ELSEVIER

Gene 199 (1997) 31–37

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

A leech homolog of *twist*: evidence for its inheritance as a maternal mRNA

Julio G. Soto *, Brad. H. Nelson [1], David A. Weisblat

Department of Molecular and Cell Biology, 385 LSA, University of California, Berkeley, CA 94720-3200, USA

Received 18 February 1997; accepted 14 May 1997; Received by E. Boncinelli

Abstract

In the development of leeches such as *Helobdella robusta*, mesodermal and ectodermal fates segregate to cells DM and DNOPQ, respectively, at fourth cleavage. As one step in identifying genes that may act in mesoderm determination, we have cloned the *H. robusta* homolog to the *Drosophila* gene *twist*. This homolog, designated *Hro-twi*, exhibits high (>90%) amino acid identity with other *twist*-class genes within its basic-helix–loop–helix (b-HLH) DNA binding motif and dimerization domain. Like *twist*, *Hro-twi* contains CAX-rich stretches: three stretches 5′ to the b-HLH and one located 3′ of the b-HLH motif. RT–PCR analysis suggests that *Hro-twi* is present throughout development, beginning as a maternal transcript in the oocyte. © 1997 Elsevier Science B.V.

Keywords: Maternal genes; Mesoderm

1. Introduction

An early event in the development of most animal embryos is the formation of distinct germ layers: endoderm, ectoderm and mesoderm. The processes by which these layers arise differ between phyla, but an essential feature of gastrulation is the separation of prospective mesoderm and ectoderm. Leech embryos are useful for the study of mesoderm formation because separate precursors for segmental mesoderm and ectoderm are separated in the course of a single cell division at fourth cleavage.

In glossiphoniid leech embryos, as in many annelids, domains of specialized cytoplasm (teloplasm), deficient in yolk and enriched in organelles (Fernández et al., 1987) and mRNA (Holton et al., 1994), arise in the zygote and segregate by unequal cleavages to macromere D′ of the eight-cell embryo (Fig. 1). The fourth cleavage plane is approximately equatorial in D′, generating daughter cells of about equal size. The animal daughter, ectodermal precursor cell DNOPQ, cleaves further to form four bilateral pairs of ectodermal stem cells (N, O/P, O/P and Q teloblasts) and 13 micromeres (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). The vegetal daughter, mesodermal precursor cell DM, cleaves further to form one pair of mesodermal stem cells (M teloblasts) and two micromeres. The teloblasts are the progenitors for the segmental tissues. Their progeny form bilaterally paired germinal bands that undergo epibolic movements during gastrulation, gradually coalescing into a ventrally situated germinal plate. The germinal plate is homologous to the germ band in arthropod embryos and forms segmental ectoderm and mesoderm.

Previous studies led Nelson and Weisblat (1991, 1992) to conclude that cells DM and DNOPQ assume their distinct ectodermal and mesodermal fates at the time of their birth and do so autonomously. They further proposed that assumption of the ectodermal fate requires a short range interaction between inherited teloplasm and factors localized to the animal cortex; the mesodermal fate, by contrast, was regarded by these authors as a ‘ground state’ for blastomeres that inherit teloplasm but do not undergo this interaction.

To study the molecular aspects of ectodermal and mesodermal cell fates in *Helobdella*, we are characterizing homologs of genes involved in mesoderm formation in

* Corresponding author. Present address: Department of Biology, Station 33, Eastern New Mexico University, Portales, NM 88130, USA. Tel.: +1 505 5622494; Fax: +1 505 5622192;
e-mail: sotoju@ziavms.enmu.edu
[1] Present address: Fred Hutchinson Cancer Research Center, Seattle, WA 98104, USA.

Abbreviations: b-HLH = basic-helix–loop–helix.

0378-1119/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0378-1119(97)00327-2

