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Original Article

Expression of actin mRNA in embryos of the leech *Helobdella triserialis*

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ABSTRACT A cDNA clone (Htr-actin) containing a 1.48 kb insert corresponding to actin was isolated from a stage 10-11 *Helobdella triserialis* cDNA library by cross hybidization to a *Drosophila melanogaster* actin clone. The cDNA is equivalent in size to the adult actin mRNA detected by Northern analysis. Genomic Southern blot analysis revealed that Htr-actin is a single copy gene and is one member of a family of actins in *H. triserialis*. Protein sequence comparisons revealed that Htr-actin was most similar to actin-1 of the earthworm and like other invertebrate actins, Htr-actin was found to be more similar to mammalian cytoplasmic actins than to mammalian muscle-specific actins. *In situ* hybridization revealed that at early stages the actin mRNA was distributed primarily in the yolk-deficient cytoplasm of all cells. At later stages, elevated levels were detected over the germinal bands and plate. Following muscle formation, a subset of the actin mRNA expression pattern resembled the immunostaining pattern obtained with the muscle specific antibody, Lan3-14. The Htr-actin transcript was undetectable in the segmental nervous system. These studies lay the groundwork for any future ectopic gene expression studies using the Htr-actin promoter in the leech embryo.

KEY WORDS: *actin, leech, Helobdella triserialis, in situ hybridization*

Introduction

Actins are highly conserved eukaryotic proteins associated with essential cell functions such as maintenance of cytoskeleton, cell motility, cell division, and contraction of muscle cells. In chordates, there are at least six different actin isoforms which have been classified as either muscle-type or cytoplasmic-type (Vandekerckhove and Weber, 1978,1979a,b,1981). These isoforms are encoded by actin gene families. In humans there are at least 25 actin cross-hybridizing regions in the genome however most of these are likely to be pseudogenes (Ng *et al.*, 1985). Actin gene families are also present in most invertebrates, ranging in size from four to seventeen members (Zechel and Weber, 1978; Fyrberg *et al.*, 1980,1983; Files *et al.*, 1983; Vandekerckhove and Weber, 1984; Fisher and Bode, 1989; Adam *et al.*, 1991) but some fungi and protists contain single actin genes (Uyemura *et al.*, 1978; Gallwitz and Seidel, 1980; Hirono *et al.*, 1987; Mertins and Gallwitz, 1987; Fidel *et al.*, 1988; Dudler, 1990).

Following the vertebrate nomenclature, in most invertebrates, actin isoforms are classified as either cytoplasmic- or muscle-specific. It is likely that both forms evolved from a cytoplasmic actin in a common ancestor to vertebrates and invertebrates. Several lines of evidence support this notion. First, the sequences of both forms are more similar to the cytoplasmic- than to the muscle-type actins of vertebrates (Vandekerckhove *et al.*, 1983). Second, protist actins are more similar to the vertebrate cytoplasmic-type actin. Third, muscle-specific actins in insects are different from both cytoplasmic- and muscle-type vertebrate actins at key amino acid positions. Thus it is likely that these muscle-specific actins arose independently in the arthropod line (Mounier *et al.*, 1992).

Among protostomes, spatial and temporal patterns of actin gene expression have been best characterized in the arthropods, *Drosophila* (Fyrberg *et al.*, 1980,1983; Tobin *et al.* 1990) and *Artemia*. *Drosophila* contains four muscle isoforms and two cytoplasmic isoforms. *Artemia* has 8-10 actin genes (Macias and Sastre, 1990). Of these, a single muscle isoform and three cytoplasmic isoforms have been characterized (Ortega *et al.*, 1992). As their name implies, muscle-specific actins are restricted to muscle tissue. However, when more that one isoform is present, each can be expressed in a subset of the musculature and these subsets often overlap. While cytoplasmic actins are named for their uniform expression throughout the organism, at

Abbreviations used in this paper: Htr, *Helobdella triserialis;* Hro, *Helobdella robusta;* Dm, *Drosophila melanogaster.*

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A

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GACGAAGACGTTACTGCCCTAGTTGTCGACAACGGCTCAGGTCTCTGCAAGGCCGGGTTT   63
 D E D V T A L V V D N G S G L C K A G F   21

GCCGGAGATGATGCCCCCAGAGCTGTTTTCCCATCAATTGTTGGACGTCCCCGTCATCAG  123
 A G D D A P R A V F P S I V G R P R H Q   41

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 G V M V G M G Q K D S Y V G D E A Q S K   61

AGAGGTAGCCTAACTCTCAAATACCCAATTGAGCACGGTATCGTCACAAACTGGGACGAT  243
 R G S L T L K Y P I E H G I V T N W D D   81

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 M E K I L H H T F Y N E L R V A P E E H  101

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 P V L L T E A P L N P K A N R E K M T Q  121

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 T V P I Y E G Y A L P H A I L R L D L A  181

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B

actin gene	amino acid position 2	3	4	5	6	10	16	17	76	89	103	129	153	162	176
leech	D	E	D	V	T	V	L	C	V	T	V	S	L	T	L
sea urchin (Cy1)	-	D	-	-	-	I	M	V	-	-	-	-	F	-	-
scallop (muscle)	-	D	E	-	-	-	M	-	-	-	-	A	-	-	-
Hydra	-	D	E	-	A	-	M	-	-	-	L	-	-	-	I
C. elegans (gene 1)	-	D	E	-	A	-	M	-	-	-	-	T	-	-	-
mammalian (β-cyto)	-	D	-	I	A	-	M	-	-	-	-	T	M	-	-
mammalian (skel.)	E	D	E	T	-	C	-	V	I	-	T	V	L	N	M

actin gene	amino acid position 201	225	259	266	271	278	286	296	298	357	366	number of identical amino acids
leech	T	Q	S	L	A	F	V	T	M	S	S	
sea urchin (Cy1)	-	-	A	-	-	Y	V	-	-	-	-	18
scallop (muscle)	-	N	-	-	-	Y	-	-	-	-	-	20
Hydra	-	E	E	I	-	-	-	-	L	-	-	16
C. elegans (gene 1)	-	-	A	-	-	Y	I	-	L	-	-	17
mammalian (β-cyto)	-	-	A	-	C	-	-	-	L	-	-	17
mammalian (skel.)	V	N	T	I	-	Y	I	N	-	T	A	5

Fig. 1. Htr-actin cDNA nucleotide and translated amino acid sequence. **(A)** *The 1,484 nucleotide sequence of the Htr-actin clone (p9A.4) is shown with the ORF in capital letters and deduced amino acid sequence underneath. The sequence begins at the 5' end of the clone which corresponds to codon 2. The putative polyadenylation signal is indicated in bold and underlined and the polyA sequence is bolded.* **(B)** *Diagnostic amino acids for cytoplasmic- versus muscle-type actins are compared between Htr-actin, and a partial list of invertebrate actins [sea urchin (Cooper and Crain, 1982); scallop, (Vandekerckhove and Weber, 1984); hydra (Fisher and Bode, 1989); C. elegans (Krause et al., 1989)], and mammalian b-cytoplasmic and a-skeletal isoforms (Vandekerckhove and Weber, 1979b). Amino acids identical to those of Htr-actin are indicated as hyphens. The number of amino acids among this diagnostic group that are identical to those of Htr-actin is indicated to the right of each sequence.*

some time during development they are generally found at high levels in unique, non-muscle, tissue-specific patterns.

