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Expression of H-twi protein in Helobdella leech embryos

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EXPRESSION OF H-TWI PROTEIN IN *HELOBDELLA* LEECH EMBRYOS

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By

Christine L. Nelson

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INFORMATION TO USERS

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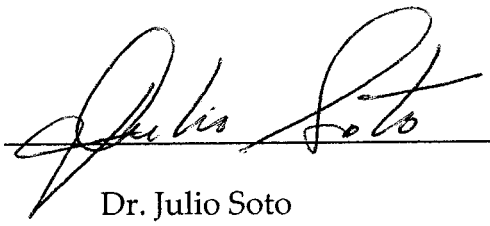

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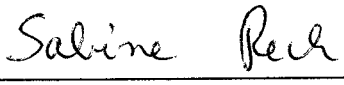

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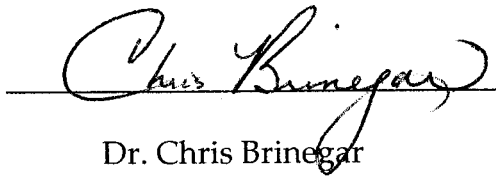

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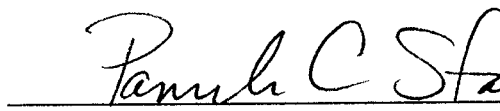

ABSTRACT

EXPRESSION OF H-TWI PROTEIN IN *HELOBDELLA* LEECH EMBRYOS

By Christine L. Nelson

Studying the distribution of H-twi protein is the first step toward understanding its function. The *Hro-twi* gene was sub-cloned into the expression vector pGEX-KG and induced to produce the Hro-twi recombinant protein. This protein was used for affinity purification of anti-Hro-twi polyclonal rabbit antibodies. Purified anti-Hro-twi antibodies were then used to target the H-twi protein in *Helobdella triserialis* leech embryos during embryogenesis up to mid gastrulation. H-twi protein was detected in all embryonic stages examined. The amount of H-twi detected cytoplasmically in the earliest stages under maternal control (stages 1 and 2) decreased during early cleavage stages (stages 3 and early 4b), then increased from late cleavage through mid gastrulation (late stage 4b to mid stage 8). By mid gastrulation, H-twi protein became nuclear. The spatial embryonic H-twi protein distribution was similar to that of *Drosophila* embryonic twist protein distribution, except that *Drosophila* twist was exclusively detected in the nuclei.

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Thank you to Dr. Julio Soto for giving me the opportunity to work on this project. Thank you to my fellow lab mates and other grad students for their sense of humor, discussions during difficult stages of research, and the fun we had. Thanks especially to Stephanie Mandel for helping me get started in the lab and to Hoan Tran for always listening and providing new insights. Thank you to the professors and technicians of the department for their helpful discussions and technical assistance, especially Dr. Sabine Rech, Dr. Chris Brinegar, Darcy Levee, Linda Olson, Dr. Jeff Honda, Dr. Steven White, Larry Young, Carol Selter, everyone in the Microbiology Service Center, Randy Kirchner, and Dr. Bill Murray. Thank you to my family and friends who gave me their support despite not completely understanding what I was doing. Finally, thank you to my partner, Heather Sadlon. Without her love and support over countless long days and nights of research and study, it would have been impossible to finish.

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Introduction

The presence of conserved domains in genes leads to the discovery of homologs in different species. However, similar domains do not necessarily mean that gene homologs will have the same specific functions. Differences in function are often found. Comparing these differences or similarities can lead to a greater understanding of evolution. For example, the Toll-Dorsal/IL-1R-NF-κB pathway is evolutionarily conserved between fruit flies and mammals for innate immunity but is also used for different developmental functions between the two taxa (reviews: Belvin and Anderson, 1996; Silverman and Maniatis, 2001). Fruit fly development has been studied extensively, allowing for the discovery of the functions of many developmental genes.

This study uses the leech embryonic model to study the protein product of the *twist* gene, found to be essential to mesoderm development in the fruit fly. Because the mesoderm is one of the three germ layers formed in early development, an understanding of its formation is an important problem in the field of developmental biology. Glossiphoniid leeches are useful for embryonic study because they have a highly stereotyped pattern of spiral development, forming predictably into 32 body segments (Irvine and Martingdale, 1996; Fernández, 1980). The main advantages to studying early development of leech

embryos are an adequate yolk supply for maintaining development, larger than average teloblasts displayed at the embryo surface, and the presence of easily traceable teloplasm (Fernández, 1980).

The formation and differentiation of the germ layers during embryogenesis is a complicated process which varies across species. The mechanism of embryonic development of segments in *Drosophila* (derived from a blastoderm synctium) differs from that in leeches (generated from blast cells), which may change the way in which homologs function in fruit flies and leeches. It is unknown whether the evolutionary origins of segmentation in the fruit fly (arthropods) and the leech (annelids) arose separately or from a common ancestor (Davis and Patel, 1999). Therefore, in a comparison of the mechanisms of segmentation between leeches and insects, one must keep in mind the evolutionary significance, as there are conflicts based on using comparative development versus molecular phylogeny for organizing the clades (Adoutte *et al.*, 2000; De Robertis, 1997).

Homologs of the gene *twist* have been studied in many species, including human (Wang *et al.*, 1997), mouse (O'Rourke and Tam, 2002), jellyfish (Spring *et al.*, 2000), fruit fly (Thisse *et al.*, 1987a, 1987b), and leech (Soto *et al.*, 1997). All *twist* genes studied have a conserved basic helix-loop-helix (bHLH) region that is

essential for regulation (Castanon and Baylies, 2002). However, the functions of *twist* across species appear to vary widely, from dorsoventral polarity determination and mesodermal fate determination in the fruit fly (Thisse *et al.*, 1987b) to neural tube closure and proper formation of head mesenchyme, somites, and limb buds in the mouse (Chen and Behringer, 1995).

Determining the protein expression pattern is the first step toward determining a gene's function. The objectives of this study were to determine the twist protein distribution in *Helobdella triserialis* embryos (H-twi) using whole-mount *in situ* hybridization of affinity-purified anti-Hro-twi polyclonal antibodies.

Review of Literature

Glossiphoniidae leech development

Leeches are invertebrates from the phylum Annelida and subclass Hirudinea. The genus *Helobdella* is within the Glossiphoniidae family. Leech embryos divide bilaterally and are convenient for study because they undergo fewer cell divisions before gastrulation, compared to the other organisms with radial symmetry. Therefore, it is easier to follow the fate of each blastula. Leeches are part of the *Bilateria* group, in the *Protosome* category. Organisms in this group develop via spiral cleavage (vs. radial cleavage for vertebrates). In spiral cleavage, the blast cells divide at slight angles to the polar axis of the embryo (Gilbert, 1997-for review).

The body of the adult leech consists of 32 segments and a prostomium (the head region). A characteristic of Glossiphoniid leech species is that they carry their embryos exteriorly under their dorsoventrally flattened bodies. When the eggs are fertilized, they stay at metaphase in meiosis I until they are moved to the cocoon under their mother (Fernández and Olea, 1995). Once in the external cocoon, meiosis is completed along with pronuclear fusion (Fernández and Olea, 1995). After the second polar body is released, the female pronucleus moves to

the center of the egg (Fernández and Olea, 1995).

Soon after polar bodies are extruded from the fertilized *Helobdella* egg, yolk-deficient cytoplasm (teloplasm) begins forming at the surface of the animal and vegetal poles (Holton *et al.*, 1994; Fernández *et al.*, 1987; Fernández, 1980). Teloplasm contains a high concentration of mitochondria, endoplasmic reticulum (Fernández *et al.*, 1987), and mRNAs (Holton *et al.*, 1994). The vegetal teloplasm (ventral side) forms before the animal teloplasm (dorsal side) (Fernández *et al.*, 1987; Fernández, 1980). During stage 1e to 1f, the teloplasm moves to the side of the egg from which cell CD will form, then the teloplasm starts moving from the outer part of the egg toward the internal part. By stage 2 (Figure 1), cell CD contains most of the teloplasm (Fernández *et al.*, 1987; Fernández, 1980). During the first three cell divisions, teloplasm segregates to cell D, then to DM and DNOPQ in late stage 4b (Figure 1).

In the developing animal embryo, one pole of the egg, the vegetal pole, is rich in yolk concentration, while the other pole, the animal pole, is low in yolk concentration. Cellular divisions tend to occur at a faster rate in the animal pole. In annelids, molluscs, mammals, and other species, yolk distribution is isolecithal; consequently, the cleavage pattern is holoblastic, or complete. Therefore, the cleavage furrow extends throughout the entire cytoplasm of the

egg, including any yolk-containing areas.

When fertilization occurs in animals, embryogenesis follows and is divided into the following characteristic stages of development. Directly upon fertilization in the multicellular animal, cleavage rapidly occurs, dividing the cytoplasm of the zygote into smaller cells, or blastomeres. Once mitosis slows down, gastrulation occurs. In this phase of development, the primary germ layers are formed as the blastomeres start to change their positions. In the leech embryo, as in all multicellular animals, there are three layers: ectoderm, mesoderm, and endoderm. Generally the ectoderm differentiates into the epidermis and nerve tissue, the mesoderm differentiates into blood cells, connective tissue, kidney, and muscle, and the endoderm differentiates into the gut and associated organs. In the leech, nephridia are mesodermally derived, as are genital primodia (Fernández, 1980). Once the germ layers are formed, organogenesis occurs.

Early experiments with *Helobdella* leech embryos showed that factors in the teloplasm were essential to the formation of mesoderm (Astrow *et al.*, 1987; Nelson and Weisblat, 1991). Experiments by Holton *et al.* (1994) showed that this may be mRNA. By performing in situ hybridizations with ^{3}H-polyuridylic acid (^{3}H-polyU), these researchers found that while cytoplasmic mRNA was initially

homogeneously distributed, it became concentrated in cells DM and DNOPQ at stage 4b when teloplasm was formed and remained concentrated in the teloplasm during early embryogenesis of the *Helobdella triserialis* leech.

Early development proceeds as follows in the *Helobdella* leech (Figure 1). Initially, the fertilized egg divides into two cells, AB and CD, at stage 2 and four cells at stage 3: A, B, C, and D (Bissen and Weisblat, 1989; Fernández, 1980). The endoderm arises from the three macromeres, A, B, and C, and eventually forms the luminal layer of the mature gut wall and the visceral mesoderm (Wedeen and Shankland, 1997). During stage 4a four micromeres, a, b, c, and d, are born. These cells give rise to the supraesophageal ganglion, the ectodermal coverings of the prostomium, muscle fibers of the proboscis, the proboscis sheath, and the epithelial covering of the proboscis (Weisblat *et al.*, 1984; Huang *et al.*, 2002). In stage 4b of leech embryogenesis, cell D, which is the largest of the four cells thus far, divides into the mesodermal and ectodermal precursor cells, DM and DNOPQ, respectively (Bissen and Weisblat, 1989; Huang *et al.*, 2002). This is the step where the fate of cell D diverges from cells A, B, and C. DNOPQ is closer to the animal pole than DM; consequently, it divides into pairs of ectodermal stem cells N, O, P, and Q, plus 13 micromeres. DM merely divides into one pair of mesodermal stem cells (mesoteloblasts) and two micromeres (Sandig and Dohle,

1988). Each teloblast then unequally divides into smaller blast cells, forming a chain or bandlet of these cells, the germinal bandlet, which occurs in stage 7 (Zackson, 1982). Also during this stage, the cells derived from the original micromere quartet begin moving around the animal pole, eventually grouping into d'/c' and a'/b' clones while lengthening and separating symmetrically around the area of the germinal plate (Nardelli-Haefliger and Shankland, 1993). At the start of stage 8, the germinal bandlets merge, forming the germinal bands (Fernández, 1980). These two chains of blast cells join together down the ventral midline in early stage 8, starting at the head, forming the germinal plate (Zackson, 1982). The germinal bands are the precursors to the segmental tissues (Weisblat *et al.*, 1984) and are covered by the provisional squamous epithelium in the process of epiboly that starts in stage 7. This temporary covering is derived from the micromeres and is lost by the end of stage 10 (Kostriken and Weisblat, 1992). By the end of stage 8, segmentation first appears in the mesoteloblasts soon after the germinal band coalesces, and somites begin to appear (Zackson, 1982; Fernández, 1980). The anterior segments start forming before the posterior segments (Weisblat *et al.*, 1984). Formation of the endoderm begins after segmentation (Zackson, 1982). In addition, organogenesis of the prostomium occurs when the micromere-derived clones surrounding the prostomium move

and become the outer epidermal layer and internal neural tissue (the supraesophageal ganglion) (Nardelli-Haefliger and Shankland, 1993). During stages 9-10, the embryo lengthens and the germinal plate grows to surround the midgut primordium (Nardelli-Haefliger and Shankland, 1993). By the end of stage 9, the 32 blocks of mesodermal tissue are formed (Fernández, 1980). By stage 10, the intestine and rectum are formed (Nardelli-Haefliger and Shankland, 1993).

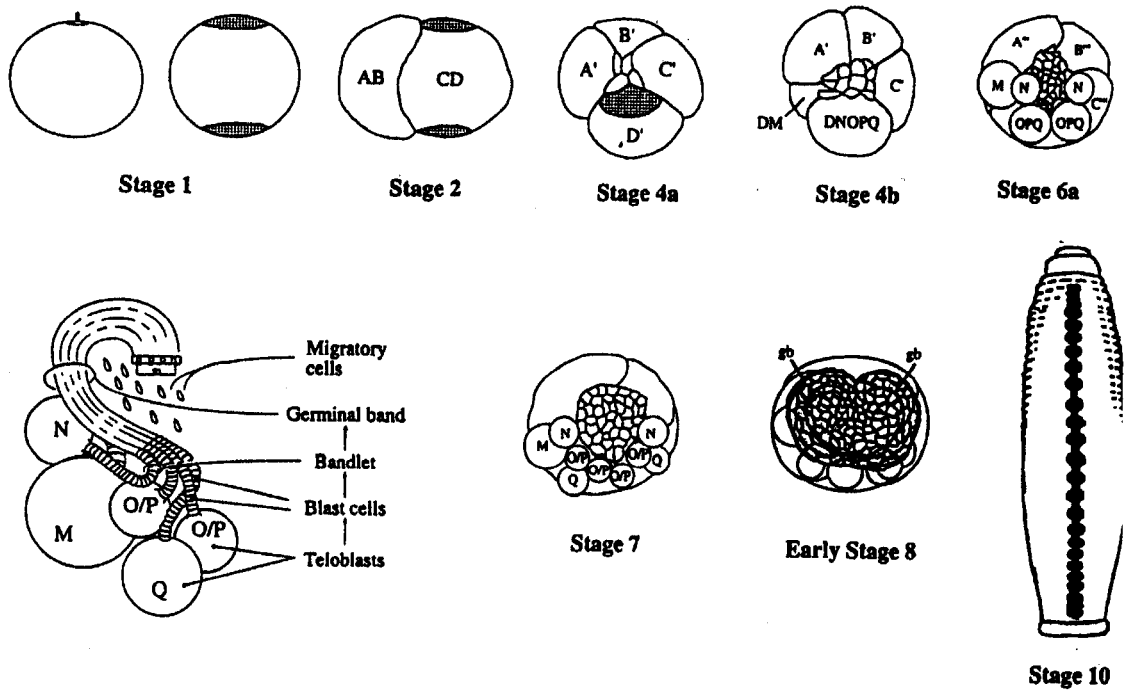

Figure 1. Schematic representation of relevant stages of development in early embryogenesis of the leech *Helobdella* (Stages 1-early 8 shown from the animal pole/dorsal view; stage 10 shown from the ventral view. Teloplasm shown as shaded regions). Stage 1: teloplasm formation at animal and vegetal poles after polar body formation. Stage 2: two cell stage with teloplasm in cell CD. Stage 4a: 8 cell stage with four macromeres and micromere quartet. Stage 4b: DNOPQ ectodermal precursor and DM mesodermal precursor are born with early micromere cap on top. Stage 6a: NOPQ divides into OPQ and N paired teloblasts, 25 micromeres (16 from cell D). Stage 7 early: O/P teloblasts are formed and provisional epithelium begins spreading (epiboly). Early stage 8: germinal bands and provisional epithelium are growing and expanding. Stage 10: coelomic cavities are forming, segmental tissues arise during differentiation of germinal plate, body tube is enclosed by germinal plate (diagram used with permission by J.G. Soto).