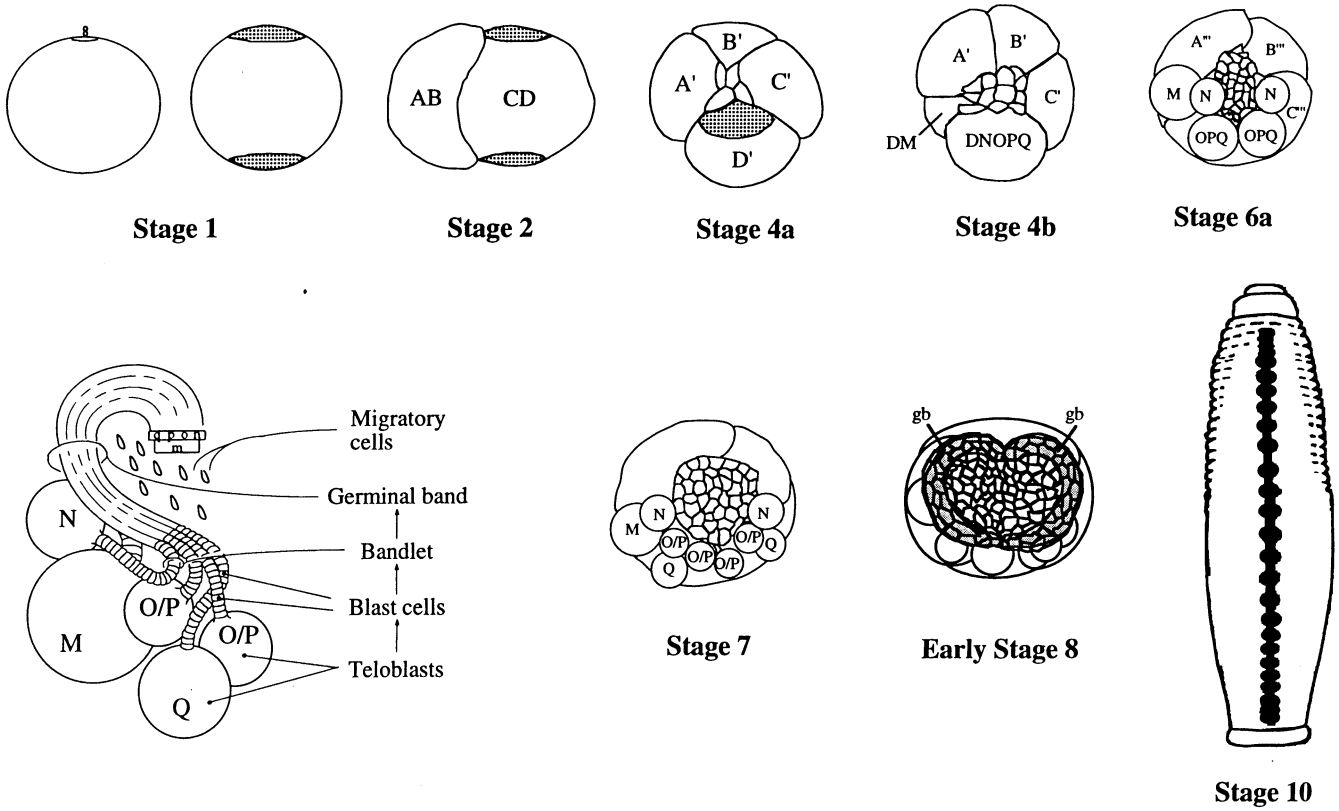


Fig. 1. Schematic representation of relevant stages of *Helobdella* embryogenesis. Stages 1–2 are equatorial views, with the animal pole up; stages 4–8 are views from approximately the animal pole which corresponds to the prospective dorsal aspect; stage 10 is viewed from the ventral aspect. After polar bodies are formed at the animal pole (stage 1, left), cytoplasmic rearrangements generate animal and vegetal domains of yolk-deficient cytoplasm called teloplasm (shading; stage 1, right). Teloplasm is partitioned asymmetrically during early cleavages, being inherited largely by macromere D′ at stage 4a. At fourth cleavage, cell D′ generates mesodermal precursor DM and ectodermal precursor DNOPQ (stage 4b); the other macromeres are endodermal precursors. The D quadrant derivatives produce five bilateral pairs of teloblasts plus additional micromeres (stages 6–7; not all teloblasts are visible in these views). During stages 7–8, the teloblasts on each side generate columns of segmental founder cells (blast cells) in coherent columns (bandlets) that merge ipsilaterally into left and right germinal bands (enlarged view shows left-hand teloblasts and germinal band formation). During gastrulation (not shown) the germinal bands coalesce along the ventral midline, giving rise to the germinal plate, from which segmental tissues arise (stage 10).

Drosophila, such as *twist* and *snail*. *Twist* was identified as a zygotic gene product whose function is essential for mesoderm formation (Thisse et al., 1987a). *Drosophila* embryos with a homozygous null mutation in the *twist* gene lack mesoderm (Simpson, 1983). Examining *twist* and *snail* double mutants, Leptin et al. (1992) suggested that cell movements in the first step of gastrulation in *Drosophila* embryos (ventral furrow formation) are controlled by products of genes transcribed under the control of *twist* and *snail*. *Twist* is required to maintain its own transcription and that of *snail*; it also activates the transcription of other early (*msh*-2, Bodmer et al., 1990) and late (β_3-tubulin and *pox meso*, Leptin, 1991) mesoderm-specific genes. Analysis of the twist protein indicates that it acts directly to regulate those genes. *Twist* encodes a transcription factor with a basic-helix–loop–helix (b-HLH) motif. The basic DNA binding subdomain is amino terminal to the HLH domain. The HLH domain contains two segments capable of forming an amphipathic α-helix connected by a 'loop' region (Murre et al., 1989). The b-HLH domain enables *twist* to form homodimers, at least in vitro, and to bind to chromosomes in vivo (Thisse and Thisse, 1992). *Twist* homologs have also been identified in the frog (*X-twi*, Hopwood et al., 1989) and the mouse (*M-twi*, Wolf et al., 1991) and are expressed in the mesoderm of these animals.

