore chose these genes as candidates for regulators of similar processes in spiralians. Here, we have identified sequences from *dorsal*- and *snail*-class genes of some spiralians. We show that Dorsal- and Snail-class proteins in the leech *Helobdella* do not show an expression pattern that would suggest a role in dorsoventral axis and mesoderm specification. Instead, after a widespread expression pattern in early development, they show a segmentally iterated pattern, after other signs of segmentation have appeared, raising the possibility that they may play a role in elaborating cell diversity within each segment.

Materials and methods

Animals

Helobdella robusta (abbreviated as "Hro" in gene and protein names; Shankland et al. 1992) was obtained from a laboratory colony, and *Helobdella stagnalis* (Hst) was obtained from a commercial supplier. Standard culture conditions (Blair and Weisblat 1984) and embryo staging criteria (Fernandez 1980) were used. The gastropod mollusk *Lymnaea stagnalis* (Lst) was cultured by the methods of Lundelius and Freeman (1986). Adults of the echiuran *Urechis caupo* (Uca) were kept in sea water at 15°C; animals were spawned and embryos were raised by the methods of Rosenthal and Wilt (1986). Adults of the sipunculid *Phascolosoma agassizii* (Pag) were collected in the intertidal zone at Hopkins Marine Laboratory, Monterrey, and were kept in the laboratory in sea water at 15°C.

Fig. 2A–D *Helobdella robusta* Dorsal-class protein (*HRO-DL*; **A**, **B**) and Snail-class proteins (*HRO-SNA*; **C**, **D**). Diagrams of predicted proteins (**A**, **C**) represent full-length or partial sequence, with *black sections* marking the rel-homology domain of *Drosophila* Dorsal and five zinc fingers of *Drosophila* Snail. The most identical regions are labeled with *percent amino acid identity*. The ends of incomplete coding sequences are indicated by *zig-zags* (for HRO-DL and HRO-SNA2). Sequences show rel-homology domain of Dorsal-class proteins (**B**) and four conserved zinc fingers (*numbered 2–5* above sequences) and C-terminal sequence of Snail-class proteins (**D**). Primer sites are indicated in *gray*. Amino acids are labeled *black* if identical, and *gray* if similar, in at least two-thirds of the sequences. The last six amino acids of LST-SNA are omitted because they were not conclusively determined. *Asterisks* before names of predicted proteins represent sequences newly reported in this paper. *LST Lymnaea stagnalis, HST Helobdella stagnalis, PAG Phascolosoma agassizii, UCA Urechis caupo*

A

HRO-DL

56%

rel-homology domain   Dorsal

B

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*HST-DL      primer>RSAGSIPGEHSTSDRKTYPSIKIHNFDGPAAIVVVSCV
*HRO-DL      primer>RSAGSIPGEHSTSDRKTYPSIKIHNPEGAAIVVVSCV
Fly Dorsal   EYVKITEQPAGKALRFRYECEGRSAGSIPGVNSTPENKTYPTIEIVGYKSR-AVVVVSCV
Frog Xrel1   PPVBIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDTSKTHPTIKINNYQGF-ARIRISLV
Human NFKB   PLIFPAEQPKQRGMRFRYKCEGRSAGSIPGERSTDTTSKTHPTIKINGYTGF-GTVRISLV
Fly Relish   PQLRIVEQPVEKFRFRYKSEMHGTHGSLNGANSKRTPKTFPEVTLCNYDGP-AVIRCSLF

*HST-DL      TKEKEYHPHPHNLVGQD-CRDGVCTVKVKNPNS---VITFPNIGIQCCKRHDVEDNIK--
*HRO-DL      TKEKEYYHPHPHNLVGQD-CKDGVCTVKVKNPSS---VITFPNIGIQCCKRHDVEDNLK--
Fly Dorsal   TKDTPYRPHPHNLVGKEGCKKGVCTLEINSETM---RAVFSNLGIQCVKKKDIEAALK--
Frog Xrel1   TKDSPHKPHPHELVGKD-CKDGYYEAELSPDRS---IHSFQNLGIQCVKKREVEDAVA--
Human NFKB   TKDPPHRPHPHELVGKD-CRDGFYEAELCPDRC---IHSFQNLGIQCVKKRDLEQAIS--
Fly Relish   QTN-LDSPHSHQLVVRKDDRIVCDPHDLHVSKERGYVAQFINMGIIHTAKKYLFEELCKK

*HST-DL      ----------IREKIRVDPFTTGYPTSN--SNIDLNSV<primer
*HRO-DL      ----------IREKIRVDVSTGYPSAN--NNIDLNSVRLCFQVFIPLANK-KFTHIVPP
Fly Dorsal   ----------AREEIRVDPFKTGFSHRFQPSSIDLNSVRLCFQVFMESEQKGRFTSPLPP
Frog Xrel1   ----------HPIRTNNNPRNVSPEELK--ADYDLNIVGLCFQVFIPDQAAGRMLPLPPFV
Human NFKB   ----------QPIQTNNNPRQVPIEEQR--GDYDLNAVRLCFQVTVRDPSG-RPLRLPPV
Fly Relish   KQDRLVFQMNREELSHKQLQELHQETEREAKDMNLNQVRLCFBAFKIBDNG-AWVPLAPP