To summarize, the DNOPQ cells produce segmental epidermis, neurons, and glia. The DM (or M) cells produce segmental muscle, nephridia, and some neurons (Gliezer and Stent, 1993). The micromeres eventually develop into the components of the head such as the epidermis covering the prostomium and other nonsegmental tissues (Ho and Weisblat, 1987; Weisblat *et al.*, 1984;

Nardelli-Haefliger and Shankland, 1993). The origins of the reproductive system in the leech have yet to be determined; however, a recent study by Kang *et al.* (2002) provides evidence that adult leech testisacs arise from cell clusters derived from segmental mesoderm. For a recent overview of leech development, see Weisblat and Huang (2001).

Mesoderm development in invertebrates

Normal mesoderm development proceeds in a typical manner in *Drosophila,* beginning with the formation of the ventral furrow, which contain the precursors of the mesoderm. This movement occurs during gastrulation when the ventral cells move internally, caused by apical constriction of the cells. Concurrent basal migration of the nuclei completes the formation of the ventral furrow (Leptin and Grunewald, 1990). After gastrulation, pre-mesodermal cells form a monolayer under the ectodermal layer (Campos-Ortega and Hartenstein, 1985) and spread dorsolaterally, stopping at the edge of the dorsal ectoderm and amnioserosa (Shishido *et al.*, 1997). After this, segmentation occurs (Borkowski *et al.*, 1995), then the beginning of specialization of different muscle types and structures, such as heart and somatic muscles (Baylies and Bate, 1996). Examples of structures formed by mesoderm in *Drosophila* include tracheal pits, the heart,

and dorsal oblique muscles (Borkowski *et al.* 1995).

The pair of DM cells formed early in leech development (stage 4b) are the precursors for the mesodermal layer. Through the remaining cleavage stages 5, 6a, 6b, and 7, teloblast DM produces two micromeres then begins dividing into smaller cells, forming the M bandlets, which have already begun forming segments (Zackson, 1982). By stage 8, the M bandlets lie on top of the macromeres A, B, and C, and below the ectodermal bandlets within the germinal bands. As the cells continue to reproduce, lengthening the bandlets, the germinal bands become parallel to each other, developing into the germinal plate (Weisblat and Huang, 2001). The segmental mesoderm are derived from this plate (Weisblat *et al.*, 1984). During stage 9, 32 segmented somites and ventral neuromeres arise. These 32 metameres are derived from homologous pairs of mesodermal and ectodermal blast cells (Weisblat and Shankland, 1985). Through stage 11, differentiation continues, and the coelom forms after cavitation of the mesodermal hemisomites (Isaksen *et al.*, 1999; Liu, *et al.*, 1998; Nardelli-Haefliger and Shankland, 1993). Muscles, some ganglionic neurons not associated with the ectoderm, and nephridia are some of the organs derived from the segmental mesoderm (Torrence and Stuart, 1986; Kramer and Weisblat, 1985; Weisblat and Shankland, 1985). Connective tissue and muscle fibers in the head of the leech

are also derived from M cells (Gleizer and Stent, 1993).

Interaction of the three germ layers is necessary for their proper formation. For example, without contact with the ectoderm, proper dorsal mesoderm subdivision is prevented, inhibiting the formation of the heart and visceral muscles (Baker and Schubiger, 1995). The presence of an intact mesoderm is important for the formation of the endoderm, as shown by Wedeen and Shankland (1997), who found through ablation experiments with *Helobdella* that the mesoderm is needed as a substrate for endodermal migration during its formation. However, Gleizer and Stent (1993) found that in *Thermyzon rude* leech embryos the development of segmental identity in the mesoderm is due to a cell-intrinsic mechanism (lineage history), as opposed to a cell-extrinsic mechanism (interactions with surrounding cells).

Genes involved in mesoderm formation in *Drosophila*

In *Drosophila,* there are at least three major genes necessary for the proper sectioning of the mesoderm (Shishido *et al.*, 1997). These include *decapentaplegic (Dpp)* for visceral mesoderm and heart (Frasch, 1995), the contesting functions of *hedgehog (hh)* and *wingless (wg)* for anterior-posterior mesoderm development (Azpiazu *et al.*, 1996), and *twist*. *Twist,* a myogenic transcription regulator, is

necessary for mesoderm development and gastrulation and is transcribed in the zygote (Castanon and Baylies, 2002; Thisse *et al.*, 1987a; Baylies and Bate, 1996). *Drosophila* embryos lacking *twist* do not form mesoderm; therefore, they cannot form a ventral furrow (Thisse *et al.*, 1988). It appears that several other genes are also important to proper mesoderm formation. *Tin*, essential to heart and visceral muscle development, is expressed in early mesodermal cells in *Drosophila* (Shishido *et al.*, 1997; Bodmer, 1993; Azpiazu and Frasch, 1993) and is activated by *twist* (Bodmer *et al.*, 1990). In addition, Casal and Leptin (1996) used a subtractive cDNA library and found 13 genes associated with mesoderm development in *Drosophila* embryos. Evidence for regulation by genes *tailless, bicoid, twist, snail,* and *dorsal* was found.

Two other *Drosophila* genes are important to mesodermal and dorsoventral axis development, *dorsal* and *snail*. These two genes, along with *twist,* are regulated by the *toll* signaling pathway (Belvin and Anderson, 1996). *Snail* is activated when dorsal binds to its promoter (Rusch and Levine, 1996). In ventral cells, *snail* is necessary for a gene expression pattern essential to proper mesoderm development (Leptin, 1991). However, as discussed in more detail later, recent study of leech *dorsal* and *snail* do not show evidence for a similar role (Goldstein *et al.*, 2001).

Toll regulates *twist, snail,* and *dorsal* expression in fruit fly embryos. In an early study, absence of *toll* in *Drosophila* embryos resulted in dorsalized embryos, where the embryos became completely made up of structures normally found only in the dorsal region of the embryo (Anderson *et al.,* 1985). This occurred because dorsal-ventral polarity was lost. In the toll-dorsal signaling pathway, toll, a receptor protein spanning the cell membrane, is activated extracellularly, initiating a chain of events leading to translocation of dorsal to the nucleus. Toll activates tube, a cytoplasmic protein, which activates pelle, a cytoplasmic serine/threonine kinase, which activates cactus, a cytoplasmic protein that binds two dorsal molecules (Hashimoto *et al.,* 1988; Kidd, 1992; Shelton and Wasserman, 1993; Belvin and Anderson, 1996). Activation of cactus releases dorsal, allowing dorsal translocation into the nucleus. Dorsal translocation is followed by *twist* and *snail* activation (Belvin and Anderson, 1996; Gross *et al.,* 1996; Anderson, 1987; Huang *et al.,* 1997). In *toll -/- Drosophila* embryos, overexpression of *twist* occurs, resulting in a transition of ectodermal cells to mesodermal derivatives (Furlong *et al.,* 2001).

As a homolog to the mammalian interleukin-1 receptor (IL-1R)-NF-κB pathway, the toll-dorsal pathway is also implicated in the *Drosophila* immune response (Belvin and Anderson, 1996). *Dorsal* is a *rel*-domain transcription factor

homologous to NF-κB of the IL-1R- NF-κB mammalian pathway (Steward, 1987; Belvin and Anderson, 1996). Fruit fly *dorsal*, mammalian *NF-κB*, and human *c-rel* have homologous DNA-binding and dimerization motifs (Thisse *et al.*, 1991; Steward, 1987; Ghosh *et al.*, 1990).

Dorsal is one of the genes necessary for proper dorsoventral development in the *Drosophila* embryo (Roth *et al.*, 1989). *Dorsal* was cloned by Steward *et al.* (1984), who found that it was transcribed in adult *Drosophila* females in the ovary, where *dorsal* mRNA accumulates in the maturing egg but not in adult males. Steward concluded that *dorsal* was transcribed only during oogenesis, because the transcript was degraded in 2.5-5 hour embryos. Dorsal protein was detected in the nuclei during the cleavage stages on the ventral side of the embryo (Roth *et al.*, 1989)

Thisse *et al.* (1987b, 1991) found that dorsal controls the expression of *twist*, as *dorsal -/- Drosophila* embryos were lacking *twist* mRNA. *Twist* is one of the initial genes stimulated by the dorsal gradient and is not detected in the newly fertilized egg of the fly until after *dorsal* is detected (Thisse *et al.*, 1988). A high concentration of dorsal appears to activate *twist*, while intermediate levels do not activate *twist* but instead repress *zen* (Roth *et al.*, 1989). Roth *et al.* studied the protein pattern of *twist* and *zen* in dorsalized, ventralized, or lateralized

Drosophila embryos and found that high *dorsal* concentrations are needed for development of the mesoderm (ventral), intermediate concentrations for the neuroectoderm (ventrolateral), and low concentrations for the dorsal ectoderm (dorsal).

Dorsal protein binds to the *Drosophila twist* promoter as a homodimer (Jiang and Levine, 1993) at eight sites and activates *twist* expression by binding to regulatory sequences (Thisse *et al.*, 1991). Thisse *et al.* also demonstrated that the 3' region of *Drosophila twist* was not associated with activation by *dorsal*. In addition, dorsal activates *snail* expression by binding to its promoter (Rusch and Levine, 1996). Activation of *dorsal* target genes such as *snail* and *twist* may involve the entire TFIID initiator complex, and TAFII110 and TAFII60 are needed for *snail* and *twist* transcription in *Drosophila* embryos (Zhou *et al.*, 1998). For a review on *Drosophila dorsal,* see Stathopoulos and Levine (2002).

The zygotic gene *snail (sna),* a zinc finger transcription factor, is essential to the formation of *Drosophila* mesoderm, as embryos mutant for this gene are abnormal by the beginning of gastrulation and do not form the ventral furrow (Reuter and Leptin, 1994; Nüsslein-Volhard *et al.*, 1984; Grau *et al.*, 1984). In addition, *snail* contributes to dorsoventral axis development and is essential to proper neurogenesis (Simpson, 1983; Grau *et al.*, 1984; Nüsslein-Volhard *et al.*,

1984; Ashraf *et al.*, 1999). Grau *et al.* (1984) found that *Drosophila snail* mutants often died by the end of embryogenesis and failed to form normal ectoderm and internal organs or ventral furrows. Snail acts as a repressor of neuroectodermal genes, such as *sim* and *rho*. Proper mesoderm-specific gene expression occurs only by repression of ventrolateral genes in the mesoderm by snail (Hemavathy *et al.*, 1997; Leptin, 1991). Missense and nonsense mutations causing significant changes to the translation product in the zinc finger areas of *snail* lead to de-repression of target genes (Hemavathy *et al.*, 1997; Boulay *et al.*, 1987).

Dorsal and twist synergistically activate *snail* expression in the mesoderm. Ip *et al* (1992) found that the area around the *snail* promoter has dorsal and twist binding sites. Further study by Jiang and Levine (1993) showed that mutations to either of these binding sites reduced the expression of *snail*. However, other alterations to the twist and dorsal binding sites in the *snail* promoter showed that twist alone could not activate *snail;* dorsal alone can activate *snail* expression, but expression was enhanced by the presence of twist (Szymanski and Levine, 1995).

Sim is a bHLH-PAS transcription factor that is needed for mesectoderm differentiation (Nambu *et al.*, 1991; Crews *et al.*, 1988). The *sim* promoter area has dorsal, twist, snail, and daughterless::scute (Da::Sc) binding sites. Results by Kasai *et al.* (1998) indicated that dorsal, twist, and snail E-box binding sites

control mesectodermal transcription. Dorsal, combined with notch, activates *sim* (Cowden and Levine, 2002). *Sim* is also activated by snail in cells low in twist and dorsal (Morel *et al.*, 2003; Cowden and Levine, 2002). Snail probably represses *sim* transcription in the mesoderm, as three out of seven *snail* binding sites found by Kasai *et al.* contain E-boxes (Kasai *et al.*, 1998; Kasai *et al.*, 1992). Regulation of *sim* expression by snail has recently been found to depend on suppressor of hairless [Su(H)], of which there are binding sites for in the *sim* promoter (Morel, *et al.*, 2003).

Twist and *snail* may have a role in human cancer

In addition to their role in early development, *twist* and *snail* may have a role in tumor growth in humans. E-cadherin suppresses tumor growth in humans (Hirohashi, 1998). During embryonic development, the mechanism of epithelial-mesenchymal transition (EMT) is mediated by cadherin cell adhesion (Boyer *et al.*, 2000). When a fibroblastic phenotype is reached, E-cadherin is lost, allowing dissociation and consequently migration of cells, necessary for proper gastrulation but possibly leading to early growth of carcinoma cells (Boyer *et al.*, 2000). Snail and Smad interacting protein 1 (SIP1) are repressors of E-cadherin transcription (Cano *et al*, 2000; Comijn *et al.*, 2001), while twist initiates *N-cadherin*

expression in *Drosophila* (Oda *et al.*, 1998). N-cadherin is a protein that can aid movement of tumor cells (Li *et al.*, 2001). Rosivatz *et al.* (2002) found in human tumor tissue that *E-cadherin* was down-regulated, while *snail*, *twist*, and *N-cadherin* were up-regulated. They concluded that snail, as a repressor of *E-cadherin*, and twist, as an activator of *N-cadherin*, may aid in the growth and spread of certain human tumors.

Twist structure and function in *Drosophila*

Thisse *et al.* (1987b) isolated the *twist* gene in *Drosophila*. Mutagenesis experiments with 26 *twist* alleles showed that all embryos with the mutated alleles died before hatching. The dead embryos were all twisted and *twist* -/- embryos were abnormally gastrulated. *Drosophila twist* has a small intron of 120 bp at the 3′ end of the gene, and a mRNA length of 1878 bp, not including the poly(A) tail, resulting in a protein of 490 amino acids (Thisse *et al.* 1987b; Thisse *et al.*, 1988).

In *Drosophila*, *twist* is critical for proper mesoderm development, acting as a bHLH transcription factor (Thisse *et al.*, 1988). The HLH region regulates protein dimerization, whereas the basic region is used for DNA contact by the dimers (Murre *et al.*, 1989a; Murre *et al.*, 1989b). *Drosophila's twist* promoter

contains two dorsal activation binding sites. A proximal enhancer (PE) is a 250 bp region upstream to the transcription site (Thisse *et al.*, 1991; Pan *et al.*, 1991; Jiang *et al.*, 1991; Jiang and Levine, 1993). A distal element (DE) is within the 1.26 kbp promoter and is also necessary for normal *twist* expression (Thisse *et al.*, 1991; Pan *et al.*, 1991).

In flies, twist homodimerizes with itself and heterodimerizes with daughterless (Da), an E protein homolog (Castanon *et al.*, 2001). The N-terminal region of twist may activate transcription (Chung *et al.*, 1996). Because Thisse *et al.* (1988) found by Northern blot and later, anti-twist antibody detection, that *Drosophila* embryos accumulate twist protein at the same time as *twist* transcripts, the *twist* gene is probably not post-transcriptionally regulated.

In *Drosophila, twist* is required for mesoderm formation and further specialized development (Simpson, 1983; Leptin, 1991), and its RNA and protein is found in the ventral furrow and other areas of the mesoderm (Thisse *et al.*, 1987a). Depending on which protein it dimerizes with, twist can either activate or repress somatic muscle development (Castanon *et al*, 2001). Furlong *et al.* (2001) found that in *Drosophila* embryos, twist increased the activity of 130 genes, while 150 genes were repressed by twist. For example, twist homodimers activate *heartless (htl),* a fibroblast growth factor (FGF) receptor necessary for

migration of the mesoderm in embryonic development (Shishido *et al.*, 1997; Castanon *et al.*, 2001). Twist homodimers also activate *tinman (tin),* needed for visceral muscle and heart development (Bodmer, 1993; Castanon *et al.*, 2001) and *Mef2,* needed for muscle differentiation (Taylor *et al.*, 1995; Castanon *et al.*, 2001). On the other hand, twist and daughterless (Da) heterodimers repress *Mef2,* inhibiting myogenesis (Castanon *et al.*, 2001). These heterodimers also repress *tin* and *htl* (Castanon *et al.*, 2001).

A study by Baylies and Bate (1996) showed that the regulation of twist protein levels in the mesoderm controlled the development and differentiation of particular mesodermal derivatives. When twist levels were forced to be high in cells that normally maintained low levels, somatic muscle development remained normal; however, heart and visceral muscle development became abnormal (i.e., heart cells were missing). When twist levels were forced to be low in cells that normally maintained higher levels, the reverse occurred. Somatic muscle differentiation was abnormal, but heart and visceral development was comparable to wild-type development.