Here we report the isolation of a gene designated *Hro-twi* from *Helobdella robusta* that is a homolog of *twist* and take the first steps toward describing its expression in normal leech embryos. We find that, in contrast to the other known *twist*-class genes, *Hro-twi* mRNA is both maternally inherited and present throughout embryonic development.

2. Materials and methods

2.1. Embryos

H. robusta embryos were obtained from a laboratory colony and maintained in HTR medium (Blair and

Weisblat, 1984). Staging criteria were those described by Fernández (1980) and Bissen and Weisblat (1989). Oocytes were obtained surgically from gravid adults.

2.2. PCR and cloning

One degenerate deoxyoligonucleotide, oligo A (shown below), was designed to be complementary to the antisense strand corresponding to the conserved amino acid sequence RVMANV which is found at residues 364–369 in *twist* (Thisse et al., 1988), residues 74–75 in *X-twi* (Hopwood et al., 1989) and residues 509–517 in *M-twi* (Wolf et al., 1991). Another degenerate deoxyoligonucleotide, oligo C (shown below), was designed to be complementary to the sense strand coding the conserved amino acid sequence KIQTLK, which is found at residues 399–404 in *twist*, residues 109–114 in *X-twi* and residues 617–622 in *M-twi*. In the sequences shown below, degenerate sites are indicated by nucleotides in parentheses.

oligo A:
5′ CCCTCGAG(C/A)G(G/A/T/C)GT(G/A/T/C)-ATGGC(G/A/T/C)AA(T/C)GT 3′

oligo C:
5′ CCGTCGAC(T/C)TT(G/A/T/C)A(G/A)(G/A/T/C)GT(T/C)TG(G/A/T)AT(T/C)TT 3′

Amplifications from genomic DNA were carried out as described by Kamb et al. (1989). Amplification was carried for 3 cycles consisting of 1 min at 94°C and 3 min at 60°C, followed by 30 cycles consisting of 1 min at 94°C and 2 min at 65°C. The PCR product (139 bp) was gel purified and converted to a blunt ended form, after which it was ligated into the *Sma*I site of pBluescript (Stratagene). The 139-bp fragment was sequenced in both directions using vector-specific primers.

2.3. cDNA and genomic screening

A cDNA λ ZAP library made from stage 10 *H. robusta* embryos was probed with the 139-bp PCR fragment that encodes the DNA binding and dimerization domains of *Hro-twi*. Hybridization was carried out at high stringency conditions (Sambrook et al., 1989). Washes were done at 68°C in 0.2 × SSC, 0.1% SDS. Four clones were isolated. About 300 nucleotides at both ends of each insert were sequenced using vector-specific primers. Restriction sites were identified in the cDNA inserts and used for further subcloning. The truncated cDNA clones (1087 bp) were sequenced in both directions using standard procedures. The four cDNA clones were identical and do not correspond to a full-length transcript.

Genomic DNA containing the *Hro-twi* gene was isolated from a partial Sau3A *H. robusta* genomic λ DASH II library. The average size insert of this library is 15 kb. Five genome equivalents were screened at high stringency. Two lifts were probed with either the *H. robusta* partial 1.087-kb cDNA fragment or with the PCR fragment as described herein. Two overlapping clones were obtained from this screening. The clones were mapped and the restriction fragments containing the *Hro-twi* gene were subcloned into pBluescript and sequenced in both directions using primer walking. Sequence analysis was performed using MacDNAsis 3.0 software (Hitachi).

2.4. Northern analysis

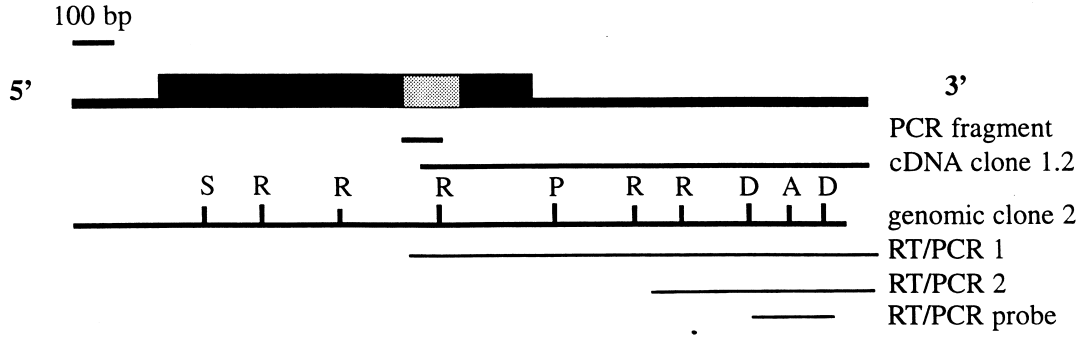
Total RNA from adult leech tissue was prepared by a modification of the Chomczynski and Sacchi (1987) method. For Northern analysis, 40 μg of total RNA were loaded on a 1% agarose–formaldehyde gel and run under denaturing conditions. RNA was transferred to a Nylon membrane under high salt conditions and the filter baked at 80°C for 1 h. The *H. robusta* partial 1.087-kb cDNA fragment was labeled with ^{32}P using random primed DNA polymerization and used as a probe. Hybridization was carried out overnight at 68°C in 5 × SSC, 5 × Denhardt's, 50 mM $NaPO_4$ (pH 7.0), 200 μg/ml denatured salmon sperm DNA. Washes were done at 68°C with 0.2 × SSC, 0.1% SDS.