We have isolated and characterized an actin cDNA from an annelid, the leech, *Helobdella triserialis*. DNA sequence analysis reveals that, like other invertebrate actins, this actin more closely resembles vertebrate cytoplasmic- than muscle-type actins. To characterize the expression pattern of the mRNA, we have done *in situ* hybridization with antisense RNA probes on various stages of *H. triserialis* embryos. Unlike the expression patterns, restricted to cytoplasm or to muscle in other protostomes, our probe detects transcripts in the cytoplasm of all cells at early stages and transcripts in muscle and other tissue at later stages of development.

Results

Sequence analysis

The 1,484 nucleotide sequence of the Htr-actin cDNA clone is shown in Figure 1A. This clone represents a nearly full length cDNA, missing any 5' untranslated region and probably the first few codons, although it is uncertain exactly how many, if any, codons are missing. The beginning of the Htr-actin clone aligns with most vertebrate actin sequences at a position corresponding to the second codon and with most invertebrate actin sequences at a position corresponding to the third codon, just behind the Met-Cys sequence that is found at the amino terminal of most invertebrate actins. But, while many actin sequences have three acidic amino acids near their amino terminal, as does this cDNA (Asp-Glu-Asp), some actins have more than three acidic amino acids (Glu or Asp) at their N-termini. The length of the putative actin protein encoded by the cDNA is 375 amino acids. A 336 nt 3' untranslated region, excluding the 14 nt polyA sequence, is present. A polyadenylation signal, AATAAA, is located 18 nt upstream of the polyA sequence.

Classification of Htr-actin

A BLASTp search was performed on the translated Htr-actin sequence and identified an actin from another annelid, the earthworm, *Lumbricus terrestrius* (actin1; JC5227) as the best match, with 367/376 identical amino acids. In second place were three different actins with equal sequence similarity to Htr-actin, two from invertebrates and one from a vertebrate. These were an actin from the humus earthworm, *Lumbricus rubellus* (YO9623), an actin from the adductor muscle of the sea scallop, *Placopcten magellanicus* (U55046), and cytoplasmic type 5 actin from the chicken, *Gallus gallus* (P53478).

In addition to the sequence comparisons described above, in another type of comparison, Htr-actin more closely resembled vertebrate cytoplasmic- than muscle-specific actins. There are 27 key amino acid positions which are diagnostic of cytoplasmic- versus muscle-type actins (Collins and Elzinga, 1975). Invertebrate actins are more similar to the mammalian cytoplasmic actin at these diagnostic positions. As expected, comparison of the amino acid residues in these positions in the translated Htr-actin protein with those of other actins revealed greater similarity with mammalian cytoplasmic actins and invertebrate actins than with mammalian skeletal actin. For example, among the invertebrate actins selected, 16-20 out of 27 diagnostic amino acids (59-74%) were identical whereas only 5 out of 27 diagnostic amino acids (18%) were identical in Htr-actin and the mammalian skeletal muscle actin (Fig. 1B). In addition, the sequence of Htr-actin was compared with those of muscle-specific actins from *Drosophila*, *Bombyx mori*, .and *Artemia* (Mounier *et al.*, 1992). None of the muscle-specific residues in these arthropods was present in Htr-actin (not shown).

Genomic southern blot analysis

To address whether actin is present as a single copy or a gene family in the leech, an Htr-actin cDNA probe was hybridized to restriction digested genomic DNA of *H. triserialis* and *D. melanogaster*, using high and low stringency conditions (Fig. 2). The DNA was digested with EcoRI or HindIII. The recognition site for neither of these enzymes is present in the probe.

Under high stringency the Htr-actin probe hybridizes to a single 11.6 kb *Htr* EcoRI fragment (lane 1) and an *Htr* HindIII fragment larger than 23 kb (lane 4). It also hybridizes to a single 4.3 kb fragment of HindIII digested *Hro* DNA (lane 3). As expected, the probe does not detect any bands in the EcoRI digested (lane 2) or the HindIII digested (lane 5) *Dm* DNA. Thus, under high stringency conditions, the probe appears to recognize a single *H. triserialis* gene, since it hybridizes to single fragments in both EcoRI and HindIII digests. In addition, in the closely related leech species, *H. robusta*, the probe appears to detect a single actin gene, since it hybridizes to one HindIII fragment.

Under low stringency, the bands described above are intensely labeled, in addition to a second intensely labeled *Htr* EcoRI fragment of 9.5 kb which appears as a doublet with the 11.6 kb band (lane 1), and a second *Htr* HindIII fragment (lane 4) of 8.5 kb. Additional faint bands are observed in all lanes, including those containing *Dm* DNA. The molecular weights of those bands detected in the *Dm* HindIII digest (lane 5) correspond to the expected sizes for restriction fragments containing the six genes in the *Drosophila* actin family (Fyrberg *et al.*, 1980).

These results reveal that an actin gene family is likely to be present in *Helobdella*. As expected, the bands detected with high stringency conditions are also labeled intensely using low stringency conditions. The additional, equally intensely labeled bands observed in the EcoRI and HindIII digests of *Htr* DNA, indicate that there is potentially one other actin gene in *H. triserialis*, closely related to Htr-actin. The faintly labeled bands in each of the digests indicate that a number of more distantly related genes probably also compose the leech actin gene family.

Northern analysis

H. triserialis actin RNA was characterized by Northern analysis. When hybridized to adult *H. triserialis* polyadenylated RNA, the Htr-actin probe detects a single band of approximately 1.5 kb, the same size as the Htr-actin cDNA (Fig. 2C). Thus, in the adult, we do not detect multiple transcripts in the form of different sized actin RNA species, as have been described for the cytoplasmic actin, 5C, transcripts in *Drosophila* development (Burn *et al.*, 1989). However the presence of several actin mRNAs of indistinguishable length cannot be ruled out.