*HST-DL      IVSQPIIDKES--VHDLVICRLSPQSGYAVGGDEVFLL<primer
*HRO-DL      
Fly Dorsal   MVSEPIFDKRA--MSDLVICRLCSCSATVFGNTQLILLCEKVAKEDISVREFEEKNGQSV
Frog Xrel1   VS-QPIYDNRAPNTAELKICRVNKNSGSCLGGDEIFLLCDKVQKEDIEVIFGLGNWEARG
Human NFKB   IS-HPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPGWEARG
Fly Relish   WYSNAINNRKSAQTGELRIVRLSKPIGGVMGNDEIILLVEKVSKKNIKVREFEEDELGET
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C

| | HRO-SNA1 |

66%

Snail

77%

HRO-SNA2

D

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              2                           3
*LST-SNA      primer>IRTHT--LPCKCQVCGKAFSRPWLLQGHIRTHTGEKPFRC
*PAG-SNA      primer>IRTHT--LPCKCKLCGKAFSRPWVLQGHIKTHTGDKPFKCQ
*UCA-SNA      primer>IRTHT--LPCKCQLCGKAFSRPWLLQGHIRTHTGEKPFQCF
*HRO-SNA2     primer>SLKMHIRTHT--LPCKCKLCGKAFSRPWLLQGHLRTHTGERPFGCE
*HRO-SNA1     NSCNYCGRKYNTTGALKMHIRTHT--LPCRONLCGKSFSRPWLLQGHLRTHSGEKPFKCN
Fly Snail     HSCEECGKLYTTIGALKMHIRTHT--LPCKCPICGKAFSRPWLLQGHIRTHTGEKPFQCF
Frog Xsna     FSCKYCEKEYVSLGALKMHIRTHT--LPGVCKICGKAFSRPWLLQGHIRTHTGEKPFSCF
Danio snal    FFCKHCPKEYNSLGALKMHIRSHT--LPGVCPTCGKAFSRPWLLRGHIRTHTGERPFSCE
Fly Glass     NLCRLCGKTYARPSTLKTHIRTHSGEREYKCPDCNKSFSQAANETAHVRTHTGCKPFRCE

*LST-SNA      HCGRA<primer
*PAG-SNA      HCGRA<primer
*UCA-SNA      HCARAFADRSNL<primer
*HRO-SNA2     TCCRAFADRSNLRAHMQTHEDVKKYNCNKCGKTFSRMSLLHKHRENNTC*
*HRO-SNA1     FCQRAFADKSNLRAHLQTHAQIKRYCCSHCGKSFSRLPLLLKHGASCGFL*
Fly Snail     DCPRSFADRSNLRAHQQTHVDVKKYACQVCHKSFSRMSLLNKHSSSNCTITIA*
Frog Xsna     HQNRAFADRSNLRAHLQTHSDVKKYQCKNCSKTFSRMSLLHKHEESGCCVAH*
Danio snal    HQNRAFADRSNLRAHLQTHSDVKKYQCSTCSRTFSRMSLLQKHSAAGCCPSTANVQ*
Fly Glass     ICDRRFSQSSSVTTHMRTHSGERPYRCSSCKKSFSDSSTLTKHLRIHSGEKPYQCKLC
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Degenerate PCR of new genes

Primers used in this study were as follows (I is inosine; N is A/G/C/T):

- dor1: 5′-CGGAATTCCGTTYMGITAYIIITGYGARGG-3′, corresponding to the sequence FRYECEG (Fig. 2) plus an *Eco*R1 adapter.
- dor2: 5′-cgggatcccgtcyttIkSIacyttItcrca-3′, corresponding to the sequence CEKVAKE plus a *Bam*HI adapter.
- dor3: 5′-CGGGATCCCGAAIacytGraarcaIarIc-3′, corresponding to the sequence RLCFQVF plus a *Bam*HI adapter.
- sna1: 5′-GTAAGCTTGGNGCNYTNAARATGCAYAT-3′, corresponding to the sequence GALKMHI plus a *Hind*III adapter.
- sna2: 5′-GTGAATTCAANGGYTTNTCNCCYGTRTG-3′, corresponding to the sequence HTGEKPF plus an *Eco*R1 adapter.
- sna3: 5′-CGC GGATCCGGIDSIYTIAARATGCA-3′, corresponding to the sequence GALKMH plus a *Bam*H1 adapter.
- sna4: 5′-CCGCTCGAGCGGRTTISWIYKRTCIGCRAA-3′, corresponding to the sequence FADRSN plus an *Xho*1 adapter.
- sna5: 5′-CCGCTCGAGCGGRTGIGTYTGIIIRTGIGCIC-3′, corresponding to the sequence RAHQQTH plus an *Xho*1 adapter.