2.5. 3′ RACE-RT–PCR

Total RNA was isolated, using a modification of the method by Chomczynski and Sacchi (1987), from 100 oocytes or embryos representing stages 1–2, 4b, 6a and 8–9. RNA was reverse transcribed using an oligodT primer modified by the inclusion of additional 5′ sequence (designated SH1) as follows:5′ CTC-ATTCCTGTTAGGCTTACCT$_{12}$ 3′.

Reverse transcription was carried out at 42°C using AMV reverse transcriptase (Gibco BRL). The 3′ end of *Hro-twi* cDNA was amplified from the first strand cDNA in a two-step PCR reaction. The oligonucleotide primers used in the first PCR reaction were Tw3 (corresponding to residues 730–750 of the *Hro-twi* gene) and SH1. The oligonucleotide primers used in the second PCR reaction were Tw15 (corresponding to residues 1278–1298 of the *Hro-twi* gene) and SH1. Both PCR reactions consisted of 25 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 2 min. Total RNAs from oocytes and embryos were used as negative controls in both PCR reactions. The amplified products of the first PCR reaction were purified using a Qiaquick column (Quiagen) prior to the second PCR reaction. A tenth of the cDNA products from the second PCR reaction corresponding to oocytes and embryos were loaded in a 1% agarose gel, transferred to nitrocellulose and probed with a 262-bp *Dra*I