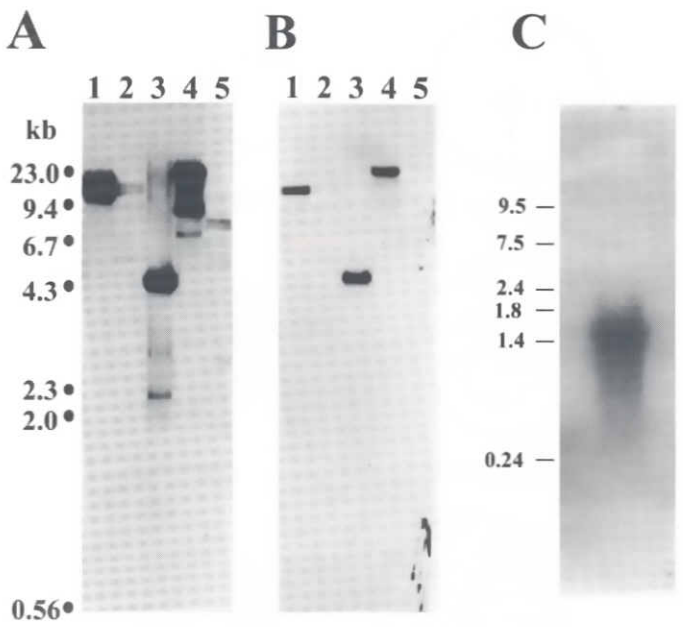

Fig. 2. Genomic southern and northern blot analysis. (A and B) *Genomic Southern blots were prepared as described in Materials and Methods. The blot shown in (A) was hybridized using low stringency conditions; (B) was hybridized using high stringency. Molecular weights of the marker for both blots (HindIII digested lambda DNA) are indicated in the left margin of (A). Lanes 1 and 2 contain EcoRI digested Helobdella triserialis (Htr), and Drosophila melanogaster (Dm) DNAs, respectively. Lanes 3-5 contain HindIII digested Helobdella robusta (Hro), Htr, and Dm DNAs, respectively.* **(C)** *Northern blot. The Htr-actin probe was hybridized to a blot containing adult poly-A RNA, identifying a diffuse band of approximately 1.5 kb. The positions of the molecular weight markers are indicated in the left margin.*

Actin mRNA expression during development

The expression pattern of Htr-actin was determined from the earliest developmental stages when the embryo consists of large yolky blastomeres through the time when muscle development is underway. A partial summary of *Helobdella* development, from stages 1 through 9, is presented in Figure 3.

The distribution of actin transcript was examined in embryos at stages 1-10 using an ^{35}S- labeled antisense Htr-actin probe (Materials and Methods; Fig. 4). As controls, embryos from each batch were hybridized in parallel to sense and antisense probes generated from a *Helobdella triserialis* Elongation Factor 1 alpha (EF1α) cDNA (R. Streck, unpublished; data not shown). Sections of embryos hybridized with EF1α sense probe showed uniform low background, indicating that the signal observed with the Htr-actin probe is not due to nonspecific sticking of probe. Sections of embryos hybridized with the EF1α antisense probe revealed patterns resembling those observed with the Htr-actin antisense

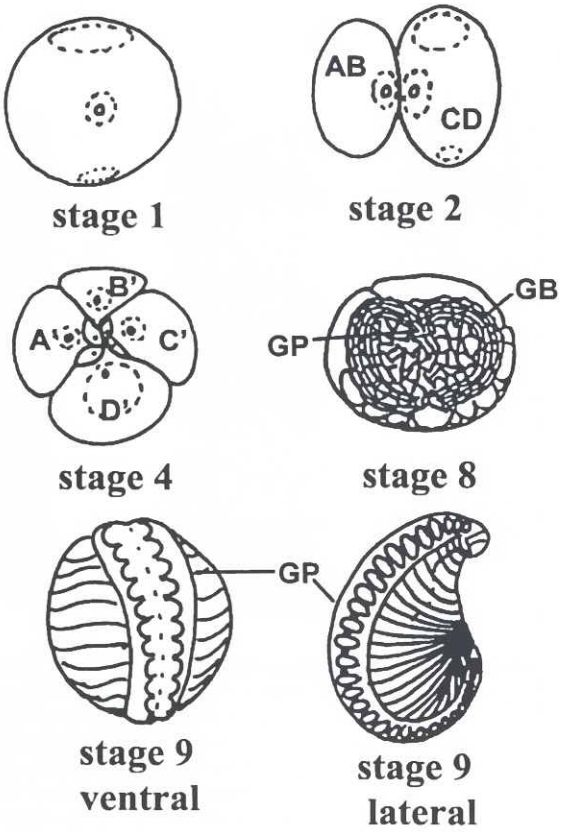

Fig. 3. Leech development. *Diagrammatic views of embryos at progressively increasing stages are shown. Stages 1 and 2 are shown as meridional sections through the center of the embryo. Stages 4 and 8 are viewed from the animal pole. Ventral and lateral views are shown for stage 9. Two pools of yolk-free cytoplasm , or teloplasm (areas designated by dashed lines) are situated at the animal (upper) and vegetal (lower) poles of the stage 1 (single cell) embryo. In addition, an area of yolk-free cytoplasm, or periplasm (also designated by dashed lines), surrounds the nucleus. At stage 2, the embryo consists of two cells, a smaller AB cell and larger CD cell. Both animal and vegetal pools of teloplasm are distributed primarily to the CD cell. Periplasm also surrounds the nuclei of AB and CD. Cells AB and CD divide to form the macromeres A, B, C, and D (not shown). In this animal pole view of a stage 4 embryo, the macromeres have divided producing A', B', C', and D' and one micromere each, a', b', c', and d' (at the center, unlabeled). At this stage, most of the teloplasm has been inherited by cell D'. Cell D' gives rise to most of the segmental tissues. By stage 8, it has produced two sets of 5 teloblasts (bottom of embryo) each of which divides repeatedly giving rise to a series of blast cells arranged in a bandlet. The five bandlets on each side of the embryo adjoin to form a germinal band (GB). The two germinal bands begin to fuse anteriorly at the ventral midline to form the germinal plate (GP). Division of the micromeres and the mesodermal teloblast gives rise to the provisional integument layer, a temporary layer, composed of a provisional epithelium and an underlying provisional muscle layer. The provisional integument lies over the germinal bands and plate and the area surrounded by them and is eliminated by the time of dorsal closure at stage 10. (Outlines of the provisional epithelial cells are shown.) In both views of the stage 9 embryo, anterior is up. By stage 9 the formation of the germinal plate (GP) is complete. In the ventral view (bottom left), neurogenesis is apparent, proceeding in an anterior to posterior direction along the ventral midline (dotted line). Lateral to the germinal plate, the provisional muscle component of the provisional integument is shown. In the lateral view (bottom right), developing segmental ganglia are shown in the germinal plate. As for the ventral view, the provisional muscle layer, but not the provisional epithelium is shown lateral to the germinal plate.*

probe at early stages, although with heavier accumulation of silver grains. By stage 10 the Htr-actin pattern was distinct from that of EF1α, which was present at high levels in all tissues (see below).