Hro-dl amplification was carried out with the primers dor1 and dor2. *Hst-dl* was amplified using the dor1 and dor3 primers. *Hro-sna* genes were amplified using the primers sna1 and sna2. *Pag-sna* and *Lst-sna* were amplified using the sna3 and sna4 primers, and *Uca-sna* was amplified with the sna3 and sna5 primers. During work aimed at producing an antibody to a leech *twist* homolog, *Hro-twi*, a sequence previously reported as 3′ UTR (Soto et al. 1997) was found to have sequence similarity to a protein-encoding sequence in *twist*. The *twist* homolog was therefore amplified from genomic DNA and the published sequence was found to contain errors, one of which produced a premature stop codon. The corrected sequence may encode a putative protein with a molecular weight of 40 kDa. During one attempt to amplify *Hro-dl*, a fragment of a leech homolog of the ascidian gene *macho-1* (Nishida and Sawada 2001) was fortuitously amplified; we call the gene represented by this fragment *Hro-macho*. All of the sequences identified or corrected in this work (*Hro-dl*, *Hst-dl*, *Hro-sna1*, *Hro-sna2*, *Hro-macho*, *Hro-twi*, *Pag-sna*, *Lst-sna*, *Uca-sna*) have been submitted to GenBank.

Simple methods for DNA preparation were adapted, with slight modifications, from a protocol developed for small amounts of *C. elegans* tissue by Barstead et al. (Barstead et al. 1991; Williams 1995), and were used for the echiuran, sipunculid, and molluscan DNA templates. A piece of tissue with a volume of roughly 0.1 mm³ (or between one and ten larvae for *U. caupo*) was added to 2.5 μl lysis buffer (5°mM KCl, 2.5 mM MgCl₂, 1°mM Tris HCl pH 8.3, 0.45% Nonidet P40, 0.45% Tween-20, 0.01% gelatin, 0.1 mg/ml proteinase K) in an Eppendorf tube, covered with mineral oil, and frozen at –80°C for at least 10 min. Several differently-sized pieces of tissue were tried for each sample. Each tube was incubated at 60°C for 1–2 h, heated to 95°C for 15 min to denature the proteinase K, and cooled to 4°C. These were used as DNA templates by adding 47.5 μl PCR cocktail [1 unit of *Taq* polymerase and 1× *Taq* buffer with MgCl₂ (Perkin Elmer Cetus), 25 μM dNTP and 10 μM primer] directly to each tube.

Expression constructs and antibody preparation

The entire Hro-dl fragment (Fig. 2) and a Hro-sna1 GALKMH to AHLQTH fragment were cloned into pQE-30 (Qiagen) to generate N-terminally 6× His-tagged polypeptides. Clones were tested for expression on protein gels and expressing clones were sequenced and then used for protein purification on nickel-NTA columns. The resulting partially purified proteins were gel-purified. Polyclonal rabbit antisera against these proteins were then raised commercially (Babco). Antisera were affinity-purified on columns against the antigens above bound to Affigel (BioRad). These affinity-purified antisera were used for all the HRO-DL and HRO-SNA

immunostaining experiments described in this paper. Attempts were also made to generate antibodies that might recognize Dorsal-, Snail-, or Twist-class proteins in multiple phyla; for Dorsal, this was attempted by expressing full-length *Drosophila* Dorsal, purifying the expressed protein as above, and injecting it into rabbits, boosting twice with this protein, and then boosting twice more with the conserved HRO-DL fragment above. Neither this, nor antisera generated against Snail-class proteins by the same method, gave the predicted patterns by immunostaining *Drosophila* embryos, nor by immunostaining leech embryos with antisera or affinity-purified antibodies (purified against the leech sequence-derived polypeptides above). Antisera and affinity-purified antibodies generated against the Twist-derived peptides QRVMANVRERQRTQSLN or DKLSKIQTLKLATRYID (Research Genetics) also failed to produce a clear pattern of nuclear localization in leech embryos.

Fixation, removal of vitelline envelope, and immunostaining

Embryos were collected from adults, fixed in 4% formaldehyde in one-quarter strength phosphate-buffered saline (PBS) for 90 min, and then rinsed in PBS plus 0.1% Tween-20 (PBT). All steps were carried out at room temperature unless otherwise noted, and all further incubations were carried out in Eppendorf tubes filled with fluid (to prevent damage resulting from bubbles) and rotating at a speed sufficient to keep the embryos from settling (about 5 s per rotation).

To efficiently remove the vitelline envelope from large numbers of embryos, Pasteur pipettes are pulled over a flame, and then cut with watchmakers forceps under a dissecting scope to a bore diameter just smaller than the diameter of the vitelline envelope, using intact embryos for comparison. Useful pipettes are selected by testing some on fixed embryos: drawing groups of embryos into the pipette, using a mouth pipette to carefully control suction, results in removal of the vitelline envelope with visible damage to fewer than 10% of the embryos. An excessively small bore size will result in embryos getting stuck or damage to a greater percentage of the embryos, and an excessively large bore size will result in failure to remove the vitelline envelope. Effective pipettes are rinsed and saved for reuse.