Twist homologs in other organisms

Twist has also been studied in many other organisms. This research

shows that twist is often concentrated in the ectoderm and mesoderm of developing embryos. For example, in the developing jellyfish, podocoryne *twist* is expressed in the entocodon, a cell layer with similarities to the mesoderm that differentiates into smooth and striated muscle, and is also expressed in the cell layers which differentiate into non-muscle tissues (Spring *et al.*, 2000). Wang *et al.* (1997) have shown human twist to be expressed most strongly in the placenta. They found human twist also expressed in mesodermally-derived tissues, such as the adult heart and skeletal muscle, not in ectodermally-derived tissues such as the brain. A study on *Caenorhabditis elegans twist, Ce-Twist,* suggests that the protein is involved in postembryonic mesoderm differentiation (Harfe *et al.*, 1998). For example, Ce-Twist was detected in some "neuron-like" cells in the head and muscles associated with the intestine.

A *twist* homolog in the leech

Hro-twi is the *Helobdella robusta* leech homolog of the *twist* gene, isolated and cloned from genomic DNA by Soto *et al.* (1997). By using degenerate deoxyoligonucleotides corresponding to the conserved amino acid sequences of *twist* homologs in fruit fly, frog, and mouse, the *Hro-twi* gene was amplified from cDNA using PCR. Analysis by Southern blot showed that there is only one *Hro-*

twi gene.

Hro-twi, like the other *twist* homologs, encodes a bHLH transcription factor and shares a 78% identity with *Drosophila twist* in the bHLH region (Thisse *et al.*, 1988; Soto *et al.*, 1997; Castanon and Baylies, 2002-review). Also, *Hro-twi* has comparable potential glycosylation sites, an Arg/Lys-rich region, and CAX lengths (Soto *et al.*, 1997). However, *Hro-twi* has a third CAX area that is not found in *Drosophila* (Soto *et al.*, 1997). The bHLH domain is highly conserved among the other *twist* class genes studied (Soto *et al.*, 1997; Adjaye *et al.*, 1999; Spring *et al.*, 2000). As mentioned earlier, proteins with bHLH domains form homodimers and heterodimers with other bHLH proteins (Thain and Hickman, 1996). They activate the transcription of genes by binding to the E-box consensus sequence (CANNTG) in muscle gene promoters and enhancers (Olson and Klein, 1994).

Staining of *H. robusta* embryos showed that *Hro-twi* mRNA was present in all leech embryonic stages examined in the study, including the oocyte, indicating that *Hro-twi* is maternally inherited (Soto, 1994). *Hro-twi* mRNA localized to the teloplasm at its formation in stage 1 and remained associated with the teloplasm until early stage 8, when it was found in the mesodermal germinal bands and some micromere derivatives. By stage 10, *Hro-twi*

transcripts were detected in ganglionic fibers, hemisomites, and in the mesenchymal tissue of the head region (Soto, 1994).

Leech genes isolated and studied

Many *Helobdella* homologs to *Drosophila* genes have been isolated. While some leech homologs may have functions similar to fruit fly genes, most show evidence for diverging functions. In addition, some leech homologs show more similarity in function to nematode homologs.

Two genes closely associated with *Drosophila twist* and mesoderm development have been isolated in *Helobdella.* Goldstein *et al.* (2001) cloned a dorsal-class gene (*Hro-dl)* and two snail-class genes (*Hro-sna1* and *Hro-sna2)* from *Helobdella robusta* genomic DNA. In *Drosophila, dorsal* regulates *snail* and plays a role in dorsoventral axis and mesoderm development, as well as innate immunity (Belvin and Anderson, 1996; Rusch and Levine, 1996; Manfruelli *et al.*, 1999). Snail plays a role in dorsoventral axis, mesoderm and nervous system development (Simpson, 1983; Nüsslein-Volhard *et al.*, 1984; Ashraf *et al.*, 1999). Goldstein *et al.* (2001), however, did not find evidence for a similar role by the leech homologs, but instead found evidence that both *dorsal* and *snail* function in mesoderm development and cell diversification during segmentation. In

addition, their results do not exclude the possibility that *Hro-dorsal* regulates *Hro-snail,* as with *Drosophila dorsal* and *snail.* The *dorsal* product they amplified, 525 bp and 175 amino acids, showed a significant (56%) homology to the conserved *rel*-domain of *Drosophila dorsal.* Their *snail* product, amplified from genomic leech DNA, was 2.0 kbp and 203 amino acids, with four putative zinc fingers. This sequence showed significant (66%) homology to the conserved regions of *Drosophila snail* as well as significant (77%) homology between the N-region of the snail protein in *Drosophila.*

Protein staining patterns for leech dorsal and snail were identical except that staining for snail occurred after dorsal staining in early- to post-gastrulation (stages 7-9) in blast cell nuclei and the germinal plate, supporting the possibility that dorsal activates *snail,* as shown by temporal expression patterns in stages 1-10 of the *Helobdella* leech embryo (Goldstein *et al.,* 2001). High levels of dorsal protein were seen in pre- to early-gastrulation (stages 6b-7), as nuclear staining in all micromeres; dorsal detection in pre-gastrulation stages occurred in the teloplasm in all stages but was weak. Through gastrulation stages, dorsal protein was detected in all blast cell nuclei and their descendants. By post-gastrulation (stage 9), detection of dorsal protein in blast cell nuclei decreased; however, stripes of cytoplasmic staining in the germinal plate appeared in cells

adjacent to the putative nervous system. In addition, cytoplasmic staining in the provisional integument shifted to nuclear staining in older cells (more developmentally advanced). Snail protein detection was identical, except that staining in the blast cell nuclei for snail was not apparent until hours later than dorsal staining, and staining for snail in the germinal plate did not occur after dorsal nuclear staining was seen. This supports results by Rusch and Levine (1996) for *Drosophila dorsal* and *snail,* where dorsal activates *snail;* however, their data did not support a function for *dorsal* and *snail* in dorsalventral axis development. On the other hand, a function in mesoderm development is not refuted (nor is a function in innate immunity). In addition, their findings support the idea that leech dorsal and snail may function in cell diversification within segment primordium, because stripes of nuclear staining were seen during segmentation.

Song *et al.* (2002) isolated leech homologs to *Drosophila's* pair-rule *even-skipped (eve)* from *Helobdella robusta* and *Theromyzon trizonare,* and found a 73-91% homology to *Drosophila eve.* In early *Drosophila* embryos *eve* is expressed in a repetitive pattern of 14 transverse stripes, as well as neuronally in later embryogenesis (Frasch *et al.,* 1987; Duman-Scheel and Patel, 1999). Song *et al.* did not find evidence to support the pair rule function, because chromatin-associated

Hro-eve mRNA was evenly dispersed in primary blast cells and micromeres during gastrulation and not in any alternating repetitive pattern. Their RT-PCR experiments showed that cytoplasmic *Hro-eve* was expressed repetitively in segmentally-derived neurons of the ventral nerve cord of stage 9-11 embryos. In addition, blocking *Hro-eve* transcripts disrupted neuronal differentiation in late embryogenesis (stages 10 and 11). Other knockdown experiments in early development showed that normal development of teloblast and primary blast cells was disturbed. Therefore, separate roles for *Hro-eve* in early development as well as neurogenesis are possible.

The *hunchback (hb)* gene encodes for a zinc finger transcription factor and is needed for anteriorposterior segmental development in the *Drosophila* embryo (Tautz *et al.*, 1987). *Hb* is anteriorly activated by bicoid (Simpson-Brose *et al.*, 1994; Driever *et al.*, 1989) and repressed by nanos and pumilio posteriorly (Murata and Wharton, 1995). In the *Drosophila* embryo, *hb* RNA is expressed in a gradient during segmentation. The *H. triserialis* leech homolog to *hb*, renamed Leech Zinc Finger II (LZF2) has 5 zinc finger motifs and shows a 69% homology to the conserved zinc finger region in *Drosophila hb* (Savage and Shankland, 1996; Iwasa *et al.*, 2000). Iwasa *et al.* (2000) subcloned the leech homolog *LZF2* into an expression vector to generate polyclonal anti-LZF2 antibodies. Immunostaining

of leech embryos did not show the gradient pattern seen in fruit fly embryos. Rather, although *LZF2* mRNA was found in ectodermal and mesodermal precursors during early development and through gastrulation, the pattern was uniform. Interestingly, LZF2 protein was found only in ectodermal precursors in early development through gastrulation, such as in the provisional epithelium and prostomium in stage 8. In addition, it was detected in the cytoplasm as well as the nucleus (the expected site). During organogenesis (i.e., stage 10), LZF2 was detected in neuronal cells. A similar expression pattern is also seen in the *hunchback-like* gene, *hbl-1*, found in nematodes (Fay *et al.*, 1999). Although *LZF2* function in the leech is currently unknown, evidence from this research does not support a function in anterior-posterior development, as found in *Drosophila.*

Nanos, which controls anterior-posterior polarity in fruit flies (Curtis *et al.*, 1995) and represses *hunchback* to allow abdomen development (Irish *et al.*, 1989), was initially cloned from *Helobdella robusta* leeches by Pilon and Weisblat (1997). In *Drosophila* embryos, *nanos* also is needed for proper migration of germline cells to the somatic gonad and for germline stem cell maintenance in later stages of development (Forbes and Lehmann, 1998). Pilon and Weisblat (1997), finding a 66% homology of *Hro-nanos* to *Drosophila nanos,* gathered evidence to support a function for *Hro-nanos* in embryonic polarity. Their specific findings showed that

Hro-nanos was maternally produced, with the transcript detectable up to late cleavage (stage 6a), then it was zygotically transcribed from late gastrulation to organogenesis (stages 8-10). These transcripts were detected as paired spots in segments M8-18 in stages 9-10, which are derived from segmental mesoderm. Segments M8-13 may develop into adult testisacs.

Analysis by western blot of the nanos protein showed a slightly different pattern, that it was present in all stages through gastrulation (stages 2-8), peaking at stage 4b, where the ectodermal and mesodermal teloblasts are born (Kang *et al.*, 2002). The protein was present in a higher concentration in the ectodermal precursor (DNOPQ) than the mesodermal precursor (DM). A possible explanation for the higher concentration of protein in cell DNOPQ is that the inheritance of a larger pool of teloplasm leads to higher activation of translation, possibly also due to cortically associated maternal mRNA (Nelson and Weisblat, 1991). To clarify whether *Hro-nanos* functions in embryonic polarity development, more characterization is necessary with the leech gene *pumilio (pum),* because in *Drosophila,* this homolog works with nanos during anterior-posterior embryonic development (Barker *et al.*, 1992).

Master *et al.* (1996) isolated the muscle segment homeobox gene homolog from *H. robusta* and *H. triserialis* embryos, *Le-msx,* finding a 90% homology in the

conserved homeodomain to fruit fly *msh.* In *Drosophila, msh* transcripts are found in the early embryo in the ectoderm and in muscle and peripheral nervous system precursors. In the later embryo, *msh* transcripts are found in the central nervous system and the somatic mesoderm (i.e., in segmental muscle cells) (Lord *et al.,* 1995). *Le-msx* mRNA was detected in mesodermal and ectodermal precursors, including pre-epidermis and central nervous system cells. This general pattern of expression was not seen in *Drosophila;* therefore, protein expression data are necessary, because the protein distribution may differ.

Materials and Methods

Embryos

Helobdella triserialis leeches were collected from ponds at a sturgeon farm near Sacramento, California. Embryos were obtained from gravid leeches kept in 1% seawater at room temperature. If further development after extraction was required, embryos were allowed to develop in 1% sterile seawater at room temperature until the desired stage was reached. Standard staging criteria were used (Weisblat and Huang, 2001; Fernández, 1980). The following steps of the project used genomic DNA from *H. robusta (Hro)* and polyclonal antibodies produced using *Hro* antigen. The species (*triserialis* vs. *robusta)* difference was considered nominal based on former studies that found similar results irrespective of leech species (Kostriken and Weisblat, 1992; Master *et al.*, 1996; Kourakis *et al.*, 1997). However, Huang *et al.* (2002) found some differences in micromere lineage between two leech species originally thought to be the same species, *H. robusta* (Sacramento) and *H.* sp. (Galt). This may be pertinent because H-twi is detected in the micromeres in this study.

Polyclonal serum production

Polyclonal rabbit serum was obtained from J. Soto (Babco labs, Berkeley,

Caliornia). He had injected three rabbits with full-length Hro-twi recombinant protein and obtained three bleeds. The polyclonal antibodies used for this experiment were obtained from the second bleed. Before affinity purification, these antibodies were tested against Hro-twi to verify their specificity (data not shown).

Subcloning of the *Hro-twi* gene into an expression vector

The full-length gene was subcloned directly from *Helobdella robusta (Hro)*-genomic DNA, using *PfuUltra Hot-Start Polymerase* (Stratagene), a high fidelity polymerase, to PCR-amplify the sequence. The forward primer (TW16/R1; 31 bp) had the following sequence (underlined area shows *EcoRI* restriction site: 5'CCGGAATTCTAATCATGTCACCATCTTTTCC3'. The reverse primer (TW2/HindIII; 30 bp) had the following sequence (underlined area shows *HindIII* restriction site): 5'ACGCAAGCTTTACCCCAACTTGTCCGAGGA3'. The forward primer started at nucleotide #79 (Soto *et al.*, 1997), the first methionine, and contained an *EcoRI* linker. The reverse primer started at nucleotide #1006 so that the total number of nucleotides amplified was 927 bp. The reverse primer, with a linker for *HindIII*, allowed for directional cloning.

PCR conditions were: 1X *PfuUltra* buffer (contained $MgCl_2$), 0.2 mM each

dNTP, 0.4 μM each primer, 220 ng *H. robusta* genomic DNA, and 1.25 units *PfuUltra Hot-Start Polymerase* in a 25 μl volume. PCR amplification was carried out at 94°C for two minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for one minute, 72° for 75 seconds, and a final extension of 72° for 10 minutes.

The *Hro-twi* PCR product was ligated into the expression vector, pGEX-KG, then plasmid constructs were transformed into DH5α *E. coli* cloning cells that had been made competent using the $CaCl_2$ chemical method. Colonies grown on LB agar plates with 50 μg/ml ampicillin were screened for the insert using PCR (*Taq polymerase,* Promega). Prior to the PCR screening, colonies were picked, dropped in LB broth with 50 μg/ml ampicillin and grown with shaking at 225 rpm overnight at 37°C. PCR was performed the next morning under the following conditions: 1X *Taq polymerase* buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer, ~1 μl overnight culture (pipet tip dip), and 1.25 units *Taq polymerase* in a 25 μl volume. PCR amplification was carried out at 94°C for 30 seconds, 55°C for 30 seconds, 72° for one minute, 25 cycles. Two plasmids with the insert were randomly chosen for the induction step. Prior to induction, the plasmids were isolated from *E. coli* using the Wizard® *Plus* DNA Purification System (Promega).

Hro-twi induction

Synthesis of full-length GST/Hro-twi fusion protein was induced using BL21 *E. coli* cells (Stratagene) and 1 mM isopropyl-beta-D-thiogalactoside (IPTG). After transforming the two randomly chosen constructs into BL21 cells, the DNA was amplified and induced in the following manner: a colony of plasmid-containing BL21 cells grown on a plate of LB agar with 50 μg/ml ampicillin was dropped into 100 ml of pre-warmed 2xYT (BioExpress) broth containing 50 μg/ml ampicillin and allowed to grow 12-16 hours at 37°C with shaking at 225 rpm. Next, 20 ml of the overnight culture was placed into 180 ml fresh 2xYT broth at 26-27°C, and allowed to grow with shaking at 225 rpm until the absorbance at 595 nm reached approximately 0.25. IPTG was then added to the culture, and the incubation continued for two more hours before harvesting the cells.

Sequencing of the chosen construct

The construct was sequenced using three primers, TW8 (5'TGTTGCTGTCATTGC3') to verify that translation was in frame, and PCR primers TW16/forward (sequence in the subcloning section) and TW2/reverse (sequence in the subcloning section) for the majority of the coding region. The cycle sequencing reactions were carried out using Big Dye v.1 (Applied

Biosystems, Perkin-Elmer Corporation) chemistry and read on the Applied Biosystems 310 Genetic Analyzer. There were 25 cycles consisting of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

DNA sequence alignment and translation

Data analysis was carried out using the following websites: National Center for Biotechnology Information (NCBI), Biology Workbench, and ExPASy Proteomics tools (Gasteiger *et al.*, 2003).