A



B

```
1   TAACCTACCTCGTCTTCCTACCAGAACACTTCAGTCAGCTCTCACGACATCATCGAATTTCCGGCAGCAACGCGGACG
79  ATGATGTCACCATCTTTTCCAACTACGTCATCCTCTCATCATTTGCGTCGGCTAACTCAGCAGCAGCAACGGCAGCGA
 1    M  M  S  P  S  F  P  T  T  S  S  S  H  H  L  R  R  L  T  Q  Q  Q  Q  R  Q  R

157 CGACAACCGGCGTCAACTGATTCTGTCATTAAAAATTGTCTCTTCAACAATAAAAATGACACGTCGACACACTTAACT
 27   R  Q  P  A  S  T  D  S  V  I  K  N  C  L  F  N  N  K  N  D  T  S  T  H  L  T

235 ACGCATTTACAAGGCGTTAACAAGATTTATTTTCACAACAACAACAACTGTAGCAATGACAGCAACAACAACAGCGTC
 53   T  H  L  Q  G  V  N  K  I  Y  F  H  N  N  N  N* C  S  N* D  S  N  N* N  S  V

313 GACCTCAAGCCTGCTACTAAAAAACGAAAGAAAAACATCAAAAAACTAACTAAATCCGATTCACGCCCAATAAGGACG
 79   D  L  K  P  A  T  K  K  R  K  K  N  I  K  K  L  T  K  S  D  S  R  P  I  R  T

391 TCTGAAAGTACTAAAAATGACTTGCAACAAAACAACGTTGTGAAAGTTGAGCTGGATCAGTCAGACAACAACACAGCG
105   S  E  S  T  K  N  D  L  Q  Q  N  N  V  V  K  V  E  L  D  Q  S  D  N  N  T  A

469 GAAAACGAAGACAGTAAATCTTCAGTTAAAGACAGCAAAAACAACAACCACTGCAACAACACAGCGCCGACGACTACA
131   E  N  E  D  S  K  S  S  V  K  D  S  K  N  N  N  H  C  N  N  T  A  P  T  T  T

547 ATTACGACGACTCCGAGCATAAATTTCAACACCCAAACAACGACATCTACCTACAGAAAACTTCCCAACGCCACCACA
157   I  T  T  T  P  S  I  N  F  N  T  Q  T  T  T  S  T  Y  R  K  L  P  N  A  T  T

625 AACAACATCGCCTACAACCACAACGGTAGCGGCAAAAACTACTACAGCAACTACAACAGCCACAACAACTCGCCACCA
183   N  N  I  A  Y  N  H  N* G  S  G  K  N  Y  Y  S  N  Y  N  S  H  N  N  S  P  P

703 CTCTCGCTACAAACGCAGCGAGTTCTTGCGAACGTCCGTGAGCGCCAAAGAACGCAATCCTTAAACGATGCCTTCCCA
209   L  S  L  Q  T  Q  R  V  L  A  N  V  R  E  R  Q  R  T  Q  S  L  N  D  A  F  P

781 CAGTTAAGAAAAATCGTTCCGACGCTCCCGTCCGACAAACTCAGCAAAATCCAAACCCTGAAACTGGCAACCAGATAT
235   Q  L  R  K  I  V  P  T  L  P  S  D  K  L  S  K  I  Q  T  L  K  L  A  T  R  Y

859 ATCGACTTTTTATACGATCAATTAGAGAACAATAAGCAGCAGCAGCAGCAACATCAGCAGCAGCAGCAACAACAACAT
261   I  D  F  L  Y  D  Q  L  E  N  N  K  Q  Q  Q  Q  Q  H  Q  Q  Q  Q  Q  Q  Q  H

937 CAAAGTATGGACGAATTTGCTAACGGTGGATATGTTTCCGGTTAAACGGAAGTCCGGTCATCCTCGGACAAGTTGGGG
287   Q  S  M  D  E  F  A  N  G  G  Y  V  S  G  †

1015 TACGATCGTTTTCAGTTTGGAGGATGGAGGGCGCGTGGCAGATGAATAAAAGTGACTTAATAATAACGGATGTTACTT
1093 ACGTAACGCACGAGTCGTGGTGGCAGCAGTAACAACGTTCGCGATGGCAGTGTCATTGGCTGACGTGTCTCTATATGT
1171 ATGCGTGTGTATGTACATATGTGTGTATGATAAAAATAATAGTAATAATAATAATATTTATTATTATTATTATTATAA
1249 CAGTAATAAGAGAAATAACAATAATATCTTTAGGTGCTTACCAAGAGACAAAAAATGCCTTTAATTTATACAATATTG
1327 TGAAGCTGTACGTATGAGTGTTTTTACAACAACAGCAACAACAACAACAACAATAATAATAATAAAATTTGGTGGTAT
1405 TATAAACAATATCATTTTTACTCGCGTTATCATTGGTTATATAAAAACTTCCATTACATCCGTTTCTCAACTGAGACA
1483 TTGATTGCGATACAAAGTATGTGCCTTGGAAAAAATCGAGTCAACCCTTTTAAATGAAGCAATGTTATTTTATATCGT
1561 CCTAATAATAACACACACACACACACTCACTCACGACATGTATACGCACACACACACTCACTCACATATATCGACACA
1639 CACACGCACAAACGCATATGCGCTCACACACACACAAAATATTCATTTATTTATTTGTGTGTGTATCACACTTTCATT
1717 GTTGTCAAAGTTAAGCAATATGTTATTTCTGGTTTTTTATTTTTTTAAATTTCTTGAGTTTATAAATTTTATTTATTG
```

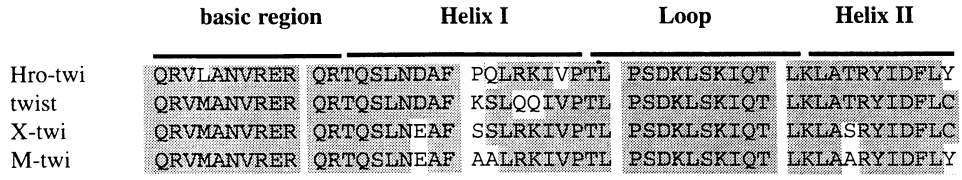


Fig. 3. Amino acid alignment of within the b-HLH domains of twist-class proteins from leech (Hro-twi), fruitfly (twist), frog (X-twi) and mouse (M-twi). Identical amino acids are shaded. Components of the b-HLH domain are indicated above the sequence.

fragment located internally to the PCR amplified 3′end *Hro-twi* fragment.

3. Results

3.1. *Cloning the* twist*-class gene (*Hro-twi*) from* Helobdella robusta

Degenerate oligos designed against the highly conserved b-HLH region of known *twist*-class genes were used to amplify an 87-bp fragment from genomic DNA of *H. robusta*. This gene fragment encodes a 29-amino-acid peptide that is homologous to the b-HLH domain of the *twist*-class genes, starting with the residues RERQ (residues 221–224 of the protein) and ending with the residues SDKLS (residues 245–249 of the protein; Fig. 2A). Southern blot analyses indicate that this sequence is present as a single copy in the genome (data not shown). The PCR fragment was used as a probe in the isolation of cDNA clones of this gene, which we designated *Hro-twi*. Four identical clones were isolated, containing the last 60 amino acids (starting with the residues PTLPS at positions 242–246) and 907 bp of 3′ untranslated sequence (Fig. 2). These cDNAs seem to represent truncated mRNAs because they have no transcription start sites. In addition, Northern analysis showed that there is a 2.4-kb transcript in the adult (Fig. 4). Thus, to obtain the entire coding region of *Hro-twi*, we isolated *Hro-twi* genomic clones from a *H. robusta* ł DASH II library (Fig. 2A).