Preceding and during the early cleavage stages, mRNA was detected at proportionately high levels in areas of yolk-deficient cytoplasm. At stage 1, the single cell embryo, transcript was detected predominantly in the perinuclear cytoplasm and in the teloplasm. The yolk deficient cytoplasm near the animal hemisphere, i.e., the animal teloplasm, can be seen clearly in the section shown in Figure 4,A,B; however, transcript was also present in the vegetal teloplasm (not shown). The density of grains over the teloplasm, perinuclearplasm, and the yolk was compared in representative sections. The number of grains in a 1 μm^2 area over the teloplasm and an equivalent sized area of the perinuclearplasm was roughly identical, with the ratio of grains over the teloplasm to grains over the perinuclearplasm equal to 0.95±0.15. (See Materials and Methods.) However, using the same method of counting grains, the levels were roughly 2-3 fold higher over the teloplasm relative to over the yolk. The grain density ratio of the teloplasm to the yolk was 2.5±0.6.

At the two-cell stage (Fig. 4,C and D) silver grains were disproportionately found over the yolk-deficient cytoplasm of both cells AB and CD. The figure shows a section through the perinuclear cytoplasm, however, equally high levels were observed over the teloplasm at this stage. Similarly, at stage 4 (Fig. 4,E and F), transcript was detected in yolk- deficient cytoplasm, with especially high levels in the teloplasm of cell D'.

Later, during stage 8 (Fig. 5,A and B), with the formation of the segmental ectoderm and mesoderm well underway, higher levels of transcript were detected throughout the embryo relative to earlier stages but a particularly high density of silver grains was observed over the germinal bands (and plate; not shown). The stage 8 embryos were mounted in blocks side-by-side with stage 1 embryos and sections were mounted onto the same slide so that the relative levels of probe hybridization could be compared, controlling for variability in emulsion thickness and potential differences in developing conditions. Based on grain counts, the probe hybridized at a 2.6±0.7 fold higher level in the yolk of the stage 8 embryo than in the yolk of the stage 1 embryo. Levels of Htr-actin over the germinal plate of the stage 8 embryo were 6.0±0.8 fold higher than those over the teloplasm of the stage 1 embryo. And within a single stage 8 embryo section, the grain density was roughly 5-7 fold higher over the germinal plate than over the yolk (i.e., the ratio of grain density over the germinal plate to that over the yolk was 6.2±1.1).

During stage 8, elevated levels of Htr-actin transcript were also detectable in the provisional integument layer. Due to the long track length from ^{35}S particle emission, it was not within the resolution of this method to determine whether the transcripts were expressed in both layers of the provisional integument or were restricted to either the provisional epithelium layer or the underlying provisional muscle layer. However, hybridization of digoxigenin labeled probe to stage 8 and stage 9 embryos revealed a pattern of transversely arranged strands in the provisional integument that was reminiscent of the provisional muscles. Thus, the pattern is consistent with the transcript being restricted to the muscle layer of the provisional integument. In addition, *in situ* hybridization revealed a segmentally iterated

pattern in the germinal plate which may have partially corresponded to mesodermally- derived circular muscles (Figs. 5C-E; 6A,B). In some cases these strands appeared to cross the midline (particularly apparent in-between the anterior-most and the next posterior, adjacent segment shown in Fig. 6B) and probably corresponded to the *primary circular fibers*, the first circular muscle fibers to form (Stent *et al.*, 1992). The pattern of Htr-actin hybridization resembled the pattern of muscle cells in the developing body wall and provisional integument labeled by injections of fluorescent tracers into the M blastomere (Torrence *et al.*, 1989; Gleizer and Stent 1993). And to some degree the pattern also resembled that obtained by immunostaining with a muscle-specific antibody, Lan3-14, which reveals the muscles of the provisional integument, the longitudinal musculature beginning to form surrounding the nervous system, and the primary circular fibers in the germinal plate (Zipser and McKay, 1981; Stent *et al.*, 1992; Fig. 6C). However, the probe hybridized to additional tissues in the germinal plate, not recognized by the Lan3-14 antibody.

The Htr-actin probe hybridized to a variety of different tissues, including muscle. However, the probe did not hybridize to the segmental ganglia of the central nervous system, as evidenced by the absence of staining in the area surrounding the ventral midline in whole-mounted embryos (Fig. 6A,B) and by the absence of silver grains over transverse sections through ganglia (Fig. 5F,G).

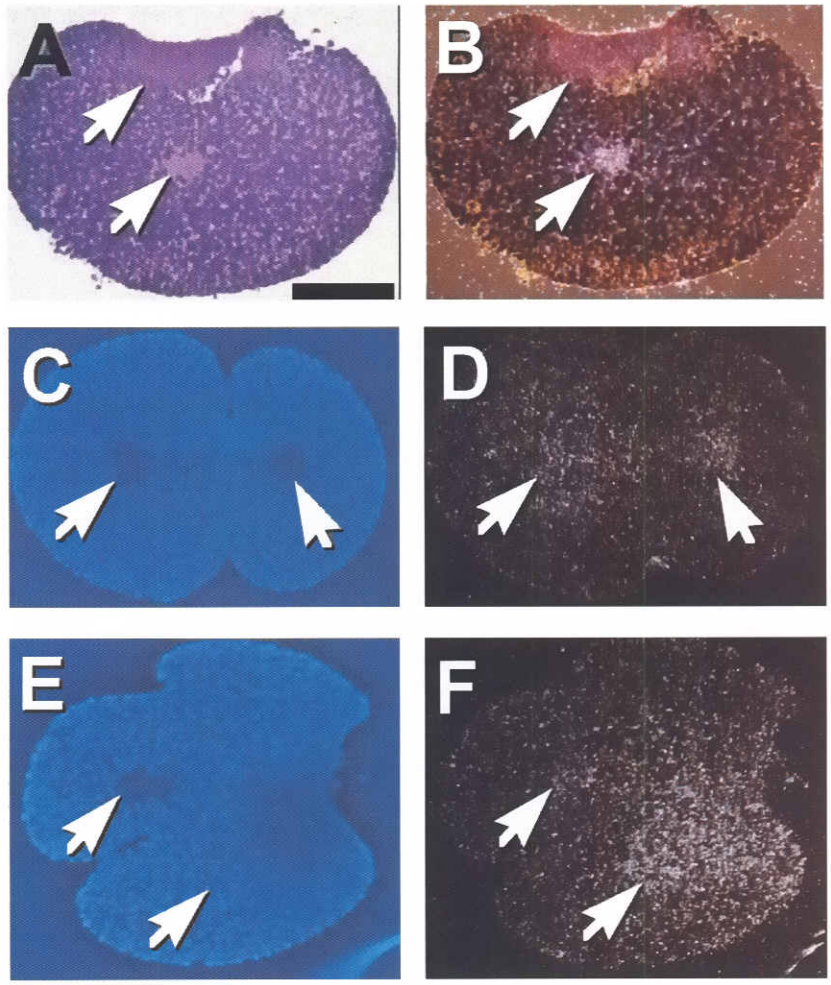

Fig. 4. Expression of Actin mRNA in early embryos as revealed by ^{35}S *in situ* hybridization. *Sections through stage 1 (near meridional), stage 2 (horizontal), and stage 4 (transverse) embryos hybridized with ^{35}S-labeled Htr-actin antisense RNA probe. The yolk of the stage 1 embryo section was counterstained with Toluidene Blue while stage 2 and 4 sections were counterstained with Hoechst 33258. The arrow in bright* **(A)** *and dark* **(B)** *field views of a stage 1 embryo section indicates silver grains concentrated over the perinuclear region. Likewise, the arrows in fluorescent* **(C)** *and dark field* **(D)** *views indicate a higher concentration of silver grains over the perinuclear regions of cells AB (right) and CD (left) in a stage 2 embryo section. The arrows in fluorescent* **(E)** *and dark field* **(F)** *views of a stage 4 embryo section point to a higher density of silver grains over the teloplasm of cell D (lower arrow) and that of cell C (upper arrow). Bar in A, 100 μm.*