Hro-twi protein enrichment using glutathione-agarose affinity chromatography

After induction, the culture containing the GST/Hro-twi fusion protein was centrifuged to pellet the *E. coli* cells. The cells were resuspended in PBS/0.2% Tween-20 (PBST)/2 mM ethylenediaminetetraacetic acid (EDTA)/1% beta-mercaptoethanol (βME), with protease inhibitor cocktail set II (CalBioChem), then disrupted by sonication to release the fusion protein. Soluble protein was isolated from the lysate using the Bulk GST Purification Module (Amersham Pharmacia). After the lysate was incubated with glutathione sepharose beads for 30 minutes (at 4°C with rocking) to allow binding to occur, the beads were washed with PBST to remove any unbound proteins. The beads containing the GST/Hro-twi fusion protein were then

washed and equilibrated with thrombin cleavage buffer (50 mM Tris-HCl pH 8.0/150 mM NaCl/2.5 mM $CaCl_2$/0.1% βME). Thrombin was added, cleaving Hro-twi from GST and releasing it into the supernatant. The supernatant was collected and dialyzed with 100 mM HEPES buffer (pH 9), to remove tris, in preparation for the polyclonal antibody purification procedure.

To verify that the protein cleaved from GST attracted the same antibodies as the fusion protein, a Western blot was run with induced lysate (containing GST-Hro-twi) and dialyzed supernatant (containing the cleaved protein, Hro-twi) using unpurified serum (1:5000 dilution) as the probe.

Western blotting

Protein samples were mixed with an equal volume of SDS-PAGE sample buffer containing 5% βME and heated to 95-100°C for 5 minutes. These samples were then loaded onto a 10% SDS-PAGE gel (Sambrook *et al.*, 1989, p. 18.53), separated (running buffer: 25 mM tris/25 mM glycine/0.1% SDS), then transferred (transfer buffer: 39 mM glycine/48 mM tris/0.037% SDS/20% methanol) to nitrocellulose using 300-500 mA current. The membrane was then incubated with agitation at 4°C for 12-18 hours in 10mM HEPES/0.5 M NaCl/3% w/v bovine serum albumin (BSA)/0.2% Tween-20/0.5% w/v nonfat dried milk to

block nonspecific binding. Next, the membrane was rinsed with wash buffer containing Tween-20 (WB-T): 10 mM HEPES/0.5 M NaCl/0.2% Tween-20, then washed three times with WB-T, 15 minutes each. The membrane was then incubated with agitation for two hours at 4°C with a 1:5000 dilution of polyclonal rabbit serum in blocker, rinsed in WB-T, then washed three times with WB-T, 15 minutes each. Next, the membrane was incubated for one hour at room temperature with 1:3000 dilution of HRP-conjugated goat anti-rabbit IgG (Bio-Rad #170-6515) in blocker followed by a rinse and three washes in WB-T, as described above, then two washes for 10 minutes each with wash buffer without Tween-20. To visualize results, the membrane was immersed in freshly-made diaminobenzidine (DAB-Bio-Rad #170-6535) color development solution (50 mg DAB/3 X 10^{-3} % H_2O_2/TBS, pH 7.5) for 1-15 minutes, until sufficient color developed. The reaction was stopped by immersing the membrane for 10 minutes in ddH_2O (with one water change).

Polyclonal antibody purification using affinity chromatography

To purify the polyclonal serum, approximately 1 mg of enriched recombinant Hro-twi protein in HEPES buffer was incubated with 0.8 ml Affi-gel 10 (Bio-Rad) for four hours at 4°C, allowing protein coupling. This gel was then

loaded into a polypropylene Econo-Column® chromatography column (Bio-Rad). One ml (45 mg/ml) of polyclonal serum was added to the column. Five one ml fractions of putative purified serum were eluted with glycine-HCl (pH 2.5) at a flow rate of approximately 1 ml/minute. To verify that the affinity-purified antibodies remained functional, they were tested in various concentrations (1:10 dilution to 1:5000 dilution) as probes in a Western blot on induced lysate.

Staining of leech embryos with affinity-purified anti-Hro-twi antibodies

Embryos were fixed and stained using a modification of protocols from Goldstein *et al.* (2001) and Nelson and Weisblat (1992). Embryos were fixed in 4% EM-grade formaldehyde (in 100 mM cacodylic acid) for one hour at room temperature. Vitelline membranes were removed with insect pins in sterile 1% seawater and split into two groups, experimentals and negative controls. Embryos were incubated in PBS/10% normal goat serum/5% BSA/0.5% Triton X-100 for 19-24 hours at 4° C to block non-specific binding. Experimental embryos were then incubated with a 1:100 dilution of the affinity-purified Hro-twi antibody in PBS/5% normal goat serum/2% BSA/0.5% Triton X-100 for 15 hours at 4°C. The undiluted concentration of primary antibody was 75 μg/ml. Unbound primary antibody was removed by rinsing the experimental embryos

three times in PBS/5% normal goat serum/2% BSA/0.5% Triton X-100 (PGT), then washing over a period of six hours with six changes of PGT at room temperature. Experimental and negative control embryos were then incubated with a 1:500 dilution of HRP-conjugated goat anti-rabbit (Bio-Rad) in PGT for 17 hours at 4°C. Unbound secondary antibody was removed by rinsing embryos three times in PGT, then washing over a period of six hours with six changes of PGT at room temperature. To visualize HRP, embryos were then transferred to a 9-well glass plate, rinsed in PBS, then covered from light and incubated for 10 minutes in 0.4 mg/ml DAB with 0.01% H_2O_2, or until sufficient color development occurred. In preparation for viewing under the microscope, embryos were dehydrated in an ethanol series (50% to 75% to 85% to 100%) then cleared in methyl salicylate. Embryos were viewed and photographed under a Zeiss Axioskop 2 microscope.

Results

Subcloning of the *Hro-twi* gene into an expression vector

Using PCR, a 927 bp sequence was amplified from *H. robusta* genomic DNA. With the addition of the linkers, the total length of the amplification product was 968 bp (Figure 2).

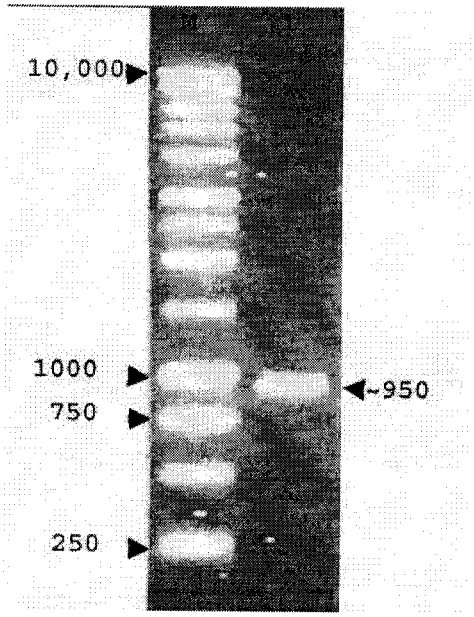

Figure 2. *Hro-twi* insert amplified from *H. robusta* genomic DNA (lane M = 1 kbp DNA ladder, measurements in bp; lane 1 = *Hro-twi* insert).

After ligation, colonies were screened for the recombinant plasmid using PCR. Out of 11 initial colonies screened, nine contained the insert. Isolation of the plasmid from the nine positive colonies, followed by digestion with *EcoRI* and *HindIII* showed that all plasmids contained an insert measuring approximately 950 bp in length.

Hro-twi induction

The initial induction of two constructs, clone G4 and clone G10, showed that both produced the protein. This was verified by a Western blot using a 1:5000 dilution of unpurified polyclonal serum as the probe (Figure 3). The negative controls (uninduced lysate and uninduced and induced expression plasmid pGEX-KG without insert) produced a couple of weakly-reacting protein bands, while the positive controls produced several strongly reacting protein bands, one with the expected molecular weight.

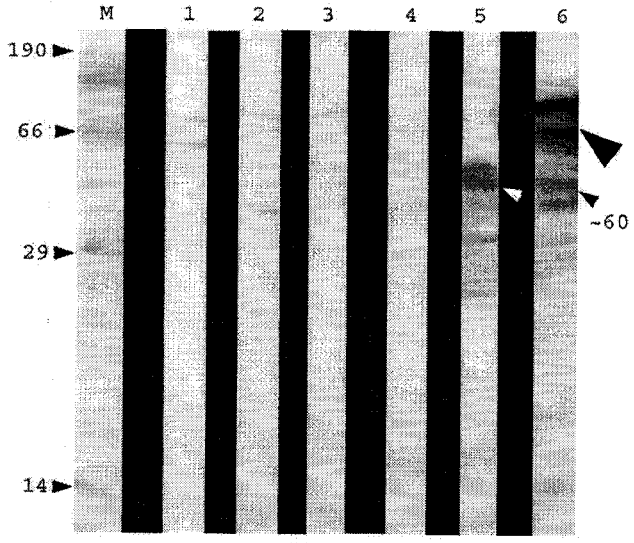

Figure 3. Verification of GST/Hro-twi fusion protein production using a Western blot with a 1:5000 dilution of unpurified serum as the probe (lane M = Bio-Rad Kaleidoscope marker, in kDa; lane 1 = clone G10 before induction; lane 2 = clone G4 before induction; lane 3 = pGEX-KG before induction; lane 4 = pGEX-KG after induction; lane 5 = clone G10 after induction; lane 6 = clone G4 after induction. Large arrowhead indicates inclusion bodies; small white/black arrowheads indicate GST/Hro-twi fusion protein).

For the next steps, the construct (clone G10) that produced fewer inclusion

For the next steps, the construct (clone G10) that produced fewer inclusion bodies was chosen. Small-scale inductions were run to optimize the GST/Hro-twi fusion protein production and minimize leaky expression as well as inclusion body formation, such as altering growth and induction temperature, varying IPTG concentration, and testing different media. After optimization, a concentration of 1 mM IPTG and an induction temperature of 27-28°C were used for subsequent inductions in 2xYT broth (Figure 4).

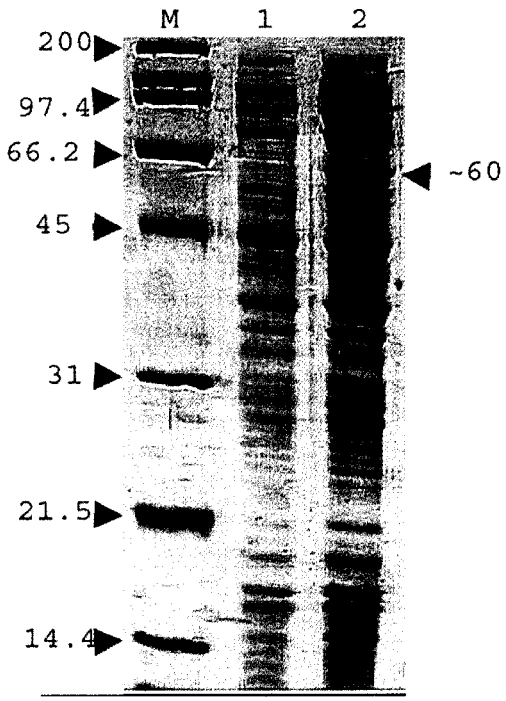

Figure 4. Coomassie-stained 10% SDS-PAGE showing GST/Hro-twi fusion protein in uninduced and induced lysate (2 hour induction at ~28°C) (lane M = Bio-Rad broad range protein marker, measurements in kDa; lane 1 = uninduced lysate; lane 2 = induced lysate).

DNA sequence alignment and translation

Nucleotide and subsequent amino acid alignment of clone G10 showed

the nucleotide sequence for clone G10, compared to the *Hro-twi* NCBI sequence (accession number AF410867), including one transition, two transversions, three insertions, and six deletions. These mutations led to 13 amino acid mutations with strong conservation between amino acid groups, 9 amino acid mutations with weak conservation between groups, and 25 mutations with no consensus between groups. However, there was 100% consensus in the conserved bHLH region.

According to Soto *et al.* (1997), the *Hro-twi* sequence amplified and cloned using the above methods should result in a product of 927 bp (not including linkers) and 301 amino acids. This sequence of amino acids has a molecular weight of approximately 33.1 kDa (Stothard, 2000). However, the *Hro-twi* sequence in the NCBI website shows that a translation of *Hro-twi* should result in an amino acid length of 359 amino acids (1077 bp), which has a molecular weight of 40.4 kDa. The longer amino acid sequence is due to an absence of a stop codon after the codon for amino acid #301. Sequence data for clone G10 supports the absence of the stop codon in the same place. However, further correlation of the sequence of clone G10 with the *Hro-twi* NCBI sequence is prevented because the insert is too short due to the position of the TW2/reverse primer used for cloning (Figure 6). Clone G10 is therefore truncated by 138 nucleotides or 46

amino acids. Consequently, data was accurate only up to the 879th bp, allowing for accurate translation up to 281 amino acids.

To make an accurate estimate of the expected recombinant Hro-twi molecular weight for this study, the thrombin linker site that remains after cleavage by thrombin and the position of the TW2 primer site should be taken into consideration, leading to a molecular weight of 37 kDa (330 amino acids). In addition, the full-length recombinant protein, GST/Hro-twi, should have a molecular weight of approximately 63.5 kDa with the addition of 26 kDa for the GST portion of the protein and 0.5 kDa for the remaining thrombin cleavage site (Guan and Dixon, 1991; Biology Workbench). The estimated theoretical isoelectric point for Hro-twi is 10.1 (Biology Workbench), comparable to the theoretical isoelectric point of 9.59 for human *twist* (Wang *et al.*, 1997), although basic compared to the theoretical isoelectric point for *Drosophila* of 6.15 (Thisse *et al.*, 1988).

Figure 5. Nucleotide and amino acid sequence of Clone G10, construct used for Hro-twi induction (blue amino acids = thrombin linker sequence remaining after cleavage; underlined ATG = starting met; black amino acids = fully conserved residues compared to NCBI sequence; green amino acids = conservation of strong groups; italicized amino acids = conservation of weak groups; red amino acids = no consensus; underlined amino acids = conserved bHLH region; *nucleotide count; **amino acid count).

```
CTGGTTCCGCGTGGATCCCCGGGAATTTCCGGTGGTGGTGGT          42*
  L  V  P  R  G  S  P  G  I  S  G  G  G  G          14**
GGAATTCTAATGATATCACCATCTTTTCCAACTACGTCATCC          84
  G  I  L  M  I  S  P  S  F  P  T  T  S  S          28
TCTCATCATTTGCCGTCGGCTAACTCAGCAGCAGCAACGGCA          126
  S  H  H  L  P  S  A  N  S  A  A  A  T  A          42
GCGACGACAACCGCGTCAACTGATTCTGTCATTAAAAATTGT          168
  A  T  T  T  A  S  T  D  S  V  I  K  N  C          56
CTCTTCAACAATAAAAATGACACGTCGACACACTTAACTACG          210
  L  F  N  N  K  N  D  T  S  T  H  L  T  T          70
CATTTACAAGGCGTTAACAAGATTTATTTTCACAACAACAAC          252
  H  L  Q  G  V  N  K  I  Y  F  H  N  N  N          84
AACTGTAGCAATGACAGCAACAACACCAGCGACGACCTCAAG          294
  N  C  S  N  D  S  N  N  T  S  D  D  L  K          98
CCTGCTACTAAAAAACGAAAGAAAACATCAAAACTAACTAAA          336
  P  A  T  K  K  R  K  K  T  S  K  L  T  K          112
TCCGATTCAACGCCCAATAAGGACGCTGAAAGTACTAAAAAT          378
  S  D  S  T  P  N  K  D  A  E  S  T  K  N          126
GACTTGCAACAAAACAACGTTGTGAAAGTTGAGCTGGATCAG          420
  D  L  Q  Q  N  N  V  V  K  V  E  L  D  Q          140
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TTAAAGACAGCAAAAACAACCACTTGCAACAACACAGCGCCG          504
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CAAACAACGACATCTACCTACAGAAAACTTCCCAACGCCACC          588
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TACTACAGCAACTACAACAGCCACAACAACTCGCCACCACTC          672
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TCGCTACAAACGCAGCGAGTTCTTGCGAACGTCCGTGAGCGC          714
  S  L  Q  T  Q  R  V  L  A  N  V  R  E  R          238
CAAAGAACGCAATCCTTAAACGATGCCTTCTCACAGTTAAGA          756
  Q  R  T  Q  S  L  N  D  A  F  S  Q  L  R          252
AAAATCGTTCCGACGCTCCCGTCCGACAAACTCAGCAAAATC          798
  K  I  V  P  T  L  P  S  D  K  L  S  K  I          266
CAAACCCTGAAACTCGCAACCAGATATATCGACTTTTTATAC          840
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GATCAATTAGAGAACAATAAGCAGCAGCAGCAGCAACATCAG          882
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CAGCAGCAGCAACAACGAACATCAAAGTATGGACGAATTTGC          924
  Q  Q  Q  Q  Q  R  T  S  K  Y  G  R  I  C          308
TAACGGTGGCATATGTTTCCGGTTTAAACGGAAGACCGGTCT          966
  X
```