Devitellinized embryos were blocked in PBS with 1% Tween-20 and 10% heat-inactivated normal goat serum (PTN) for 3–4 h, then incubated in primary antibody in PTN plus 0.02% sodium azide overnight. Affinity-purified anti-HRO-DL was used at 1:100, affinity-purified anti-HRO-SNA at 1:50, and mouse anti-histone monoclonal antibody MAB052 (Chemicon) at 1:1,000. Embryos were rinsed in several changes of PBS with 1% Tween-20 and then washed over 12 h through several more changes. Embryos were then incubated in 1:1,000 HRP-conjugated anti-rabbit IgG secondary antibodies (Jackson Immunoresearch Laboratories) in PBT with 10% heat-inactivated normal goat serum overnight at 4°C. Embryos were washed for 6 h through six changes of PBT. HRP localization was revealed by incubating embryos in 0.4 mg/ml DAB with 0.01% hydrogen peroxide in PBS for 1–5 min, watching under a microscope, with a white surface behind the embryos, until the reaction developed sufficiently. Embryos were then washed several times in PBT, passed through an ethanol series and left in 100% ethanol for 3–5 min, and then cleared in 3:2 benzyl benzoate:benzyl alcohol (BBBA). Sectioned embryos were stained as whole mounts and then sectioned. Unless otherwise noted, the observations discussed for whole-mount embryos are based on seeing the pattern invariably in a minimum of ten embryos of each stage; the observations for sectioned embryos are based on complete sections of three embryos. None of the patterns found here were found after staining embryos from the same clutches with polyclonal antibody to a different leech protein (HRO-NOS; Pilon and Weisblat 1997) or with any of the pre-immune sera.

Results and discussion

Identification of leech homologs of *dorsal* and *snail*

We designed primers corresponding to sequences conserved between each fly gene and its vertebrate homologs, and performed degenerate PCR, using genomic DNA from the leech *H. robusta* as template, to identify leech homologs of these genes.

The *dorsal* primers amplified a 525-bp PCR fragment encoding 175 amino acids of polypeptide (Fig. 2); this stretch showed 56% amino acid identity to most of the *Drosophila* Dorsal protein's rel-homology domain, a domain involved in DNA binding, dimerization, and binding to Cactus, a regulator of Dorsal localization and DNA-binding activity (Isoda et al. 1992; Govind et al. 1996). We refer to the gene represented by this fragment as *Hro-dl*. A fragment of a *dorsal* homolog was also amplified from a second leech, *H. stagnalis*; we refer to the gene represented by this fragment as *Hst-dl*. The *Hro-dl* predicted gene product shares more sequence identity with Dorsal than it does with the corresponding fragments of divergent rel-homology domain proteins, such as the *Drosophila* proteins Dif (41%) or Relish (31%), suggesting it is a true Dorsal homolog. The same is true for the *Hst-dl* predicted gene product.

The *snail* primers amplified two different 126-bp fragments, both of which had sequence similarity to the *Drosophila snail* sequence. We refer to the genes represented by these fragments as *Hro-sna1* and *Hro-sna2*, and we refer to the two genes together as *Hro-sna* genes. In the course of this work, fragments of *snail* homologs were also identified from three other spiralian phyla by PCR; we refer to the genes represented by these fragments as *Pag-sna* (from the sipunculid *P. agassizii*), *Uca-sna* (from the echiuran *U. caupo*) and *Lst-sna* (from the mollusk *L. stagnalis*). To identify more sequence of each of the leech genes, the *Hro-sna1* and *Hro-sna2* PCR fragments were used to screen a *H. robusta* genomic DNA library. A 2.0-kb Hro-sna1 genomic clone was obtained. This clone contained sequence that may encode a 203-amino-acid polypeptide with four predicted zinc fingers and a predicted molecular weight of 23 kDa. The 107 amino acids that may encode four zinc fingers had 66% sequence identity to zinc fingers 2–5 of *Drosophila* Snail and similar percent identities to the *Drosophila* Snail paralogs Worniu (64%) and Escargot (67%). A 0.8-kb *Hro-sna2* genomic clone was obtained. This clone contained sequence with the potential to encode the N-terminal 93 amino acids of a protein; this 93-amino-acid stretch showed 77% sequence identity to the N-terminal part of the *Drosophila* Snail protein and similar percent identities to the *Drosophila* Snail paralogs Worniu (77%) and Escargot (78%). Both *Hro-sna1* and *Hro-sna2* share more sequence identity with *snail*, these *Drosophila* paralogs and vertebrate orthologs than they do with divergent zinc finger genes, such as the *Drosophila* genes *scratch* and *glass*, suggesting they are true *snail*-class genes (Fig. 2).
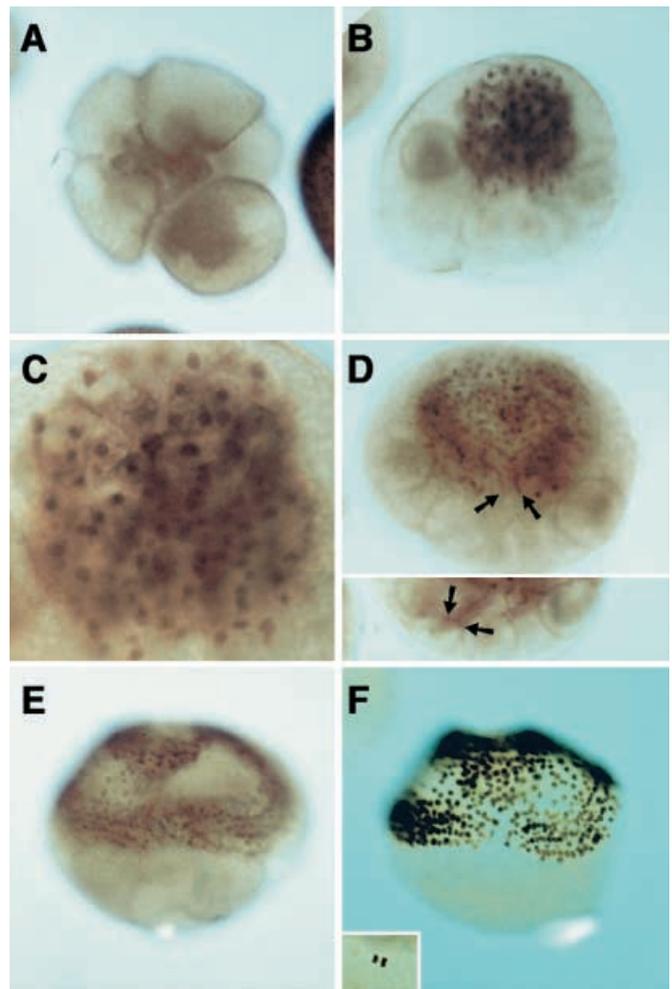
Fig. 3A–F HRO-DL protein localization in early embryos (dorsal views unless otherwise indicated). **A** Weak teloplasmic staining at stage 4b. **B** Micromere nuclei staining at stage 6b. **C** Higher magnification view of embryo in **B**. **D** Micromeres and lines of blast cells stain at stage 7 (and early stage 8; *inset*). *Arrows* mark some of the earliest-staining blast cell nuclei. **E** Blast cells, their derivatives, and micromeres stain at early stage 8. **F** Similar stage as **E**, stained with an anti-histone antibody to show the locations of nuclei at this stage. *Inset* shows a macromere nucleus from another embryo stained with an anti-histone antibody. Scale: embryos are about 400 μm in diameter