Discussion

We have isolated and sequenced a nearly full length actin cDNA from a stage 10-11 *Helobdella triserialis* cDNA library. The cDNA is encoded by a single copy gene, Htr-actin, which in the adult is expressed as a single species of mRNA. Htr-actin appears to be a member of a family of leech actins containing at least one other closely related member and some additional, more distantly related members. By the methods used, we cannot tell whether these are expressed or if any members of the family are pseudogenes. However the absence of Htr-actin expression in the nervous system implies that at least one other actin isoform may be expressed there. Comparison of the translated Htr-actin amino acid sequence to other sequences in GenBank reveals a close similarity to an actin from another annelid, the earthworm. Comparison of the Htr-actin sequence to those of vertebrate actins reveals that, as for actins from other invertebrates, Htr-actin is more similar to vertebrate cytoplasmic- than to muscle-type actin.

Based on its expression pattern, Htr-actin cannot be categorized exclusively as a cytoplasmic or a muscle-specific actin since at different developmental stages it is found at relatively high levels cytoplasmically, in muscles, and in other cell types. Htr-actin transcripts are first detected in the yolk-deficient cytoplasm of the early blastomeres. By stage 8, transcript levels have increased significantly and transcripts are detectable in several tissue types, including ectoderm, mesoderm, and the provisional integument. Later, the Htr-actin *in situ* hybridization pattern was compared to the immunostaining pattern of the muscle-specific antibody, Lan3-14, revealing transcript in the circular muscles, in the temporary muscle cells of the provisional integument and in additional cells in the germinal plate. However, Htr-actin appears to be absent from the nervous system.

Since our results indicate that an actin gene family is likely to be present in *Helobdella*, we cannot definitively rule out the possibility that the Htr-actin probe used for *in situ* hybridization is detecting one or more additional, closely related actins. For example, it may

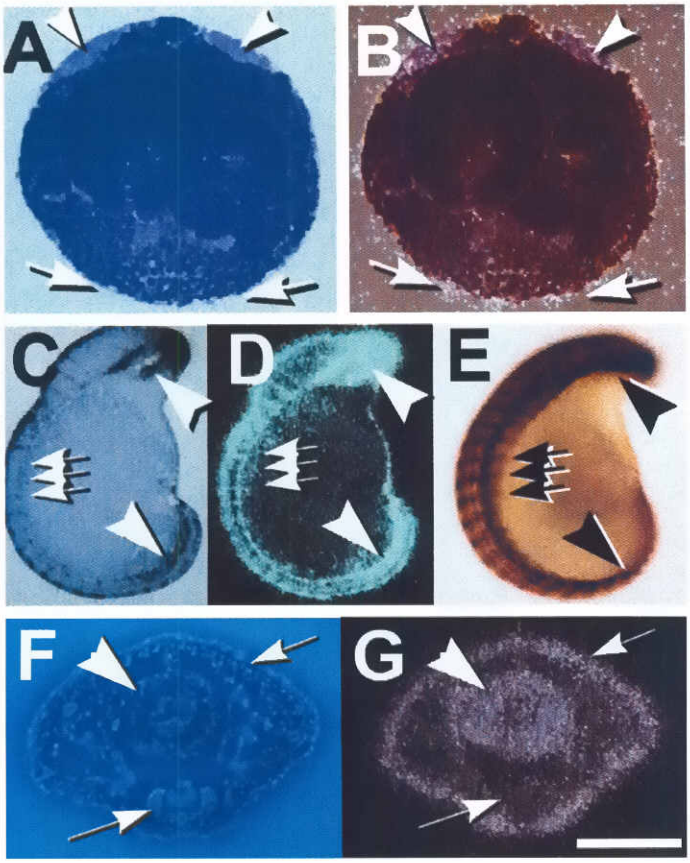

Fig. 5. Expression of Actin mRNA in late embryos as revealed by ^{35}S and digoxigenin *in situ* hybridization. *The arrow heads in bright field* **(A)** *and dark field* **(B)** *indicate high density of silver grains over the germinal bands of a stage 8 embryo section hybridized with an ^{35}S labeled probe. In addition, a high density of grains is observed over the micromere cap region between the arrows at the bottom of the embryo section shown in B. The stage 8 embryo section was counter stained with Toluidene Blue. Bright and dark fields of a parasagittal section through a stage 9 embryo hybridized with the ^{35}S labeled probe are shown in* **(C)** *and* **(D)**, *respectively. The arrowhead at the anterior end (top) indicates intense label around the pharynx. Segmentally iterated labeling occurs between each of the ganglia. Three arrows in the midbody region indicate such staining between four of the developing ganglia. Intense label is observed in broad segmentally iterated bands surrounding the neurogenic region at the posterior end in this section (bottom arrowhead points to one such band). The embryo in* **(E)** *is a whole-mount stage 9 embryo hybridized with a digoxigenin labeled antisense actin RNA probe. The hybridized probe is recognized with an HRP-antidigoxigenin antibody and detected by a DAB reaction. The pattern obtained by this method closely resembles results in C and D. The arrows designate the same regions designated in the section shown in C and D. Hoechst fluorescence and dark fields of a cross section through an anterior midbody segment of a stage 10 embryo hybridized with the Htr-actin antisense probe used in A-D are shown in* **(F)** *and* **(G)**, *respectively. Dorsal is up. Heavy accumulation of silver grains is observed over the ectodermal and mesodermal layers of the body wall (upper arrow) and the musculature surrounding the pharynx (arrowhead). The section passes through a ganglion of the ventral nerve cord (lower arrow), which is unlabeled by the probe. Bar in G, 100 μm; in A and B, 215 μm in C-E; and in F and G75 um.*

detect the transcripts from both a cytoplasmic gene and a muscle-specific gene. However, this is unlikely given the *in situ* hybridization conditions and washes. Although the hybridization temperature was well below the melting temperature for the probe, washes were done at high stringency and included an RNase step to remove any unhybridized probe. In addition, control hybridizations, done in parallel, using an *H. triserialis* EF1α sense probe gave no signal above background and the EF1α antisense probe revealed a hybridization pattern different from that obtained with Htr-actin antisense probe. Furthermore, developmentally regulated expression patterns of actin mRNAs have been observed before. For example, in *Drosophila*, transcripts from the cytoplasmic actins, Act5C and Act42A, accumulate in all cells of the blastoderm but by late embryogenesis are found at high levels in unique tissue specific patterns (Tobin *et al.*, 1990).