Figure 6. Nucleotide sequence of Clone G10 compared to NCBI *Hro-twi* sequence (accession number AF410867) and Soto *et al.* (1997) sequence (underlined ATG = starting M; underlined sequence at end = TW2/reverse primer sequence; bold type = stop sequence; *nucleotide count of NCBI sequence).

```
G10   ATGATATCACCATCTTTTCCAACTACGTCATCC-TCTC-ATCATTTGCCG
NCBI  ATGATGTCACCATCTTTTCCAACTACGTCATCC-TCTC-ATCATTTGC-G      125*
Soto  ATGATGTCACCATCTTTTCCAACTACGTCATCCATCTCGATCGATTTGCG

G10   TCGGCTAACTCAGCAGCAGCAACGGCAGCGACGACAACCG-CGTCAACTG
NCBI  TCGGCTAACTCAGCAGCAGCAACGGCAGCGACGACAACCGGCGTCAACTG      175
Soto  TCGGCTAACTCAGCAGCAGCAACGGCAGCGACGACAACCGGCGTCAACTG

G10   ATTCTGTCATTAAAAAT--TGTCTCTTCAACAA-TAAAAATGACACGTCG
NCBI  ATTCTGTCATTAAAAAT--TGTCTCTTCAACAA-TAAAAATGACACGTCG      222
Soto  ATTCTGTCATTAAAAATACTGTCTCTTCAACAAATAAAAATGACACGTCG

G10   ACACACTTAACTACGCATTTACAAGGCGTTAACAAGATTTATTTTCACAA
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G10   CAACAACAACTGTAGCAATGACAGCAACAACACCAGCGACGACCTCAAGC
NCBI  CAACAACAACTGTAGCAATGACAGCAACAACAACAGCGTCGACCTCAAGC      322
Soto  CAACAACAACTGTAGCAATGACAGCAACAACAACAGCGTCGACCTCAAGC

G10   CTGCTACTAAAAAACGAAAGAAAA-CATCAAA--ACTAACTAAATCCGAT
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Soto  CTGCTACTAAAAAACGAAAGAAAAACATCAAAAAACTAACTAAATCCGAT

G10   TCAACGCCCAATAAGGACG-CTGAAAGTACTAAAAATGACTTGCAACAAA
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G10   ACAACGTTGTGAAAGTTGAGCTGGATCAGTCAGACA-CAACACAGCGGAA
NCBI  ACAACGTTGTGAAAGTTGAGCTGGATCAGTCAGACAACAACACAGCGGAA      471
Soto  ACAACGTTGTGAAAGTGGAGCTGGATCAGTCAGACAACAACACAGCGGAA

G10   AACGAAGACAGTAAATCTTCAGTTAAAGACAGCAAAAACAACCACT--TG
NCBI  AACGAAGACAGTAAATCTTCAGTTAAAGACAGCAAAAACAACAACCACTG      521
Soto  AACGAAGACAGTAAATCTTCAGTTAAAGACAGCAAAAACAACAACCACTG

G10   CAACAACACAGCGCCGACGACTACAATTACGACGACTCCGAGCATAAATT
NCBI  CAACAACACAGCGCCGACGACTACAATTACGACGACTCCGAGCATAAATT      571
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G10   TCAACACCCAAACAACGACATCTACCTACAGAAAACTTCCCAACGCCACC
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Soto  TCAACACCCACACAACGACGTCTACCTACAGAAAACTTCCCAACGCCACC

G10   ACAAACAACATCGCCTACAACCACAACGGTAGCGGCAAAAACTACTACAG
NCBI  ACAAACAACATCGCCTACAACCACAACGGTAGCGGCAAAAACTACTACAG      671
Soto  ACAAACAACATCGCGTACAACCACAACGGTAGCGGCAAAAACTACTACAG

G10   CAACTACAACAGCCACAACAACTCGCCACCACTCTCGCTACAAACGCAGC
NCBI  CAACTACAACAGCCACAACAACTCGCCACCACTCTCGCTACAAACGCAGC      721
Soto  CAACTACAACAGCCACAA---CTCGCCACCACTCTCGCTACAAACGCAGC

G10   GAGTTCTTGCGAACGTCCGTGAGCGCCAAAGAACGCAATCCTTAAACGAT
NCBI  GAGTTCTTGCGAACGTCCGTGAGCGCCAAAGAACGCAATCCTTAAACGAT      771
Soto  GAGTTCTTGCGAACGTCCGTGAGCGCCAAAGAACGCAATCCTTAAACGAT
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 G10  CAGCAAAATCCAAACCCTGAAACTCGCAACCAGATATATCGACTTTTTAT
NCBI  CAGCAAAATCCAAACCCTGAAACTGGCAACCAGATATATCGACTTTTTAT        871
Soto  CAGCAAAATCCAAACCCTGAAACTGGCAACCAGATATATCGACTTTTTAT

 G10  ACGATCAATTAGAGAACAATAAGCAGCAGCAGCAGCAACATCAGCAGCAG
NCBI  ACGATCAATTAGAGAACAATAAGCAGCAGCAGCAGCAACATCAGCAGCAG        921
Soto  ACGATCAATTAGAGAACAATAAGCAGCAGCAGCAGCAACATCAGCAGCAG

 G10  CAGCAACAACGAACATCAAAGTATGGACGAATTTGCTAACGGTGGCATAT
NCBI  CAGCAACAAC-AACATCAAAGTATGGACGAATTTGCTAACGGTGG-ATAT        969
Soto  CAGCAACAAC-AACATCAAAGTATGGACGAATTTGCTAACGGTGG-ATAT

 G10  GTTTCCGGTTTAAACGGAAGACCGGTC----------------------
NCBI  GTTTCCGGTTTAAACGGAAGTCCGGTCATCTCCTCGGACAAGTTGGGGTA      1019
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 G10  --------------------------------------------------
NCBI  CGCG--TTTTCAGTTTGGAGGATGGAGGGCGCGTGGCAGATGAATAAAAG      1067
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 G10  --------------------------------------------------
NCBI  TGAACTTAATAATAACGGATGTTACTTACGTAACGGCAGCAGTGCTGGTG      1117
Soto  TG-ACTTAATAATAACGGATGTTACTTACGTAACG-CACGAGTCGTGGTC

 G10  ------------------------------------------------
NCBI  GCAGCAGCAACAACGTTCGCGATGGCAGTGTCATTGGCTGACGTGTCTC      1167
Soto  GCAGCAGTAACAACGTTCGCGATGGCAGTGTCATTGGCTGACGTGTCTC
```

Hro-twi protein enrichment using glutathione-agarose affinity chromatography

After the fusion protein was released from the cells, Hro-twi was cut from GST by cleavage with the endoprotease thrombin, which restricts between arginine and glycine within the thrombin linker sequence (L-V-P-R-G-S). To verify that the protein cleaved from GST attracted the same antibodies as the fusion protein, a Western blot was run using a 1:5000 dilution of unpurified serum as the probe. The Coomassie-stained gel shown in Figure 7A shows the GST/Hro-twi fusion protein in lane 3 and the cleaved protein, Hro-twi, in lane 4.

Plate B shows the Western blot, which verifies that the cleaved protein attracts the same antibodies as the fusion protein. The size of the cleaved recombinant protein measures approximately 40 kDa (range = 39-40), slightly higher than the expected molecular weight of 37 kDa. The size of the fusion protein appears to be 60 kD (range = 58-62), slightly lower the expected molecular weight of 63.5 kDa.

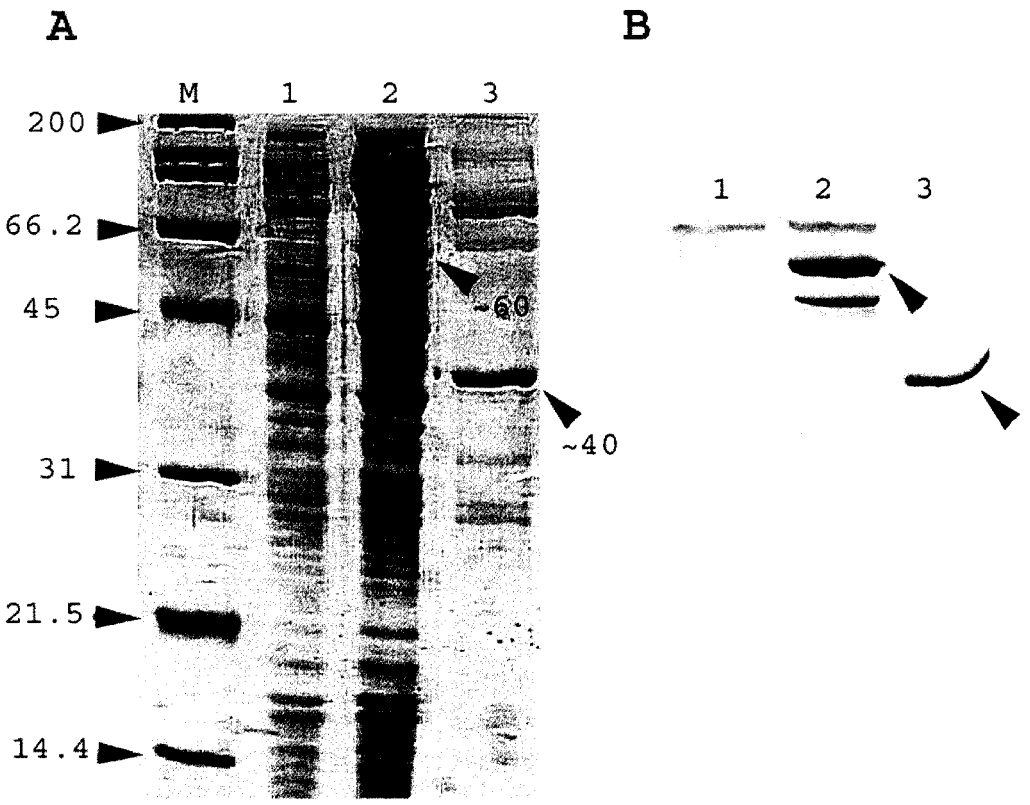


Figure 7. Verification of Hro-twi cleavage from GST and that the antibodies in the unpurified serum (1:5000 dilution) bind to Hro-twi. **A.** Coomassie-stained 10% SDS-PAGE gel. **B.** Western blot using a 1:5000 dilution unpurified polyclonal serum (lane M = Bio-Rad broad-range protein marker, measurements in kDa; lane 1 = uninduced lysate; lane 2 = induced lysate; lane 3 = supernatant after thrombin cleavage). Blue arrowheads indicate GST/Hro-twi fusion protein; black arrowheads indicate Hro-twi recombinant protein.

Polyclonal antibody purification using affinity chromatography

In preparation for affinity-purification of the polyclonal antibodies with Affi-gel 10, a 10% SDS-PAGE gel was run with Affi-gel 10 beads after incubation with Hro-twi. The gel (Figure 8) shows that Hro-twi did successfully bind to the beads after a four hour incubation. In addition, inclusion bodies did not bind, as expected, as well as a significant percentage of Hro-twi (~60%). After affinity-purification of the polyclonal antibodies, five fractions of purified antibodies were collected. Samples of each fraction were run on a 10% SDS-PAGE gel and compared to unpurified serum (Figure 9). Most albumin was removed from the serum, and the fractions appeared to contain a higher relative concentration of heavy and light chains. For the following step (using the purified antibody as probes to embryos), fraction #2 was chosen because it appeared to contain less albumin compared to fraction #1, making it even more enriched in heavy and light chain antibody.

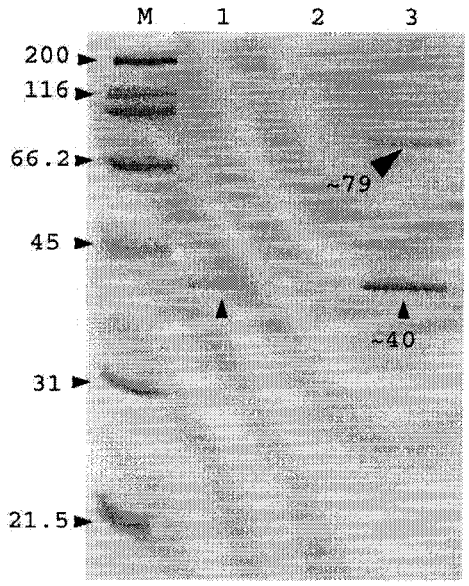


Figure 8. Verification that Hro-twi is bound to the Affi-gel 10 before antibody purification. Approximately 1 mg Hro-twi was incubated with Affi-gel 10. Results are shown in a Coomassie-stained 10% SDS-PAGE gel (lane M = Bio-Rad broad range protein marker, measurements in kDa; lane 1 = Affi-gel 10 beads only; lane 2 = empty; lane 3 = Affi-gel 10 supernatant; small arrowheads indicate Hro-twi band; large arrowhead indicates inclusion bodies).

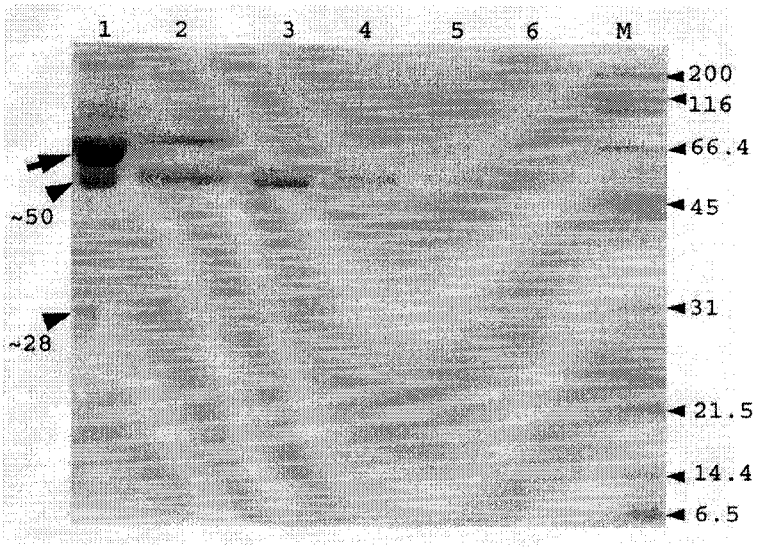


Figure 9. Affinity purification of the polyclonal serum. Results are shown in a Coomassie-stained 10% SDS-PAGE gel [lane 1 = unpurified serum (45 mg/ml); lane 2 = fraction 1 (375 μg/ml); lane 3 = fraction 2 (150 μg/ml); lane 4 = fraction 3 (50 μg/ml); lane 5 = fraction 4 (35 μg/ml); lane 6 = fraction 5 (30 μg/ml); lane M = Bio-Rad broad-range protein marker, measurements in kDa]. Arrow in lane 1 indicates albumin; black arrowheads in lane 1 indicate the heavy chain (~50 kDa) and light chain (~28 kDa). Fraction 2 was chosen for the whole-mount immunostaining of the embryos. Before use in immunostaining, fractions diluted 1:1 in glycerol.

To verify that the affinity-purified antibodies remained functional, they were tested in various concentrations (1:10 dilution to 1:5000 dilution) as probes for induced lysate in a Western blot. As shown in Figure 10, all concentrations of the affinity-purified antibody remained functional and targeted the expected protein bands. Three main protein bands were targeted, inclusion bodies of GST/Hro-twi (~95 kDa), soluble GST/Hro-twi (~65 kDa), and degraded GST/Hro-twi (~58 kD).