Localization of the *HRO-DL* and *HRO-SNA* proteins in embryos

To begin to understand how *Hro-dl* and *Hro-sna* genes might function in development, we examined where the proteins encoded by these genes localize in developing embryos. We made his-tagged versions of our fragment of HRO-DL and of a highly conserved fragment of HRO-SNA1 (see Materials and methods). Highly conserved sequences were chosen to maximize the chance of recognizing all leech Dorsal-class and Snail-class proteins with the antibodies, although it is not yet clear whether the antibodies do in fact recognize multiple Dorsal-class or Snail-class proteins. Each expressed protein
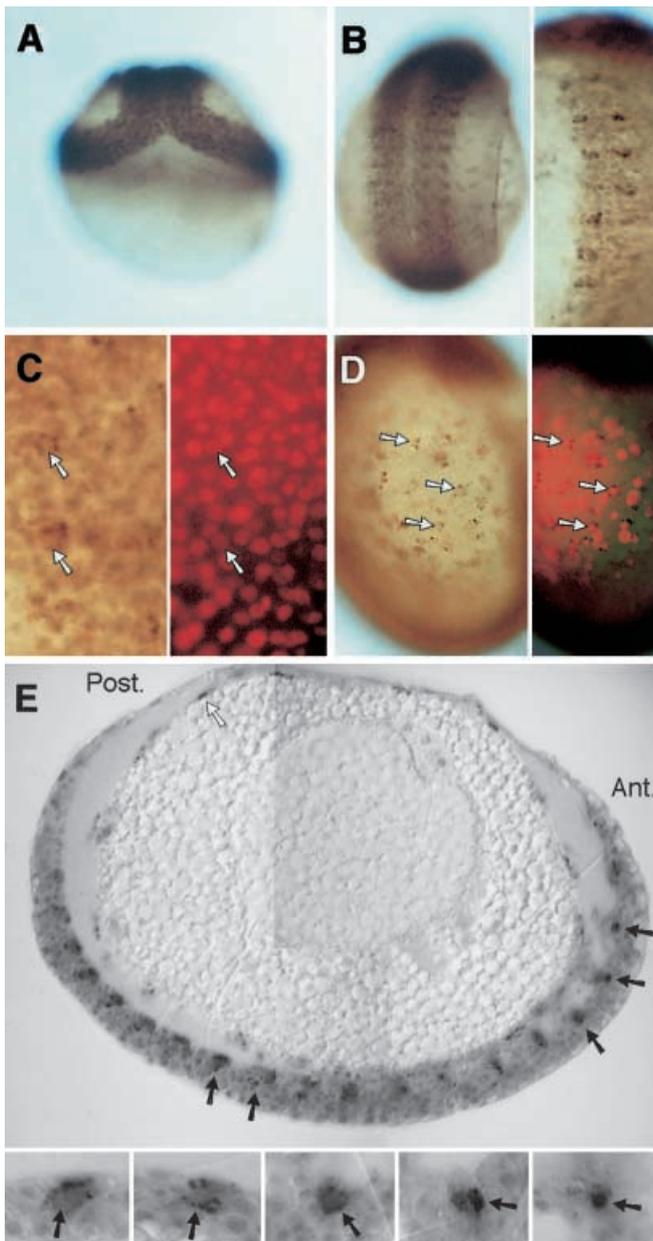
Fig. 4A–E HRO-DL protein localization in later embryos (dorsal views unless otherwise indicated). **A** All blast cell derivatives show nuclear staining at mid-stage 8 (ventral view). **B** Stripes of staining appear in stage 9, as staining of nuclei of all blast cell derivatives fades (ventral view). *Inset* shows an enlarged ventral view of another embryo at the same stage. **C** Cells of midbody stripes at stage 9 show cytoplasmic staining (*left* is anti-HRO-DL, *right* is anti-histone of same embryo; *arrows* point to the same nuclei, left and right). **D** Squamous epithelial cells of the provisional integument at stage 9 show cytoplasmic staining (lateral view; *left* is anti-HRO-DL, *right* is anti-histone of same embryo with anti-HRO-DL image overlain; *arrows* point to the same nuclei, left and right). **E** Sagittal section of a stage 9 embryo, with ventral *down* and the anterior (*Ant.*) and posterior (*Post.*) labeled, showing nuclear staining in anterior stripes (*two black arrows at right*), cytoplasmic staining in posterior stripes (*two black arrows at left*), and intermediate nuclear/cytoplasmic staining in one transitional stripe (the *remaining black arrow*). *Insets* show views of other sections of the same embryo, illustrating, from left to right: cytoplasmic staining, cytoplasmic staining, intermediate nuclear/cytoplasmic staining, two nuclei staining, and one nucleus staining. The anteri-