We found a single species of Htr-actin mRNA in the adult, which corresponded to the size of the Htr-actin cDNA cloned from the stage 9-11 library. Although not addressed here, It would be interesting to learn whether a single species of Htr-actin exists throughout embryogenesis or if there is developmental regulation of multiple transcripts as for Act5C in *Drosophila* (Burn *et al.*, 1989).

It is possible that Htr-actin is required in proliferating cells and the absence of expression in the nervous system is due to a reduced rate of cell division there. The expression patterns of some protochordate and chordate actin isoforms have been found to correlate with rates of cell proliferation. Transcripts from an ascidian actin, ScCA15, accumulate exclusively in rapidly dividing cells, in both early and late development (Beach and Jeffrey, 1990) and in chicken, β-actin mRNA levels are high in dividing myoblasts but levels decrease when the myoblasts start to differentiate into a postmitotic state. Concurrently, α-cardiac actin levels increase in the differentiating myoblasts (Taneja and Singer, 1990).

Htr-actin is unusual in that it is expressed in muscle and non-muscle tissue alike. It remains to be seen whether this sort of pattern of actin gene expression is unique to this annelid or if it will be demonstrated in members of other protostome groups as well.

We look forward to the characterization of the other members of the actin gene family in the leech. The present body of work will serve as a foundation for molecular genetic manipulations of the leech embryo. It will be worthwhile to identify the promoter region of the Htr-actin gene, for use in ectopic gene expression studies. Constructs containing a coding region of interest and a marker of choice linked to the Htr-actin promoter could then be injected into embryonic cells and their expression followed through development. Determining the expression pattern of the Htr-actin gene has provided us with some of the essential knowledge needed for such studies since it allows us to predict in what cell types gene expression driven by the Htr-actin promoter will occur during development. In particular, this promoter might be useful for examining paracrine effects of gene products on the central nervous system.

Materials and Methods

Embryo culture

Embryos were obtained from a laboratory colony of *Helobdella triserialis* and cultured under conditions described before (Blair and Weisblat, 1984). Staging conventions were as described by Fernandez (1980) and modified by Bissen and Weisblat (1989).

Isolation and characterization of Htr-actin cDNA clone

A *Helobdella triserialis* cDNA library (kindly provided by Marty Shankland), corresponding to stages 10-11 and constructed in Lambda-zap vector, was screened with a random primer generated (Amersham),

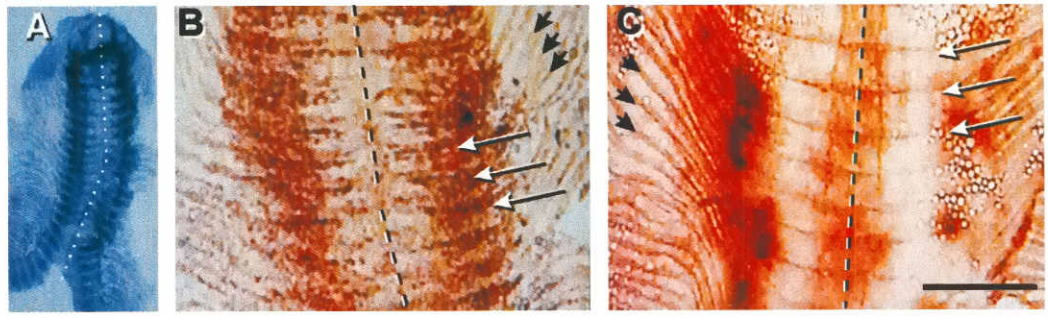

Fig. 6. Detection of Actin mRNA and muscle-specific marker in dissected stage 9 embryos. (A) *An embryo treated identically to that shown in Figure 5E except that actin message was recognized by an alkaline phosphatase conjugated anti-digoxigenin antibody and detected with an NBT/BCIP color reaction (purple). Yolk was dissected away and the embryo mounted ventral side up. In this embryo (and in B and C) anterior is toward the top of the figure. The ventral midline is indicated by a white dotted line. The posterior end of the embryo is torn along the ventral midline. Segmentally iterated staining is observed throughout the germinal plate, however the region nearest the ventral midline (neurogenic region) is devoid of color reaction product. The posterior-most 5 segments of the figure correspond to the region indicated by the lower arrowhead in Figure 5C-E. Further anterior, staining occurs in broad bands in each segment of the germinal plate. It extends ventrally between the developing ganglia and laterally in strands within the provisional integument.* **(B)** *A close-up of the anterior midbody segments of an embryo treated identically to that shown in Figure 5E. Yolk was dissected away and the embryo mounted ventral side up. The ventral midline is designated by the shadowed white dotted line. The germinal plate is heavily stained. Three white arrows point to staining in between developing ganglia, corresponding to the primary circular fiber. The black arrowheads near the upper right corner point to strands of HRP label in the provisional integument.* **(C)** *A close-up of the anterior midbody segments of an embryo treated with Lan3-14 monoclonal antibody (Zipser and McKay, 1981) and HRP conjugated goat anti-mouse secondary followed by a DAB color reaction. The ventral midline is designated by the shadowed white dotted line. On each side of the ventral midline, a longitudinal muscle strand is labeled. The white arrows indicate the primary circular fibers forming in-between the segments. The points of these arrows lie at the future dorsal midline, i.e., the lateral border of the germinal plate. Lateral to the germinal plate, muscle strands in the provisional integument are labeled with Lan3-14. Three such strands are designated by the black arrowheads (upper left). Bar, 120 μm in A and 430 μm in B and C.*

^{32}P-labeled *Drosophila melanogaster* cytoplasmic actin probe, Act5C or DmA2 (Fyrberg *et al.*, 1980), using low stringency conditions as described by Wedeen *et al.* (1989). Following plaque purification, it was determined that one positive recombinant, λ9A.4, contained an approximately 1.5 kb insert. The insert was removed from the vector by digestion with EcoRI and was subcloned into the EcoRI site of Bluescript KS (Stratagene), generating the construct, p9A.4. The cDNA sequence was obtained from p9A.4 using the dideoxy chain termination method. The Bluescript vector of the p9A.4 construct was found to differ from the sequence published by Stratagene in that it was missing a G near the KpnI site of the polylinker. The cDNA will hereafter be referred to as Htr-actin cDNA.