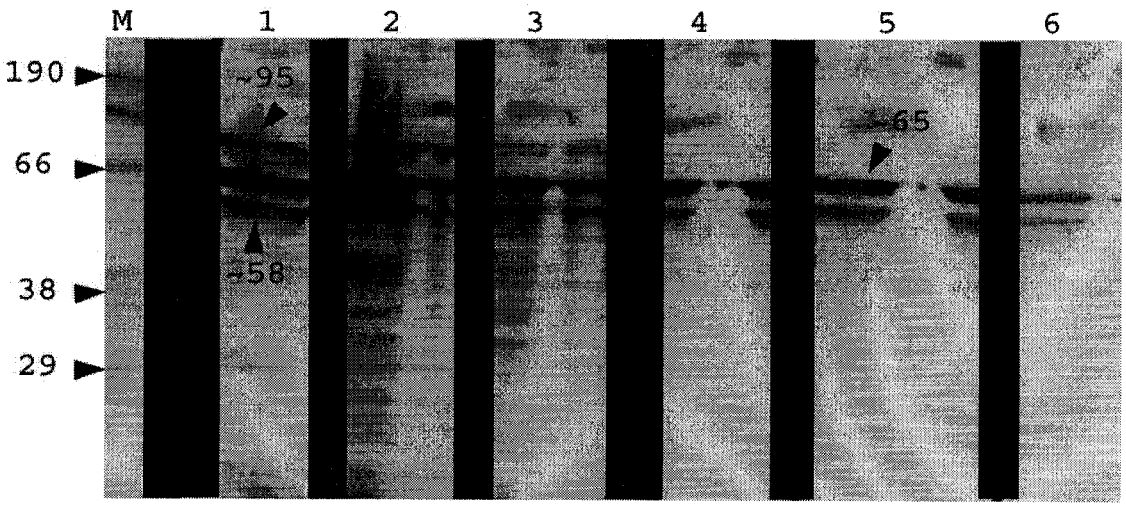


Figure 10. Verification that the affinity-purified polyclonal antibodies remain functional (lane M = Bio-Rad Kaleidoscope marker, in kDa; lanes 1-6 contain induced lysate as target. Description of the probes: lane 1 = unpurified polyclonal serum, 1:5000 dilution; lane 2 = purified serum, 1:10 dilution; lane 3 = purified serum, 1:100 dilution; lane 4 = purified serum, 1:1000 dilution; lane 5 = purified serum, 1:2500 dilution; lane 6 = purified serum, 1:5000 dilution).

Staining of leech embryos with affinity-purified anti-Hro-twi antibodies

Helobdella triserialis leech embryos were used instead of *Helobdella robusta* embryos for this study; therefore, discussion of the protein targeted in embryonic

whole-mount staining will refer to the protein as "H-twi". H-twi protein was present in embryonic stages 1, 2, 3, 4b, 5, 6, 7, and 8 (Table 1). Anti-H-twi staining was moderate in stages 1 and 2 (Figures 11-14), very faint in stages 3 (Figure 15) and early 4b (data not shown), then increased in intensity from late stage 4b to mid stage 8 (Figures 16-19). All negative controls (incubated with secondary antibody only) showed no or very little background staining (Figures 11B-13B, 15B-18B).

In stages 1-2, staining was detected in the teloplasm (Figures 11-14). In late stage 1, the protein was detected in the animal and vegetal pools of teloplasm. In stage 2 embryos, H-twi was detected in the teloplasm of cell CD, although H-twi protein was occasionally seen in cell AB (Figure 14A).

In stage 3 (Figure 15), low levels of H-twi protein were detected in the border of blastomeres A, B, C, and D, where the micromere cap will form. Similarly, in early stage 4b embryos, low levels of H-twi protein were detected at the site of the first micromeres (data not shown).

In late stage 4b (Figure 16), H-twi protein was detected in the micromeres and in blastomeres A', DNOPQ', and possibly DM. The age of this clutch of embryos was approximately 9-10 hours after zygote deposition (AZD), based on a visual estimation of the number of micromeres, a', b', c', d', c'', dnopq', dm', a'',

b'' (Weisblat and Huang, 2001; Sandig and Dohle, 1988). All micromeres contained H-twi protein, but micromeres a'' and dnopq' were stained darkest. In stages 5 and 6, moderate H-twi protein levels were detected in the micromeres (data not shown). In stage 7 (Figure 17), strong anti-H-twi staining was detected in the micromere cap. Anti-H-twi M bandlet staining (Fernández, 1980), was also detected from the vegetal side of the embryo (Figure 17D).

In early stage 8, anti-H-twi cytoplasmic staining was detected in the germinal bands (Figure 18A). Without the help of lineage tracing, it is not known whether or not the staining was occurring in the ectodermal bandlets or just the mesodermal bandlet. By mid stage 8 (Figures 18 C, D and 19A), anti-H-twi nuclear staining of some cells in the area of the micromere-derived provisional squamous epithelium, as well as cells directly adjacent to the outer edge of the germinal bands, was detected. The dark staining of the germinal bands themselves made it difficult to discern whether or not the staining is nuclear or cytoplasmic. However, the view in Figure 19B showed some probable anti-H-twi nuclear staining in the older region of the prostomial precursor.

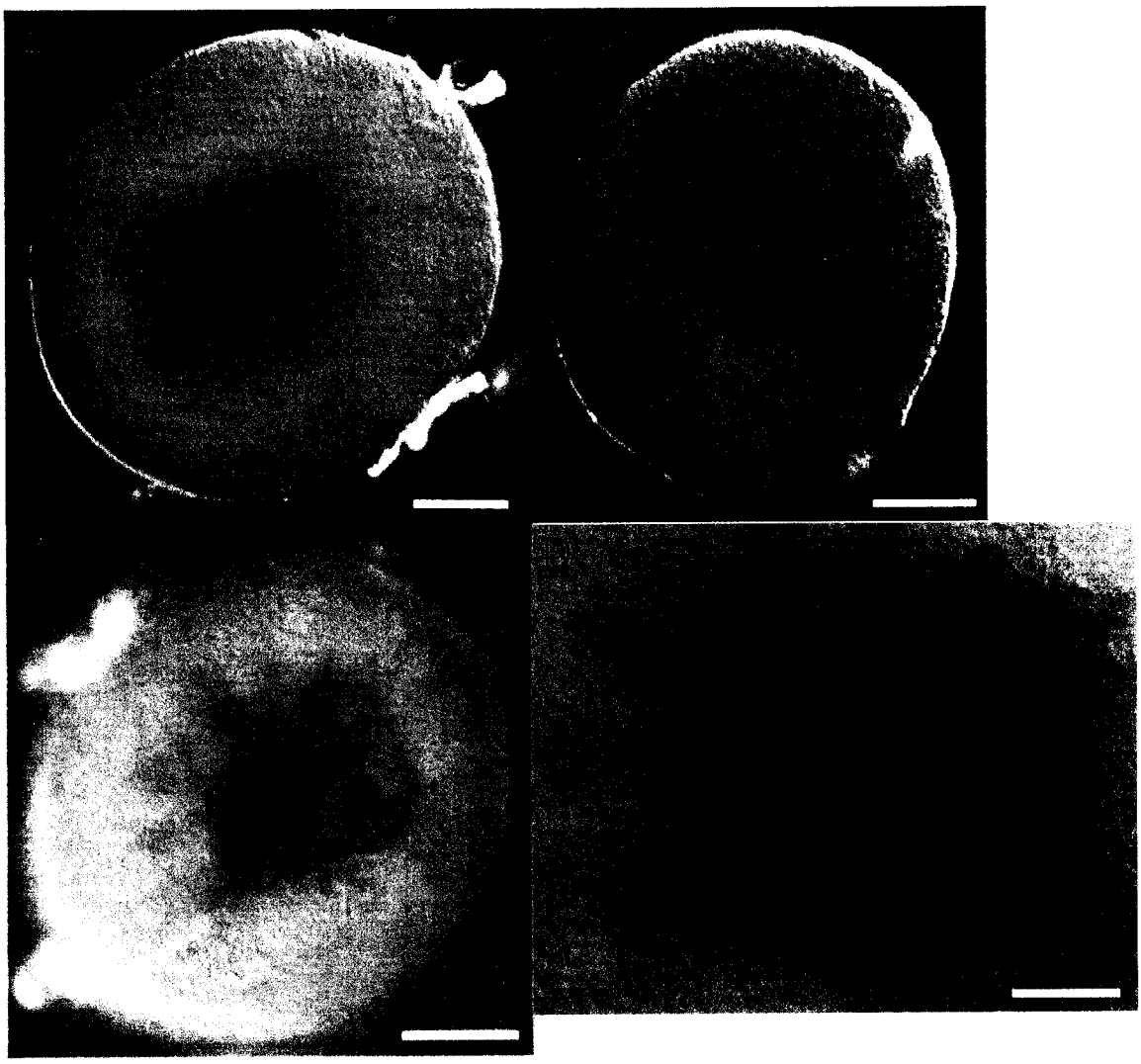


Figure 11. Anti-H-twi localization in stage 1. **A.** Staining of affinity-purified anti-H-twi antibody is visible in the teloplasm region. **B.** Negative control. **C.** Altered focus on anti-H-twi antibody staining. **D.** Same focus as in **C**, at higher magnification, 320X. Note: Scale bars represent ~100 μm for **A**, **B**, and **C** and ~50 μm for **D**. All views of embryos in all Figures are from the animal side at magnification 160X, unless stated otherwise.

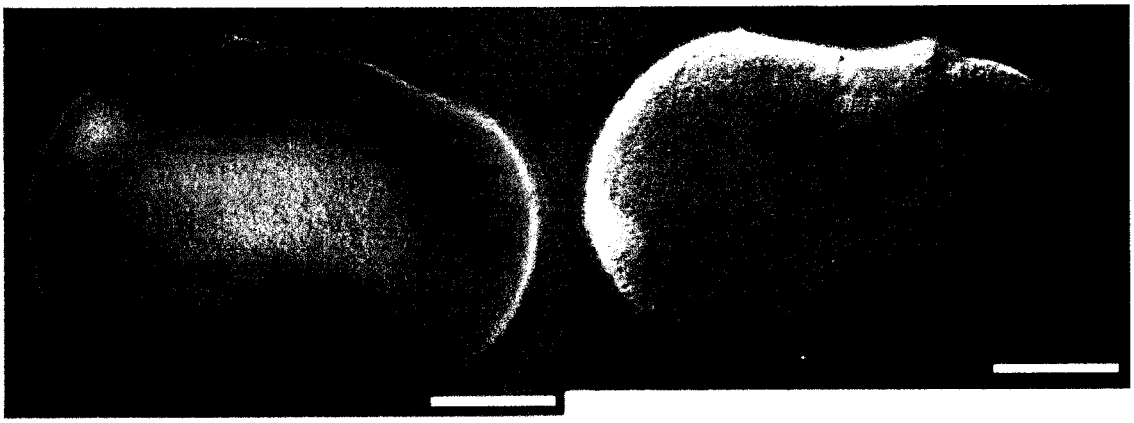


Figure 12. Anti-H-twi localization in stage 1. **A.** Anti-H-twi antibody is visible in equatorial view of late stage 1 before cleavage, in the region of the teloplasm forming at the animal and vegetal poles. **B.** Negative control. Scale bars represent ~100 μm.

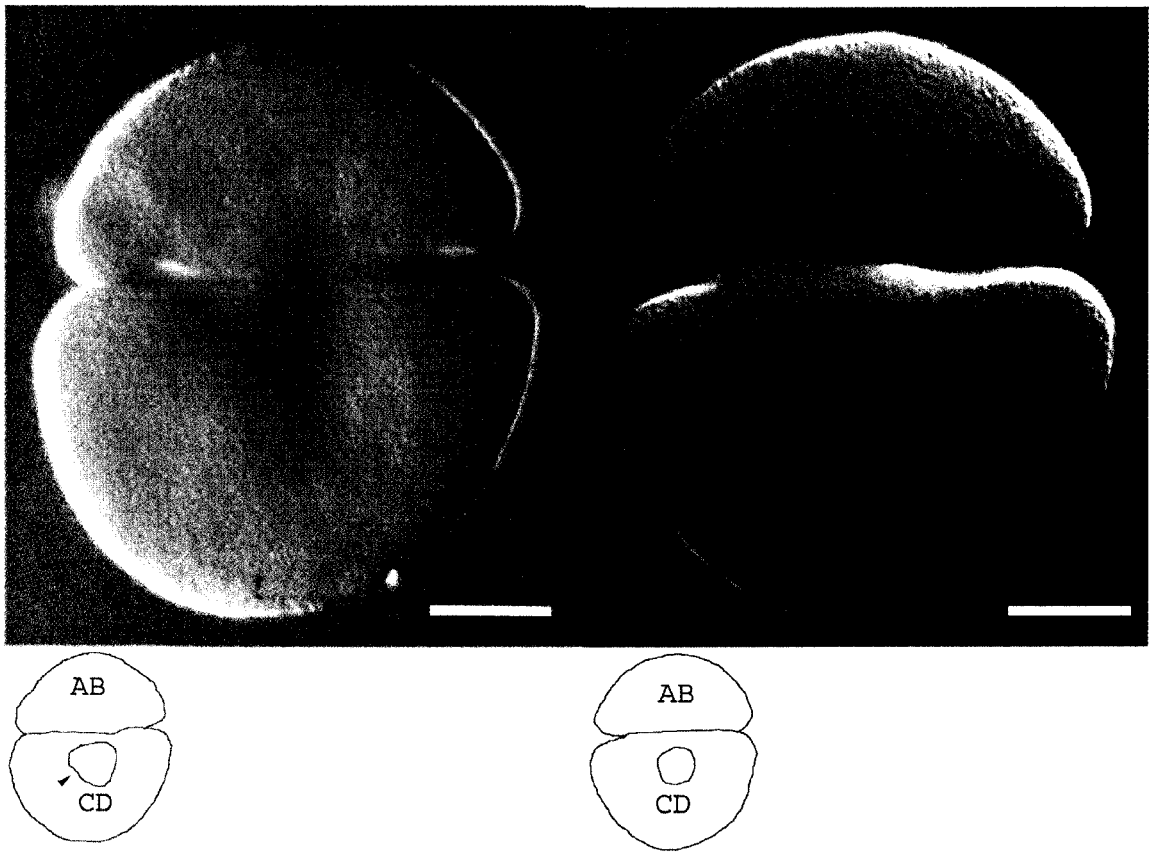


Figure 13. Anti-H-twi localization in stage 2. **A.** Light anti-H-twi staining in the teloplasm, most of which has segregated to cell CD. **B.** Negative control. Teloplasm is shown circled in cell CD of the drawing. Scale bars represent ~100 μm.

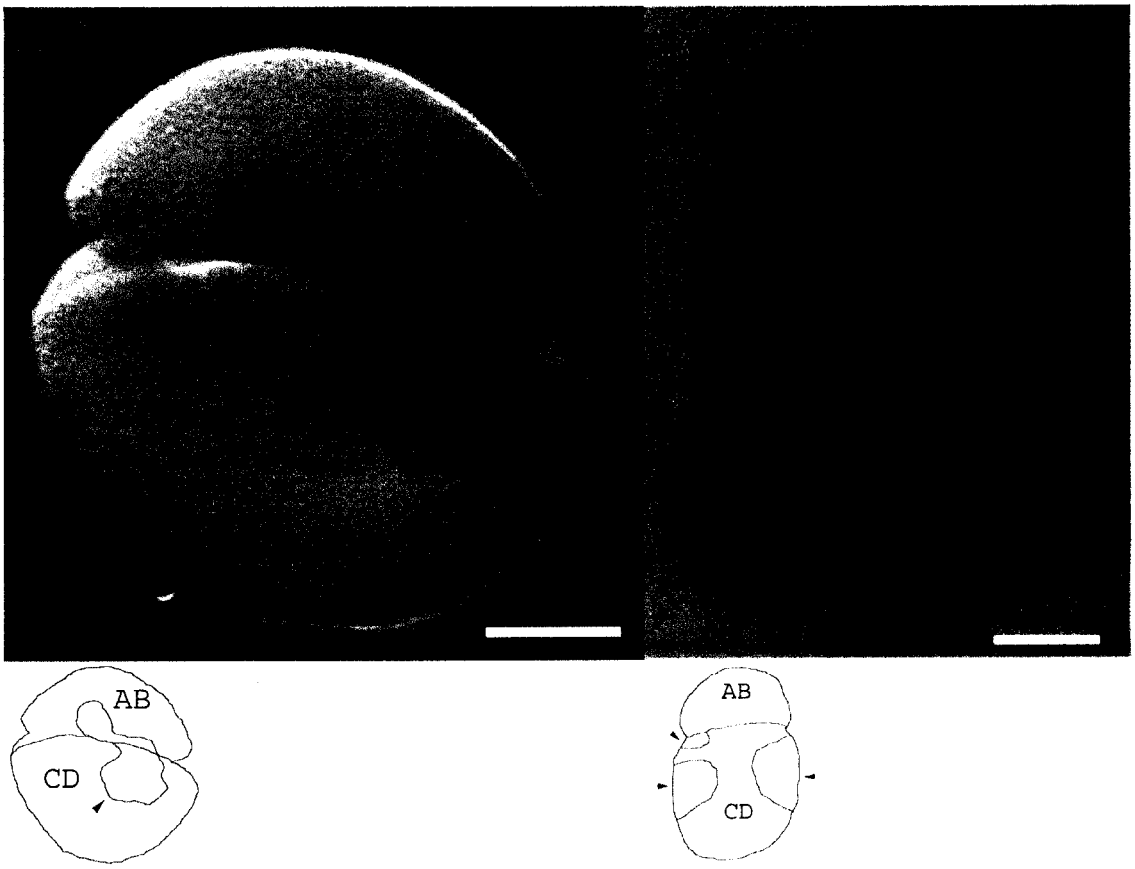


Figure 14. Anti-H-twi localization in stage 2. **A.** Medium-level staining of anti-H-twi antibody in the teloplasm, most of which has segregated to cell CD, but also partially remained in cell AB (black arrowhead in the drawing). **B.** Faint staining of anti-H-twi antibody is seen in cell CD as it prepares to divide into blastomeres **C** and **D** (black arrowheads in drawing). Scale bars represent ~100 μm.