From the sequence alignment of the cDNA and genomic clones, a single open reading frame of 900 bp is obtained. Although the exact 5′ end of the *Hro-twi* mRNA has not been determined yet, we believe that we have obtained the first methionine for two reasons. First, the two residues following the proposed initiation codon are identical to those in *twist*, and second, there is an in-frame termination codon (TAA) 78-bp upstream of the proposed initiation codon (Fig. 2B).

300 amino acids are obtained from a conceptual translation of the open reading frame. The highly conserved (>90% identity, Fig. 3) b-HLH domain is located at the C terminus as in other *twist*-class genes. The polypeptide encoded by the open reading frame exhibits additional structural motifs. These include: a region rich in Arg and Lys (nine out of 16 amino acids between residues 81 and 96); four possible glycosylation sites (Asn-X-Ser), located 5′ of the b-HLH; and four CAX-rich stretches, three located 5′ of the b-HLH motif and one located 3′ of it. The 5′ CAX-rich stretches are translated as Gln, Asn/Ser, and Asn/Ser. The 3′ CAX-stretch is the largest and it is translated as 13 Gln and two His. Glutamine-rich stretches are present in some transcription factors and have been implicated in gene activation (Dynlacht et al., 1989).

3.2. Hro-twi *is present in oocytes and throughout cleavage*

We have used PCR amplification of stage-specific cDNAs (3′ RACE) for our initial analyses of the temporal expression of *Hro-twi* mRNA. *Hro-twi* mRNA was present in all the embryonic stages examined, including the oocyte, stages 1–2, 4b, 6a and 8–9 (Fig. 4B). Expression of *Hro-twi* prior to stage 4b means that the transcript is present in cells prior to the establishment of a separate lineage for segmental mesoderm. The detection of *Hro-twi* in oocytes was unexpected because *twist*-class genes have only been detected as zygotic transcripts in other species.

The RT–PCR technique used here provides qualitative information that *Hro-twi* was present among the mRNAs from different stages. To determine its spatial

Fig. 2. Map and sequence of the *Hro-twi* gene. (A) The open reading frame (ORF) is shown as the black box, within which the gray region represents the b-HLH domain. Both 5′ and 3′ UTRs are indicated with bold lines. The three lines beneath the gene map indicate the locations of the original PCR fragment, the cDNA clone 1.2 and genomic clone 2, respectively. Restriction sites are indicated on the genomic clone (S = *SalI*; R = *RsaI*; P = *PvuI*; D = *DraI*; A = *AccI*). (B) Nucleotide sequence and conceptual translation of *Hro-twi*. Nucleotides are numbered beginning at the first TAA 5′ of the ATG; the 5′ cap site has not been determined; a single polyadenylation signal sequence is located at positions 1836–1841 (double underline). The conceptual translation sequence starts at position 79 (the presumed initiation codon ATG) and ends at the termination codon TAA ('dagger', position 979). The *Hro-twi* genomic and cDNA clones have 907 bp of 3′ UTR sequence; the deduced protein is 300-a.a. long. Also indicated are the b-HLH domain (shaded box); stretches of 'CAX' repeats (clear boxes); four possible glycosylation sites (Asn-X-Ser; asterisks); and an Arg/Lys-rich stretch (underline).

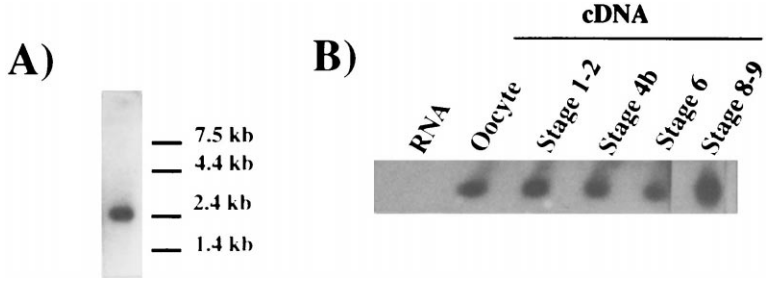


Fig. 4. Temporal expression of *Hro-twi*. (A) Northern blot analysis of total adult RNA (40 μg), probing with the *Hro-twi* cDNA clone 1.2, reveals a single transcript of approximately 2.4 kb. (B) 3′ RACE-RT–PCR was performed with reverse transcribed cDNAs from pooled embryos at specific stages of development (see Fig. 1). The amplified products were probed with an internal 3′ UTR DraI fragment. Non-reverse transcribed RNA was used as a control in the 3′ RACE-PCR reactions. Shown here is the RNA control from the oocyte sample.

distribution, in situ hybridization is desirable. Because of technical difficulties resulting from non-specific binding of digoxygenin-labeled RNA probe to teloplasm in early embryos (M. Pilon, personal communication), it is not yet possible to obtain reliable gene expression data from *Helobdella* embryos at early stages using in situ hybridization.