Genomic southern blots

Genomic DNAs of *Helobdella triserialis*, *Helobdella robusta* and *Drosophila melanogaster* were prepared as described by Wedeen *et al.* (1989). The DNAs were digested to completion with restriction enzymes and 1 ug of digested leech DNA or 10 ug of digested fly DNA were loaded per lane. Following electrophoreses in a 1% agarose gel, DNA was transferred bidirectionally to two sheets of nitrocellulose, baked 2 h at 80°C, prehybridized for two hours under hybridization conditions and then one sheet hybridized at low and the other at high stringency with a ^{32}P-labeled Htr-actin probe [generated by random prime method (Amersham)]. The low stringency conditions were designed to allow hybridization of strands containing 40% mismatch of bases, as described in Wedeen *et al.* (1989). Under the high stringency conditions, 0% mismatch was allowed.

Northern analysis

The methods used for RNA isolation and characterization are described in Current Protocols in Molecular Biology (eds, Ausubel *et al.*, 1996). Total RNA was purified from adult *H. triserialis* using the guanidinium phenol chloroform method (Chomczynski and Sacchi, 1987). Polyadenylated RNA was purified from total adult RNA by passage over an oligo-dT column. One μg of polyadenylated RNA was loaded on a 1.2% formaldehyde agarose gel next to RNA markers and electrophoresed. Using established protocols, the RNA contents of the gel were transferred to nitrocellulose. The blot was prehybridized, hybridized with the Htr-actin probe described above, washed and exposed to film.

In situ hybridization of ^{35}S-labeled probes to Helobdella triserialis embryos

Probe generation

An ^{35}S-labeled antisense Htr-actin RNA probe was generated from the p9A.4 construct. The clone was linearized with XbaI and the probe was synthesized using T3 RNA polymerase, ^{35}S-UTP, and unlabeled ATP, CTP and GTP ribonucleotides. Probe synthesis was done as described by Ingham *et al.* (1985). As controls, in parallel, sibling embryos were hybridized with ^{35}S-labeled RNA probes of the same specific activity, corresponding to sense or antisense strands of the *Helobdella triserialis* Elongation Factor-1α (clone, pEFc1) cDNA (kindly provided by Randy Streck). The sense probe was synthesized using T3 RNA polymerase and the antisense probe, using T7 RNA polymerase.

Fixation

Embryos were fixed under various conditions. Stage 1, 2, 4, 9 and 10 embryos were fixed for 10 h at 4°C in 3.7% formaldehyde/100 mM Tris-HCl (pH 8.0). Stage 8 embryos were fixed overnight in 3.7% paraformaldehyde/100 mM Tris-HCl (pH 7.4).

Hybridization

The permeabilization procedure was as described by Holton *et al.* (1994). After fixation prehatching embryos were transferred to TNE (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA) to dissect off vitelline membranes. To permeabilize, all the embryos (prehatching and posthatching) were incubated overnight with agitation (100 rpm) in TNET (TNE plus 1.2% Triton X-100) in a half full 1.5 ml microcentrifuge tube placed horizontally on a rotary shaker.

Following permeabilization, embryos were prehybridized, hybridized and washed using a protocol modified from that of Ingham *et al.* (1985). Prehybridization was done overnight in hybridization buffer [0.15M NaCl, 10 mM Tris, pH 7.6, 5 mM $MgCl_2$, 40% formamide, 500 μg/ml tRNA, 0.01% pyrophosphate, and 1% Denhardt's solution (2% Ficoll, 2% polyvinylpyrolidone, 2% BSA)] without probe at 50°C. Hybridizations were done in 0.2 ml polypropylene tubes at 50°C with probe at a concentration of 0.3 ng/μl/kb in hybridization buffer for 2 days. Posthybridization washes and RNase treatment were exactly as described by Ingham *et al.* (1985).

Sectioning and autoradiography

Embryos were embedded in Beem capsules containing JB4 plastic embedding medium (Polysciences, Worthington, PA). Blocks were sectioned with an ultramicrotome at a thickness of 4μm. Sections were placed onto gelatin subbed slides, dipped in 1:4 dilution of emulsion at 45° (Kodak NTB), and dried 4 h in dark with fan. The emulsion was bleached by overnight exposure to hydrogen peroxide vapor on a kimwipe, in a half-open slide box. Slides were stored at -80°C in the dark with drierite for at least four weeks and up to six months. After developing, slides were stained with 100 μg/ml Hoechst 33258 for 15 min, or 1% toluidine blue (0.1 M $Na_2B_4O_7$) air dried, and mounted under glass coverslips in Fluoromount (Polysciences).

Estimating qualitative differences in transcript accumulation

In order to make relative comparisons of transcript accumulation between different portions of an embryo and between different embryos, silver grains were counted over defined areas on a sampling of representative slides. Because the grain densities over any one subcellular area (i.e., yolk or teloplasm) appeared to be approximately uniform within a section and in different sections, this method could be used to estimate qualitative differences in distinct parts of one cell and between different embryos. Short exposure times were necessary in order to be sure that the emulsion was not saturated and that relationship between the number of grains over an area and the radioactively labeled transcripts was roughly linear. An example of how the detectable transcript levels in the teloplasm and the yolk were compared is provided here.

Grains over the teloplasm and the yolk of a one cell embryo, corresponding to a square with dimensions of 1.8 μm (or 3.2 μm^2) were counted. Grains appeared to be relatively evenly distributed throughout any one subcellular area. However in an effort to avoid biasing our sample, we were careful to choose a square area that extended from the center of the pool of teloplasm to the edge. Grain counts were done over the teloplasm in three serial sections on the same slide with totals of 58, 58 and 67 grains/3.2 μm^2. This corresponded to an average of 61±4.5 grains/3.2 μm^2 or 19±1.4 grains/μm^2. Similar grain counts were done for the same sized areas over the yolky regions from two of the same embryo sections, yielding a range of 22-27 grains/3.2 μm^2 (i.e., an average of 24.5±3.5 grains/ 3.2 μm^2 or 7.6±1.1 grains/μm^2). The ratio of grains over the teloplasm to grains over the yolk was 19±1.4/7.6+1.1 or 2.5±0.6.

In situ hybridization of digoxigenin-labeled probes to Helobdella triserialis embryos

The protocol used is a modification of those described by Nardelli-Haefliger and Shankland (1992), Kostriken and Weisblat (1992), and Bissen (1995).

Probe generation

The pHtr-actin clone was linearized with XbaI and digoxigenin labeled antisense strand was synthesized using the T3 MegaScript kit (Ambion). For the synthesis, 75 mM ribonucleotides were used, except for 48.75 mM UTP in addition to 25 mM digoxigenin-UTP (Boeringer Mannheim). The synthesis was carried out for $4^1/_2$ h at 37°C followed by 15 minute incubation with 4 units RNase free DNase I (T3 MegaScript kit) to remove the template. The transcript was lithium chloride precipitated by adding 1/2 volume of 7.5 M LiCl, 50 mM EDTA for 30 min at -20°C, pelleted by centrifugation and the pellet washed with 70% ethanol, dried and resuspended in RNase free water. The probe concentration was determined by absorbance at 260 nm.