was used to make rabbit polyclonal antisera, which were then affinity-purified. Both affinity-purified antibodies were found to recognize their antigens much better than they recognize unrelated proteins (approximately 1,000-fold higher affinity for anti-HRO-DL and approximately 100-fold higher affinity for anti-HRO-SNA, by testing antibodies on protein dot blots; data not shown). This, together with the parallels between the expression patterns for these proteins and homologs in other organisms (see below), suggests that our immunostaining experiments reveal the pattern of Dorsal- and Snail-class proteins in leech development.

Embryos of various stages were immunostained with each antibody using HRP-conjugated secondary antibodies. All embryos were co-stained with anti-histone antibody using a fluorescent secondary. Regions with high levels of HRP showed no fluorescence as expected (as the HRP reaction product blocks fluorescence), but double-staining allowed us to determine conclusively whether areas that were not stained with anti-HRO-DL or anti-HRO-SNA included nuclei. Anti-HRO-DL staining weakly marked all teloplasm during stages 1 to 6a (Fig. 3A). No exclusion or enhancement of staining was seen at nuclei. We cannot exclude the possibility that this may represent a background level of binding of the antibody to unrelated proteins. Nuclear staining above this level was first seen in micromeres. At stage 6b, all micromeres consistently showed high levels of nuclear HRO-DL (Fig. 3B, C). In most embryos of earlier stages, no enhancement of nuclear staining was visible, but in a small percentage of embryos, one or more nuclei stained during stages 4b–6a. Staining of all micromere nuclei remained visible throughout stage 7.

No other nuclei showed enhanced levels of HRO-DL until blast cells were first born, in stage 7. The most recently born blast cell produced by each of the ten teloblasts generally showed intermediate levels of nuclear HRO-DL, along with the next few older blast cells, and all other blast cell nuclei (both mesodermal and ectodermal) consistently showed high levels of HRO-DL (Fig. 3D, E). Teloblast nuclei had levels of HRO-DL near the cytoplasmic background levels, suggesting that blast cells start producing high levels of HRO-DL protein soon after they are born. Staining of all blast cell nuclei and the nuclei of blast cell derivatives continued throughout stage 8 and began to decrease in level in stage 9 (Fig. 4A).

As HRO-DL levels in all nuclei of blast cell derivatives decreased by stage 9, segmentally iterated stripes of HRO-DL staining began to appear Fig. 4B, C. The stripes of increased staining appeared only in the cytoplasm, in cells in the lateral parts of the germinal plate.