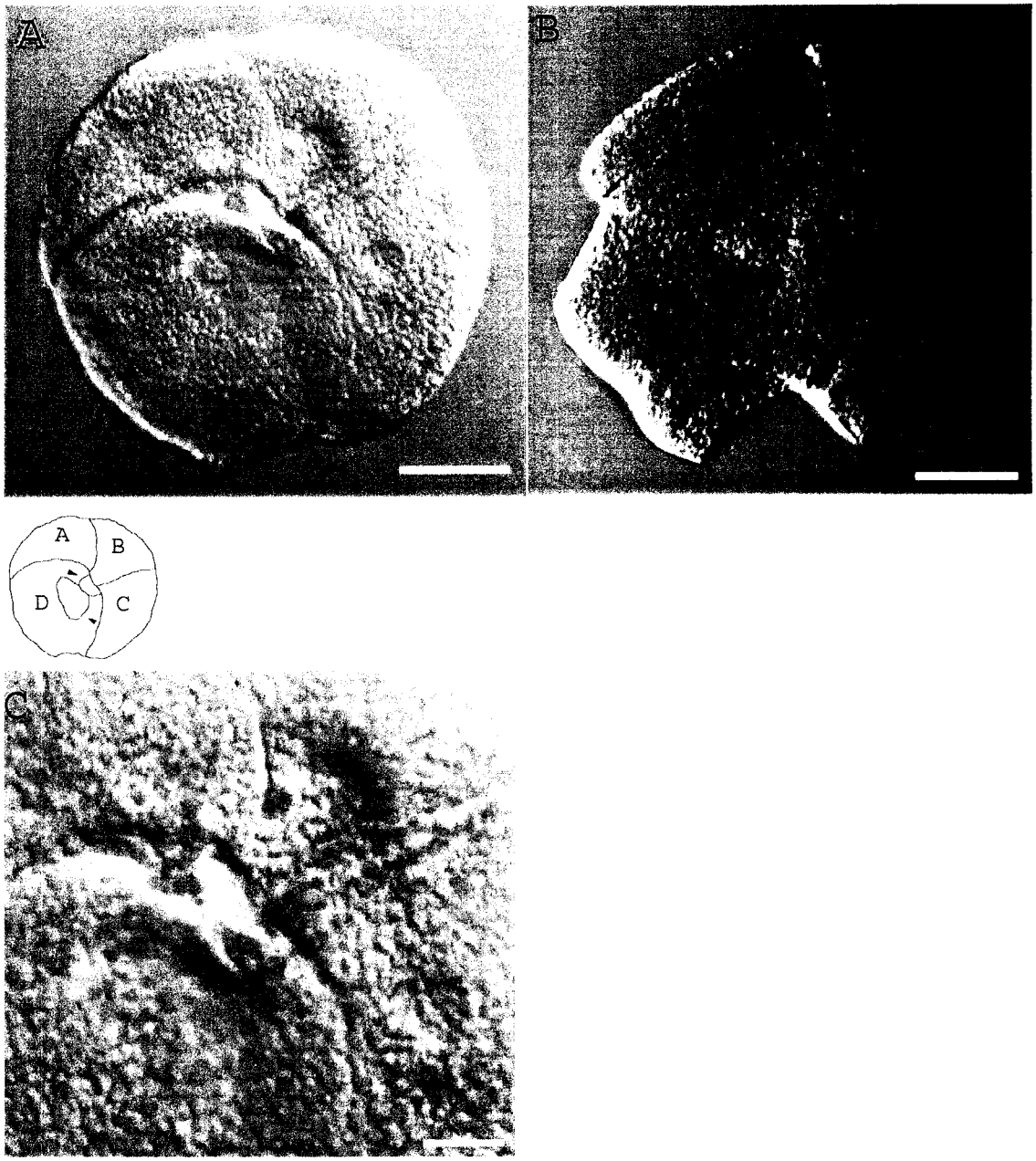
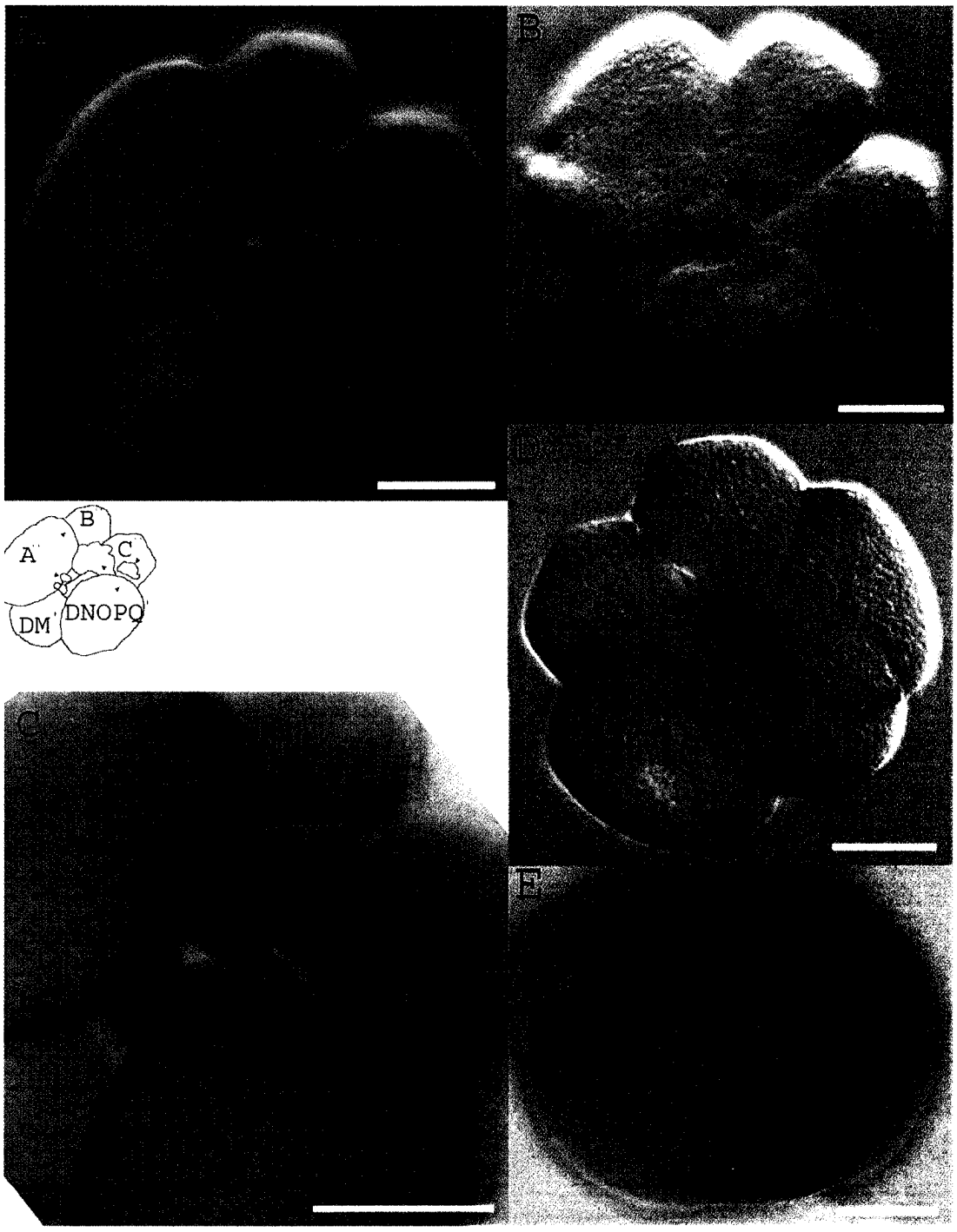


Figure 15. Anti-H-twi localization in stage 3. **A.** Faint staining is seen at the border of blastomeres A, B, C, and D, where the micromeres will form (black arrowhead in drawing). It is seen in part of the teloplasm (blue arrowhead indicates larger circle of teloplasm in drawing). **B.** Negative control. **C.** Higher magnification view (320X) of same embryo. Scale bars represent ~100 μm for **A** and **B** and ~50 μm for **C.**

Figure 16. Anti-H-twi localization in late stage 4b (approximately 9-10 hours AZD, based on number of micromeres, ~10). **A.** Strong staining is seen in the micromeres, especially a'' and dnopq'. The remaining micromeres are stained, along with blastomeres A'' and DNOPQ' (black arrowheads in drawing point to areas of strongest staining). **B.** Negative control. **C.** Increased magnification (320X) of the same embryo in **A. D.**Vegetal view, of embryo in **A** and **C**, showing that staining is not concentrated on this side of the embryo. **E.** Equatorial view, with DNOPQ' in front. Scale bars represent ~100 μm for **A, B, D,** and **E** and ~50μm for **C.**


B
A
B
C
DM
DNOPQ
C
D
E

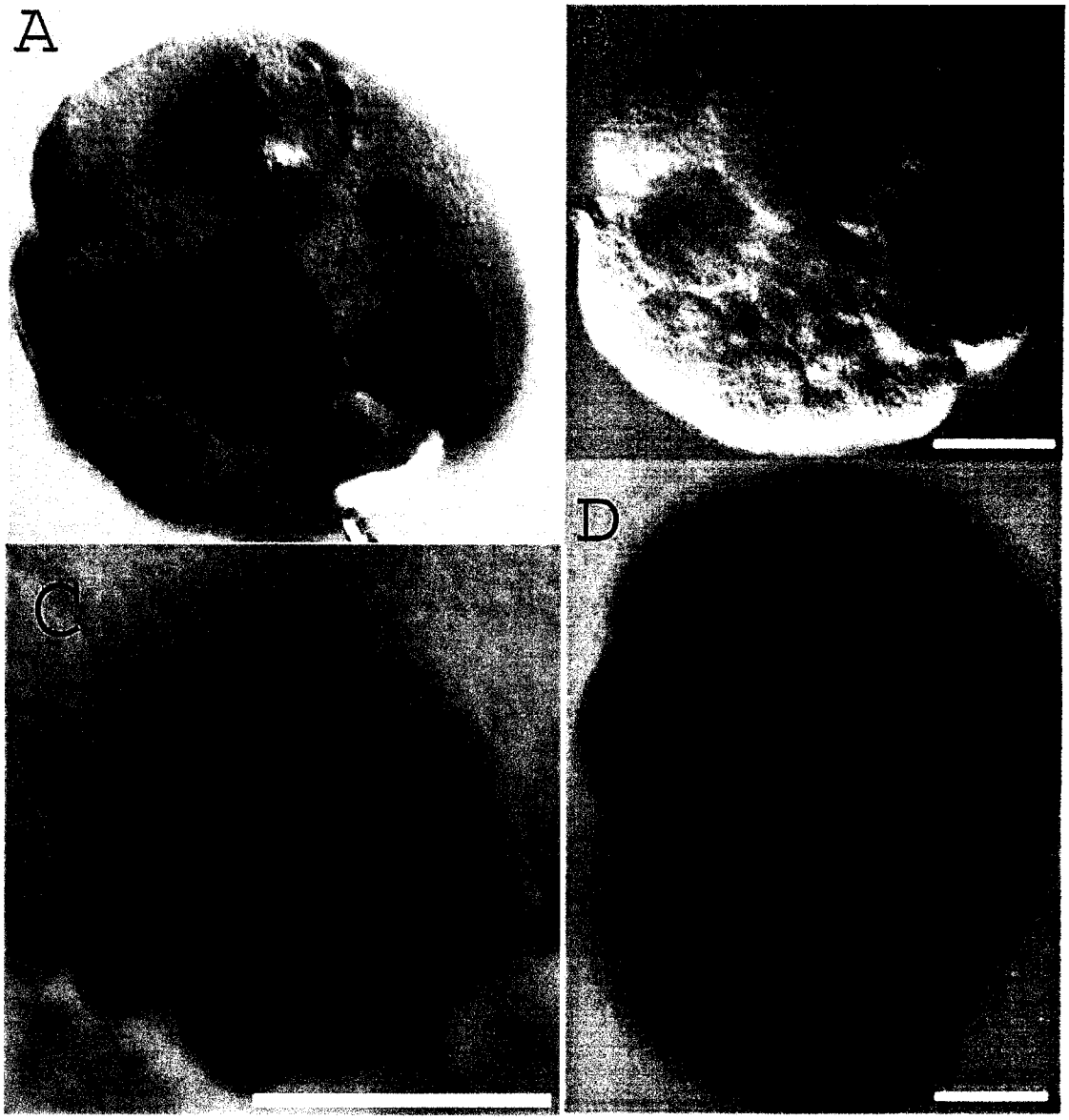


Figure 17. Anti-H-twi localization in stage 7. **A.** Strong staining in the micromere cap. **B.** Negative control. **C.** Higher magnification of embryo in **A** (320X). **D.** Vegetal view (in glycerol) showing M germinal bandlet staining (Fernández, 1980). Scale bars represent ~100 μm.

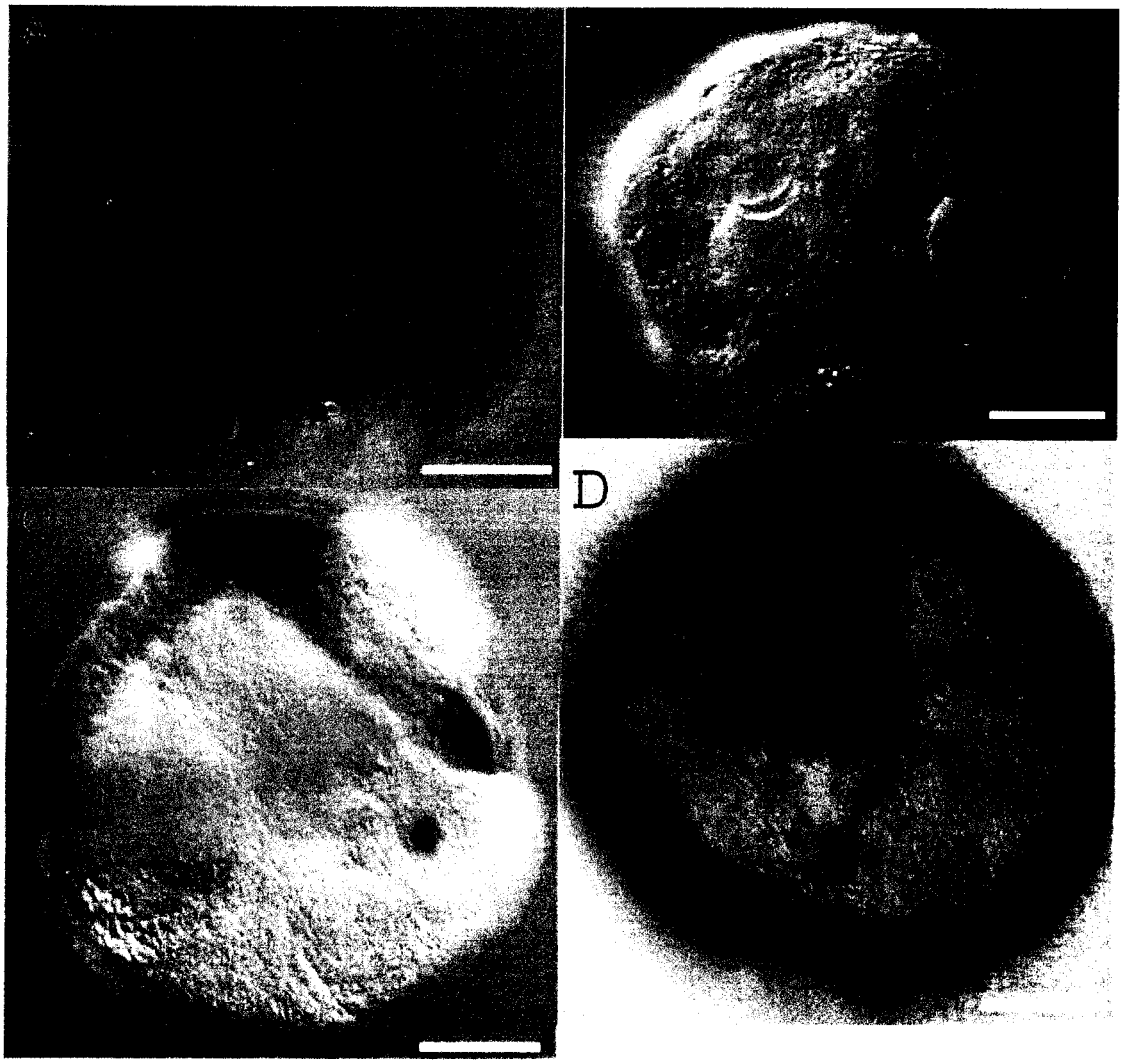


Figure 18. Anti-H-twi localization in stage 8. **A.** Early stage 8. Cytoplasmic staining in germinal bands. **B.** Negative control. **C.** Mid stage 8. Cytoplasmic staining of V-shaped germinal bands, separated by the prostomial precursor (derived from the micromere cap). **D.** Dorsal view of mid stage 8 showing that nuclear staining is detected in the area of the provisional squamous epithelium adjacent to the germinal bands. Scale bars represent ~100 μm.