4. Discussion

4.1. *A* twist-*class gene in leech*

We have reported herein the isolation and characterization of a leech gene, *Hro-twi*, that is the homolog in *Helobdella*. The size of the deduced Hro-twi protein (300 aa) is smaller than twist (490 aa), but larger than the twist-class proteins in frog (166 aa) and mouse (206 aa). Comparing the deduced amino acid sequence of Hro-twi with the other twist-class proteins, the highest similarity (>90% identity) is within the b-HLH domain at the carboxyl terminal (Thisse et al., 1988; Hopwood et al., 1989; Wolf et al., 1991). The leech and the fly proteins also share 'CAX' stretches, glycosylation sites and an Arg/Lys-rich stretch, features not conserved in the frog and mouse genes.

4.2. Hro-twi *mRNA is maternally deposited in leech oocytes*

As indicated by 3′RACE-RT–PCR, *Hro-twi* is present as a maternal transcript in leech oocytes. This result was unexpected, since *twist*-class genes in fly and vertebrates occur exclusively as zygotic transcripts (Thisse et al., 1987a; Hopwood et al., 1989; Wolf et al., 1991). Such a difference in temporal expression of homologous genes is not without precedent, however. For example, *snail* is another zygotically expressed gene that is required for mesoderm formation in *Drosophila* and for which homologs in other species have been identified. Zebrafish has two *snail*-class genes (*sna-1* and *sna-2*), both of which are present as maternal transcripts in the zygote (Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1993). Moreover, both *sna-1* and *sna-2* are widely expressed during the blastula stages and become restricted to the mesoderm only after involution.

Transcription of *twist* in *Drosophila* is activated zygotically by the product of the *dorsal* gene (Thisse et al., 1987b; Roth et al., 1989). *Dorsal* encodes a maternally inherited transcription factor involved in dorsal–ventral axis determination (Anderson, 1987; Roth et al., 1989). In ventral cells, dorsal protein is transported to the nucleus at high levels and interacts with the *twist* promoter to initiate transcription (Simpson, 1983; Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991). The maternal expression of *Hro-twi* mRNA suggests that the activation of *Hro-twi* transcription during leech oogenesis is independent of a *dorsal*-class gene.

4.3. *Implications of* Hro-twi *expression for mesoderm determination*

The presence of *Hro-twi* mRNA in embryonic stages prior to the segregation of distinct mesodermal, ectodermal and endodermal precursors is not unlike the pattern of *twist* expression in *Drosophila*. Prior to gastrulation in *Drosophila* embryogenesis, *twist* mRNA and protein are found around the anterior pole in prospective ectodermal and endodermal cells, as well as in the prospective mesoderm on the ventral side of the syncytial blastoderm (Thisse et al., 1987b, 1988). At this stage, mRNA and protein levels seem to be equal in all the cells of the ventral side of the embryo. At the onset of gastrulation, both the anterior midgut rudiment (endodermal and ectodermal tissues) and the mesodermal layer contain high levels of *twist* mRNA and protein (Thisse et al., 1988). Not until the end of gastrulation is twist localized exclusively to the mesodermal layer. Thus, the observation that *Hro-twi* mRNA is initially present in stages prior to the birth of the germ layers is reminiscent of the early *twist* expression in *Drosophila*.

Although it is possible that *Hro-twi* mRNA segregates unequally early on to one cell (CD) and at the fourth cleavage only to the mesodermal precursor (cell DM)

and to M teloblasts subsequently, our results do not eliminate the possibility of a widespread cellular distribution on both ectodermal and mesodermal precursors (cells DNOPQ and DM, respectively). To distinguish between these possibilities, elucidating the spatial distribution of *Hro-twi* mRNA and Hro-twi protein will be of great interest.

Acknowledgement

We thank D. Nardelli-Haefliger and M. Shankland for kindly providing the embryonic *H. robusta* cDNA library, and M. Leviten for assistance in constructing the *H. robusta* genomic library. We also thank M. Leviten, C. Smith, F.A. Ramirez and D. Isaksen for their helpful comments and suggestions on the manuscript. This work was supported by NSF Grants IBN 9105713 and IBN 9406141 and NIH Grant RO1 HD 23328 to D.A.W. J.G.S. was also supported in part by NIH Grant RO1 HD 23328-06A2S1.

References

Anderson, K.V., 1987. Dorsal–ventral pattern genes of *Drosophila*. Trends Genet. Sci. 3, 91–97.

Bissen, S.T., Weisblat, D.A., 1989. The durations and compositions of cell cycles in embryos of the leech, *Helobdella triserialis*. Development 106, 105–118.