or-posterior level of each inset, from left to right, matches roughly with the anterior-posterior level indicated by each of the five black arrows in the whole section above, from left to right. Some cells at the surface of the yolk also show strong staining (*white arrow*). Scale: embryos are about 400 μm in diameter
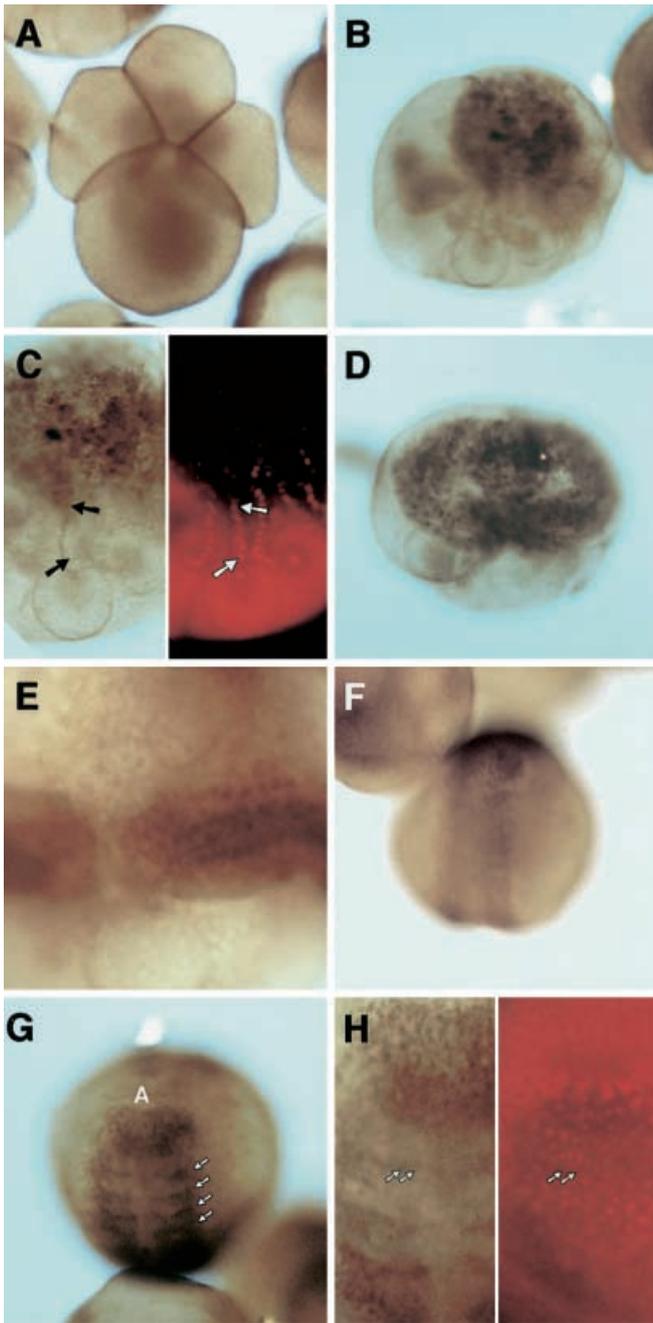
Fig. 5A–H HRO-SNA protein localization (dorsal views unless otherwise indicated). **A** Weak teloplasmic staining at stage 3. **B** Micromere nuclei stain at stage 7. **C** Enlarged view of embryo in **B**, showing anti-HRO-SNA at *left* and anti-histone at *right*. *Arrows* mark first blast cell nucleus (*bottom pair*) and first blast cell nucleus with noticeable HRO-SNA staining (*top pair*). Note that the line seen in the HRO-SNA panel to the left of the row of nuclei is the edge of a row of blast cells, not nuclei. **D** Nuclei of micromeres, blast cells, and blast cell derivatives stain at early stage 8. **E** Blast cell nuclei at mid stage 8. **F** Staining of nuclei of blast cell derivatives fades by stage 9, except nuclei in stripes in anterior (**G**), where HRO-DL is becoming nuclear (see Fig. 4). "*A*" marks anterior end of germinal plate; *arrows* point to four stripes of nuclear staining. **H** Enlarged view of embryo in **G**, showing anti-HRO-SNA at *left* and anti-histone at *right*. *Arrows* point to two nuclei of HRO-SNA stripe, and the absence of anti-histone staining nuclei in the area, as fluorescence is presumably quenched by

the HRP reaction product. Embryos in **G** and **H** are the same age (from same clutch) as are all of the stage 9 embryos in Fig. 3. Scale: embryos are about 400 µm in diameter

These cells have not yet been identified, but they clearly lay lateral to the developing nervous system. The cytoplasmic staining in these cells was punctate, similar to that observed previously for a *Xenopus* Dorsal homolog (Bearer 1994), suggesting that HRO-DL may be compartmentalized within vesicles in the cytoplasm of these cells. Exclusively cytoplasmic staining was also found in squamous epithelial cells of the provisional integument (Fig. 4D). Since the segments develop in an anterior-to-posterior temporal gradient, advanced stages of segment development can be seen by examining more anterior segments. Strikingly, staining was found to shift from cytoplasmic to nuclear localization in older (more anterior) stripes (Fig. 4E). The location in each segment where each stripe of cytoplasmic staining originates appears to be where the septa between coelomic spaces will later form, as the stripes of nuclear staining in the anterior were found to mark the ventral end of each septum (Fig. 4E).

At no point between stages 1 and 8 do any other nuclei, including for example all of the nuclei that contribute to the endoderm, stain for HRO-DL, despite these nuclei being accessible to immunostaining in general, as evidenced by the ease of marking them with even minimal concentrations of anti-histone antibody (Fig. 3F, inset). At stage 9, some cells at the surface of the yolk showed strong anti-HRO-DL staining (Fig. 4E).