Evaluation of digoxigenin incorporation into probe

Five μl volumes of 10-1000 fold dilutions of the probe (12.8 ng to 0.128 ng RNA) were slot blotted onto nitrocellulose and cross linked by UV irradiation at 256 nm for 12 sec. The blot was blocked overnight at 4°C in Blotto (1 mM $MgCl_2$, 25 mM Tris, pH 8, 137 mM NaCl, 2.7 mM KCl, 1% BSA, 2% dry milk) and incubated with 1:1000 dilution of alkaline phosphatase conjugated antidigoxigenin (Boehringer Mannheim) in Blotto overnight. The blot was washed twice (10 min each) in Blotto without BSA or milk and then twice (10 min each) with AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5). The blot was then reacted with 0.03% Nitro BT (Fisher BP108-1) [i.e., 6.6 ul/ml of 5% stock in 70% dimethylformamide] and 0.0017% 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP; Fisher BP1610-500) [i.e., 0.33 μl/ml of 5% stock in dimethylformamide] in the dark for 4 h. Color reaction product was detected as low as the 1000 fold dilution.

Probe Hydrolysis

One part probe was hydrolyzed for 30 min at 60°C with ten parts carbonate buffer (40 mM NaH_2CO_3, 60 mM Na_2CO_3). Following hydrolysis, the probe was isopropanol precipitated with 1/10 volume ammonium acetate and resuspended at 25.6 ng/ul in prehybridization solution [5xSSC (75 mM Na citrate, 0.74M NaCl), 0.2 mg/ml tRNA (Boehringer), 0.1 mg/ml heparin (Sigma H5027), 1XDenhardt's solution {0.02% acetylated BSA (Sigma B2518), 0.02% polyvinylpyrrolidone (Sigma P-5288), 0.1% Tween-20}, 0.1% CHAPS (Boehringer), and 50% deionized formamide; volume adjusted with RNase-free water] and stored at -20°C.

Fixation

Embryos were fixed for 1 h in 0.25X PBS (0.75 mM NaH_2PO_4, 1.75 Na_2HPO_4, 3.25 mM NaCl, pH 6.9) /4% formaldehyde, then dehydrated in a series of increasingly concentrated ethanol solutions (two to five min each in 30%, 50%, 70%, 80%, 90%, 95%, and two 100% ethanol washes) and transferred to methanol. Embryos were stored in methanol at -20°C for up to one year.

Embryo preparation

Embryos were rehydrated through decreasing concentrations of ethanol (solutions listed above), then washed three times in 0.25XPBS and thereafter maintained in RNase free solutions and plasticware. The vitelline membranes were removed from prehatching embryos and stage late 9-10 embryos were treated for five min with 0.5 mg/ml pronase (Sigma, Type XIV Bacterial protease, P5147) in 5XTE [50 mM Tris-HCl(pH 7.5), 5 mM EDTA]. Stage 8-early 9 embryos were treated with 0.25 mg/ml pronase and younger embryos were not treated with pronase. Pronase treatment was followed by five 1 minute washes in 2 mg/ml glycine in PBS. Embryos were washed twice (5 min each) in 500 μl 0.1M triethanolamine (pH 7.5) and acetylated twice by addition of 1μl acetic anhydride with occasional agitation for 5 min each. Following acetylation, embryos were washed three times (5 min each) in PBT (PBS, 0.1% Tween 20), then fixed a second time for 20 min in 4% formaldehyde in PBT. Embryos were then washed six times (5 min each) in PBT and incubated overnight in prehybridization solution that had been heated to 95°C for 10 min and cooled to 60°C.

Hybridization and probe detection

One part hydrolyzed probe (128 ng in 5 μl) was added to 3 parts prehybridization buffer (15 ul), heated at 95°C for 10 min and cooled to 60°C. Embryos were hybridized 12-60 h at 60°C and then washed as follows: At 60°C, 10 min in equal parts prehybridization buffer and 2XSSC (30 mM Na citrate, 297 mM NaCl), 0.3% CHAPS; 10 min in 2XSSC, 10 min in 0.2XSSC, two 30 minute washes in 0.05XSSC. At room temperature, 5 minute washes each in 3 parts 0.05XSSC to 1 part 1X PBT; equal parts 0.05XSSC and 1X PBT; 1 part 0.05XSSC to 3 parts 1X PBT. At 37°C, a 30 minute wash in 1X PBT/ 20 μg/ml RNase A, followed by three 5 minute washes in 1XPBT.

Following RNase treatment, the embryos were washed twice in PBT, incubated in 0.2 um filtered block solution (1XPBS, 0.5% Triton X-100, 2% BSA) overnight at 4°C, then treated overnight at 4°C with 1:100 dilution of horseradish peroxidase (HRP) conjugated antidigoxigenin (Boehringer Mannheim) or 1:200 dilution of alkaline phosphatase (AP) conjugated antidigoxigenin (Boehringer Mannheim) in block solution. Embryos were then washed for 5 h with 4-5 changes of block solution.

For AP detection, embryos were washed twice with 1mM levamisole (Sigma, L-9756) in AP buffer and treated with 0.03% NitroBT and 0.0017% BCIP in the dark until color appeared. For HRP detection, embryos were reacted in the dark in PBS containing 0.5 mg/ml DAB and 0.015% H_2O_2.

Lan 3-14 antibody staining

Stage 9 *H. triserialis* embryos were fixed in 4% formaldehyde/0.25XPBS, dehydrated and rehydrated as described above (see *In situ* hybridization with digoxigenin labeled probes; Fixation). Prior to placing them in 0.2 μm filtered block solution (1XPBS, 2% BSA, 0.5% Triton X-100), embryos were treated for 5 min with pronase 0.25 mg/ml (or 1/160 dilution of 40 mg/ml stock; Sigma Type XIV Bacterial protease, P5174) then washed with glycine (2 mg/ml), refixed 20 min in 4% formaldehyde/1XPBT, and washed in PBT. They were then blocked for about 16 h at 4°C. Lyophilized Lan 3-14 (Zipser and McKay, 1981) ascites fluid was resuspended and added to fresh block solution at a final dilution of 1:1000. Embryos were incubated at 4°C for 16-24 h, then washed for 5 h with 4-5 changes of block solution. They were then placed in a 1:500 dilution in block solution of HRP-conjugated goat anti-mouse secondary antibody (Jackson Laboratories) for 16-24 h, washed 4-5 times (5 h total) in block solution and color reacted in the presence of DAB and hydrogen peroxide. Embryos were dissected in 80% glycerol to remove yolk and were mounted ventral side up under coverslips for imaging in 80% glycerol, 4 mg/ml n-propyl gallate, 100 mM Tris-HCl, pH 9.

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