Blair, S.S., Weisblat, D.A., 1984. Cell interactions in the developing epidermis of the leech *Helobdella triserialis*. Dev. Biol. 101, 318–325.

Bodmer, R., Jan, L.Y., Jan, Y.N., 1990. A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation in *Drosophila*. Development 110, 661–669.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Dynlacht, B.D., Attardi, L.D., Admon, A., Freeman, M., Tjian, R., 1989. Functional analysis of NTF-1, a developmentally regulated *Drosophila* transcription factor that binds neuronal cis elements. Genes Dev. 3, 1677–1688.

Fernández, J., 1980. Embryonic development of the glossiphoniid leech *Theromyzon rude*: characterization of developmental stages. Dev. Biol. 76, 245–262.

Fernández, J., Olea, N., Matte, C., 1987. Structure and development of the egg of the glossiphoniid leech *Theromyzon rude*: characterization of development stages and structures of the early cleaved egg. Development 100, 211–225.

Hammerschmidt, M., Nüsslein-Volhard, C., 1993. The expression of a zebrafish gene homologous to *Drosophila snail* suggests a conserved function in invertebrate and vertebrate gastrulation. Development 119, 1107–1118.

Holton, B., Wedeen, C.J., Astrow, S.H., Weisblat, D.A., 1994. Localization of polyadenylated RNAs during teloplasm formation and cleavage in leech embryos. Roux Arch Dev. Biol. 204, 46–53.

Hopwood, N.D., Pluck, A., Gurdon, G.B., 1989. A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neuronal crest. Cell 59, 893–903.

Jiang, J., Kosman, D., Ip, Y.T., Levine, M., 1991. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. Genes Dev. 5, 1881–1891.

Kamb, A., Weir, M., Ruby, B., Varmus, H., Kenyon, C., 1989. Identification of genes from pattern formation, tyrosine kinase and potassium channel families by DNA amplification. Proc. Natl. Acad. Sci. USA 86, 4372–4376.

Leptin, M., 1991. *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. Genes Dev. 5, 1568–1576.

Leptin, M., Casal, J., Grunewald, B., Reuter, R., 1992. Mechanisms of early *Drosophila* mesoderm formation. Development Suppl. 2, 23–31.

Murre, C., McCaw, S., Baltimore, D., 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *Myc* proteins. . Cell 56, 777–782.

Nelson, B.H., Weisblat, D.A., 1991. Conversion of ectoderm to mesoderm by cytoplasmic extrusion in leech embryos. Science 25, 435–438.

Nelson, B.H., Weisblat, D.A., 1992. Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo. Development 115, 103–115.

Pan, D., Huang, J.-D., Courey, A.J., 1991. Functional analysis of the *Drosophila twist* promoter reveals a *dorsal*-binding ventral activator region. Genes Dev. 5, 1892–1901.

Roth, S., Stein, D., Nüsslein-Volhard, C., 1989. A gradient of nuclear localization of the *dorsal* protein determines dorso-ventral pattern in the *Drosophila* embryo. Cell 59, 1189–1202.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandig, M., Dohle, W., 1988. The cleavage pattern in the leech *Theromyzon tessulatum* (Hirudinea, Glossiphoniidae). J. Morphol. 196, 217–252.

Simpson, P., 1983. Maternal–zygotic gene interactions during formation of dorso-ventral pattern in *Drosophila* embryos. Genetics 105, 615–632.

Thisse, B., El Messal, M., Perrin-Schmitt, F., 1987a. The *twist* gene: isolation of a *Drosophila* zygotic gene necessary for the establishment of dorso-ventral pattern. Nucleic Acids Res. 15, 3439–3453.

Thisse, B., Stoetzel, C., El Messal, M., Perrin-Schmitt, F., 1987b. Genes of *Drosophila* maternal dorsal group control the specific expression of the zygotic gene *twist* in presumptive mesodermal cells. Genes Dev. 1, 709–715.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., Perrin-Schmitt, F., 1988. Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. EMBO J. 7, 2175–2183.

Thisse, C., Perrin-Schmitt, F., Stoetzel, C., Thisse, B., 1991. Sequence specific transactivation of the *Drosophila twist* gene by the *dorsal* gene product. Cell 65, 1191–1201.

Thisse, C., Thisse, B., 1992. Dorsoventral development of the *Drosophila* embryo is controlled by a cascade of transcriptional regulators. Development (Suppl.), 173–181.

Thisse, C., Thisse, B., Schilling, T.F., Postlethwait, J., 1993. Structure of the zebrafish *snail1* gene and its expression in wildtype, *spadetail* and *no tail* mutant embryos. Development 119, 1203–1215.

Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., Perrin-Schmitt, F., 1991. The *M-twist* gene of *Mus* is expressed in subsets of mesodermal cells and its closely related to the *Xenopus-twi* and the *Drosophila twist* genes. Dev. Biol. 143, 363–373.