The HRO-SNA staining pattern (Fig. 5) appeared identical to the HRO-DL staining pattern except in two respects. First, blast cell nuclei did not show high levels of HRO-SNA until several hours later than they showed high levels of HRO-DL (Fig. 5C). This could reflect a later accumulation of HRO-SNA, as might be expected if *Hro-sna* is a transcriptional target of HRO-DL, or it could simply reflect a lower affinity of the HRO-SNA antibody for its target antigens. Second, HRO-SNA staining did not appear in stripes of cells in the germinal plate until HRO-DL entered nuclei in stripes of cells (Fig. 5F–H). From the time they appeared, the stripes of nuclear HRO-SNA staining were similar in position to the stripes of HRO-DL nuclear staining, suggesting that the cells with high levels of nuclear HRO-DL acquire high levels of nuclear HRO-SNA as well, although we have not conclusively determined whether HRO-DL and HRO-SNA are at high levels in the same nuclei. We note that the entire HRO-DL and HRO-SNA embryonic localization patterns are consistent with the possibility that HRO-DL activates expression of *Hro-sna*, as is the case for *Drosophila* Dorsal and Snail (Belvin and Anderson 1996; Rusch and Levine 1996).

Expression patterns of several other developmental genes have been examined in glossiphoniid leech embryos, including homologs of *engrailed* (Wedeen and Weisblat 1991), *wingless* (Kostriken and Weisblat 1992),

msx (Master et al. 1996), *twist* (Soto et al. 1997), *nanos* (Pilon and Weisblat 1997), *hunchback* (Iwasa et al. 2000) and a large number of Hox genes (see Kourakis et al. 1997 for review). Efforts toward developing methods to test the functions of such genes in leech development by gene inactivation (see Baker and Macagno 2000, for example) or by ectopic gene expression (see Pilon and Weisblat 1997, for example) are ongoing. If HRO-DL directly activates the expression of *Hro-sna*, these genes could be useful for testing such methods, since disrupting *Hro-dl* function or ectopically expressing *Hro-dl* would give simple predictions for the effects on the distribution of a downstream gene product.

How might *dorsal-* and *snail-*class genes function in leech embryos? The expression patterns suggest some hypotheses. Fly *dorsal* and *snail* genes function in specification of the dorsoventral axis and the mesoderm. The expression pattern of *Tribolium castaneum-dorsal* suggests that beetles may use a Dorsal-class protein similarly (Chen et al. 2000). The fly *snail* gene additionally functions in nervous system development (Ashraf et al. 1999), and *dorsal* additionally functions in innate immunity (Belvin and Anderson 1996; Rusch and Levine 1996; Manfruelli et al. 1999). There is no evidence for any of these functions in leech development. It is unlikely that the HRO-DL and HRO-SNA proteins act in dorsoventral axis specification, since neither show patterns of localization consistent with this possibility; for example, neither appear to be localized in a pattern expected for a spiralian dorsoventral determinant. It remains possible that they function in mesoderm specification, although since the proteins are expressed in both mesodermal and ecotodermal precursors, this seems unlikely. The results suggest that the role of Dorsal-class proteins in specification of the dorsoventral axis and the mesoderm might have been present in the common ancestor to leeches and flies and was lost in the lineage leading to leeches, or it might not have been present in the common ancestor and was gained in the lineage leading to flies. Preliminary tests of an innate immunity function in leeches – determining whether bacterial infection can cause cytoplasmic HRO-DL to become nuclear as Dorsal does in flies (B. Goldstein, unpublished work) – have produced negative results. We have not seen specific HRO-SNA expression in the nervous system. We conclude therefore that none of the developmental functions known for these genes in flies have been found yet in leeches. Widespread expression appears transiently in leech development, in all the micromeres and teloblast derivatives between stage 6b and late stage 8. How these genes might function in these cells, or whether they have a function during this period, remains unclear.

The most suggestive parts of the leech expression patterns are the stripes of nuclear staining that arise during segmentation: this pattern raises the possibility that these genes are part of a genetic pathway that, instead of specifying the dorsoventral axis and the mesoderm as in flies, might play a role in the diversification of cell types within segment primordia during leech development. Seg-

mentally iterated stripes of staining have also been seen in *Xenopus* embryos for the *dorsal* homolog *Xrel1* (Bearer 1994). With the caveat in mind that we do not know if or how these striped patterns of Dorsal-class proteins might function in either leech or *Xenopus* embryos, these results at least raise the possibility that this may be a feature of a Dorsal pattern that was present in the ancient urbilaterians but was lost in the lineage leading to flies. Alternatively, Dorsal-class proteins might not have been expressed in iterated stripes in the common ancestor to these organisms, and might have independently acquired at least superficially similar patterns in the lineages leading to the leeches and frogs. *Snail*-class genes have been proposed to have an ancestral function in cell migration (Hemavathy et al. 2000); whether HRO-DL activates *snail* in a segmentally iterated population of cells which will later migrate remains to be determined.

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