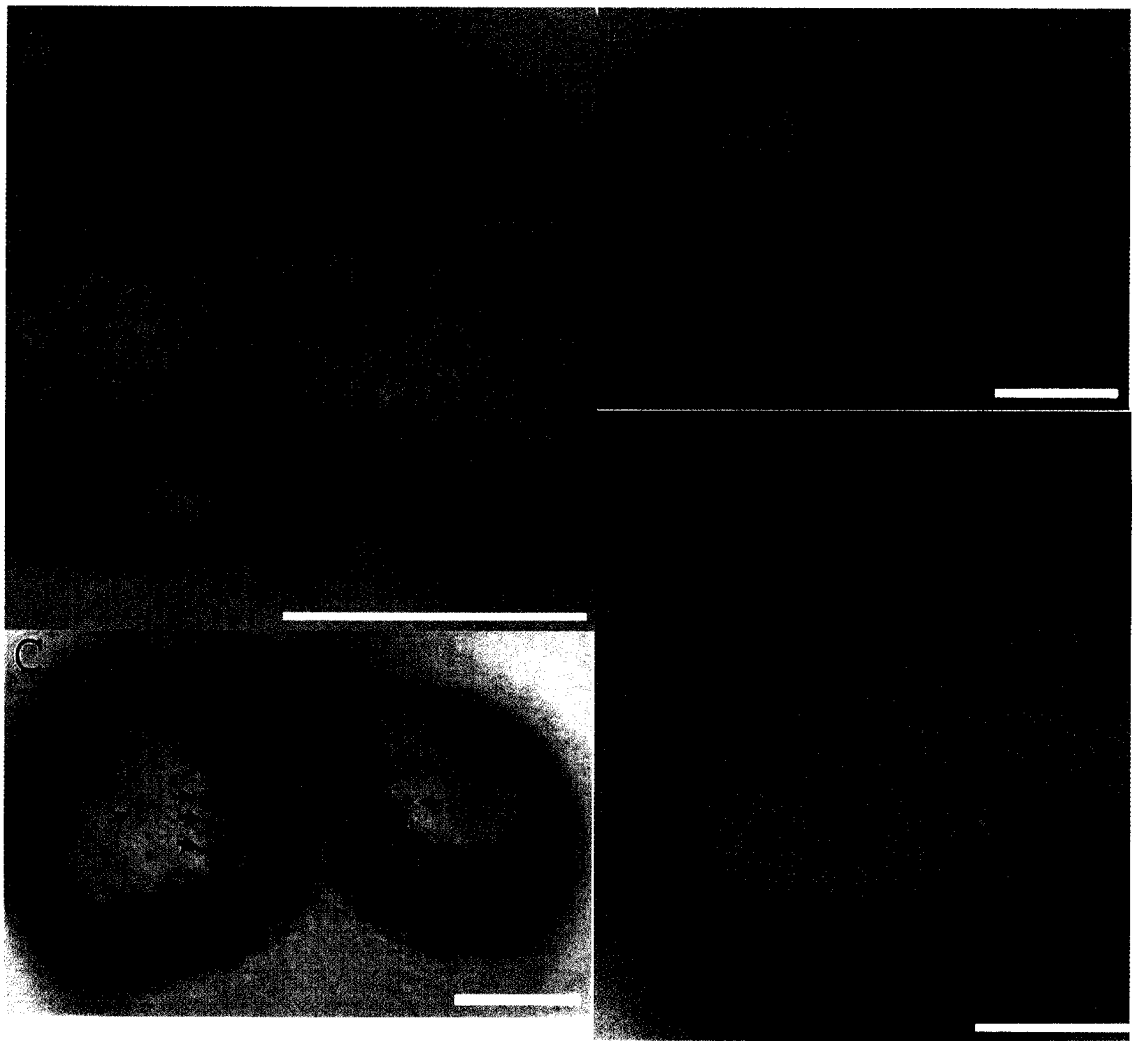


Figure 19. Anti-H-twi localization in stage 8. **A.** Mid stage 8. Dorsal view showing scattered nuclear staining and nuclear staining of cells directly adjacent to the germinal bands (black arrows). **B.** Dorsal view of mid to late stage 8, showing germinal plate. Random nuclear staining shown by arrowheads. **C.** Dorsal view of mid to late stage 8 embryo showing another view of nuclear staining of the cells adjacent to the germinal plate as well as scattered nuclear staining. **D.** Higher magnification of embryo in **C**. Scale bars represent ~100 μm.

Table 1. Total number of *H. triserialis* embryos observed expressing H-twi protein.

Stage of development	Number of embryos*
1	11
2	21
3	8
4b early	14
4b late	9
5	12
6a	8
7	9
8 early	8
8 late	26

*The data shown were collected from four experiments.

Discussion

Correlation of H-twi protein spatial and temporal expression with *Hro-twi* mRNA

A previous study on *twist* mRNA in *H. robusta* leech embryos showed similar *twist* transcript distribution (Soto, 1994) compared to the H-twi protein distribution observed in *H. triserialis* leech embryos studied here. Initially, Soto found by Southern RT-PCR blot that *Hro-twi* mRNA was present in all embryonic stages up to stage 10. This reinforces the results found in this study, where H-twi protein was found in all embryonic stages studied (stages 1-8).

Soto (1994) used non-radioactive *in situ* hybridization to study the expression of *Hro-twi* mRNA in embryos. *Hro-twi* mRNA was detected in oocytes before deposition, indicating maternal transcription. Before the first cleavage, *Hro-twi* mRNA became localized in the animal and vegetal teloplasms, as did the H-twi protein in this study (Figures 11 and 12). Throughout the cleavage stages, *Hro-twi* mRNA remained associated with the teloplasm in ectodermal (including the micromeres), endodermal, and mesodermal proteloblasts. H-twi protein expression decreased during early cleavage (stages 3 to early 4b) then increased significantly at late stage 4b when it was detected in the animal region of the embryo in the consequent cleavage stages. Because the

embryos were not sectioned, it was difficult to discern if the mesodermal precursor (DM') was stained (figure 16). At this point in development (late stage 4b), H-twi was detected cytoplasmically in ectodermal precursors (DNOPQ' and the micromeres) and an endodermal precursor (A''). H-twi expression in the micromeres was similar to that seen for the *Wnt* class gene in *H. triserialis* embryos, a signaling molecule whose protein was detected in certain micromeres in early development, then in the provisional embryonic epithelium in later development (Kostriken and Weisblat, 1992). For the next cleavage stages (5 and 6a-data not shown), H-twi staining was also similar to that seen by Kostriken and Weisblat, where H-twi expression was detected in the micromere cap. By early gastrulation (stage 7) however, the similarity ends when H-twi staining is detected in the mesodermal precursors, the M bandlets (Figure 17 for stage 7).

H-twi protein expression was very low at stage 4a and much higher at late stage 4b (approximately 3 hours later). This increase in expression suggests the beginning of zygotic transcription for *H-twi.* Experiments by Soto (1994) show evidence that *Hro-twi* transcripts are zygotically produced as early as 5 hours after zygote deposition (AZD), or by late stage 2/early stage 3. General zygotic transcription in *Helobdella triserialis* was previously found to occur by stage 4b, when macromere D divides into the mesodermal and ectodermal precursors

(teloblasts DM and DNOPQ, respectively) (Bissen and Weisblat, 1991), later than the evidence shows for *Hro-twi* transcription. However, early zygotic transcription in *H. robusta* has been seen in a recent study of the *WntA* gene, where there is evidence for zygotic transcription during stage 2 (Huang *et al.*, 2001).

By early stage 8, *Hro-twi* mRNA was seen only in the mesodermal (not ectodermal) layer of germinal bands and in some of the micromere derivatives (the future prostomium). H-twi protein was detected in the germinal bands and the future prostomium, but without sectioning the embryos or using lineage tracing, it is not known which germinal bands were stained. However, expression is probably restricted to the M germinal bands (mesodermal precursors) since these are shown to strongly express H-twi at the beginning of gastrulation (Figure 17D). The area of the future prostomium was stained (Figures 18D and 19A-C). H-twi protein staining of the germinal bands is dark, and it is difficult to determine whether the nuclei are stained, or just the cytoplasm. In this stage, the provisional squamous epithelium, a micromere derivative, is not stained. However, by mid gastrulation, nuclear staining is discernable, parallel to the germinal bands on the dorsal side (figure 18D). By mid to late gastrulation, when the germinal plate has formed, nuclear staining is

visible not only parallel to the germinal bands, but also scattered throughout the area of the provisional squamous epithelium (figure 19A-D), which are older than the cells in the germinal plate. While the provisional epithelium is derived from the a, b, c, and d micromere quartet, the contractile fibers of the provisional integument are derived from the M teloblasts (Weisblat *et al.*, 1984). Therefore, the nuclear staining seen in this region may be associated with those fibers. In addition, it appears that there may also be some nuclear staining in the prostomial precursor region, which is also derived from the micromere quartet (Figure 19B) (Weisblat *et al.*, 1984).

The results of this study do not exclude the possibility that *H-twi* is important for mesodermal development. However, they do show that further study is needed in the later stages, in addition to the current stages, using lineage tracing and sectioning to more precisely determine the position of the H-twi protein.

In general, the micromeres in the leech are fated to become part of the temporary provisional epithelium or to become nonsegmental tissues (Smith and Weisblat, 1994). Examples of cell fates for *H. robusta* micromeres include a variety of structures such as epithelial cells of the provisional integument for dnopq′, mesenchyme and muscle fibers in the proboscis for dm′, and neurons

and connective tissue for a''' (Huang *et al.*, 2002). Why is H-twi detected in ectodermal precursor cells (ie., micromeres)? A possible explanation could be that the cell fate of the mesodermal precursors is determined by cell-cell interaction. Evidence of micromere derivatives changing the fate of teloblasts has been documented. For example, Ho and Weisblat (1987) found that ablation of the epithelium changed the fate of the o blast cells. In addition, Huang and Weisblat (1996) found that the presence of a normal q bandlet was necessary for the proper future development of the p blast cells, through some sort of short range signal.

Comparison of spatial distribution of H-twist to *Drosophila* twist protein

The spatial distribution of H-twi protein expression in *H. triserialis* embryos is similar to that of fruit fly *twist* mRNA and protein, which was found in ectodermal, ventral mesodermal and endodermal cells before gastrulation (Thisse *et al.*, 1987a, 1987b, 1988). Thisse *et al.* (1988) studied the distribution of twist protein in *Drosophila* embryos using polyclonal anti-twist antibodies. In all stages that the protein was detected, it was within the nuclei.

Drosophila twist protein expression was found at the beginning of cellularization (stage 5 embryos), which is equivalent to stage 4b in leeches. At this stage, the protein was detected in endodermal, ectodermal, and mesodermal

cells. Similarly, H-twist protein was detected strongly in a similar stage (late stage 4b), mainly in proectodermal and promesodermal teloblasts and micromeres. The H-twi staining seen in the first two stages of leech embryogenesis was lighter than that detected after early stage 4b. These results support previous evidence that showed that *H-twi* has a maternal component. H-twi staining at this stage was not yet nuclear.

During early gastrulation in *Drosophila,* twist protein was detected in the ventral furrow of mesodermal cells, endodermal cells, and ectodermal cells. Similarly, in early gastrulation in *Helobdella* (stage 7), H-twi protein is detected in the micromeres (proectodermal teloblasts) and in the M bandlets (promesodermal blast cells). Staining is not yet exclusively nuclear for H-twi.

By mid gastrulation in *Drosophila,* the protein is detected in the mesodermal cells forming the ventral furrow and in the anterior midgut primordium, which are derived from endodermal and ectodermal cells. A similar trend is seen with H-twi, where at mid gastrulation (stage 8), cytoplasmic staining, possibly nuclear staining, is seen in the germinal bands. In addition, H-twi protein at this stage is beginning to localize to the nuclei of cells located in the area of the provisional squamous epithelium (ectodermally-derived), as well as the nuclei of cells that are directly adjacent to the outer edge of the germinal

bands. The nuclear H-twi observed by this stage is evidence that H-twi is a transcription factor like *Drosophila twist*.

By the end of gastrulation in *Drosophila*, twist protein can only be detected in the mesodermal layer. The protein was still detected up to stage 14, in some segmental cells and in precursor cells to somatic muscles and visceral muscles. The position of H-twi protein at the end of gastrulation has yet to be determined; however, *Hro-twi* transcripts at this stage were detected in ganglionic fibers, hemisomites, and in the mesenchymal tissue of the head region (Soto, 1994).

Regulation of H-twi

H-twi does not become nuclear until mid gastrulation, while *Drosophila* twist appears nuclear from its first detection (Thisse *et al.*, 1988). Is there a signal for H-twi at the beginning of gastrulation needed for the transport of H-twi from the cytoplasm to the nucleus? Previous studies with leech embryos show evidence that there are unknown ectodermal determinants in the teloplasm and the animal cortex that control the fate of cell DNOPQ (Nelson and Weisblat, 1992). In addition, their evidence suggests that the mesodermal fate is determined by factors in the teloplasm only. Perhaps some of these factors are preventing H-twi from entering the nucleus until mid gastrulation. This has been shown in *Drosophila* for *dorsal*, as its transfer to the nucleus is regulated by

11 genes (Roth *et al.*, 1989). Another possibility is heterodimerization. *Twist* can form homodimers and heterodimers due to its bHLH domain. Perhaps H-twist is bound in a non-functional heterodimer until it receives an activation signal. A third possibility is related to the sequence of the localization signal. In general, twist bHLH proteins usually contain a nuclear localization sequence within the bHLH domain, but this can be located elsewhere in the sequence (Jan and Jan, 1993; Castanon and Baylies, 2002-revew). Since the bHLH region of Hro-twist diverges in its amino acid sequence compared to *Drosophila* twist (78% similarity), if the nuclear localization signal is within that region, the mechanism or timing of localization for Hro-twist could be different (Soto *et al.*, 1997; Castanon and Baylies, 2002).

In *Drosophila, twist* is a transcription factor needed for proper mesodermal development (Leptin, 1991; Thisse *et al.*, 1988), known to regulate genes such as *Drosophila MEF2 (DMEF2),* a transcription factor probably involved in the differentiation of the mesoderm (Taylor *et al.*, 1995). *Drosophila* dorsal activates *twist* and *snail* transcription (Pan *et al.*, 1991; Thisse *et al.*, 1991; Ip *et al.*, 1992). These genes are involved in dorsoventral axis polarity and mesodermal development. As discussed in the literature review, homologs of *Drosophila dorsal* and *snail* studied in leeches were found to exhibit different patterns of

protein expression than that detected in *Drosophila* (Goldstein *et al.*, 2001). Their expression was not seen until *after* segmentation, when Hro-dorsal nuclear staining was detected in the micromeres in pre-gastrulation stages (stages 6b-7), in all blast cell nuclei and their descendants through gastrulation, and as stripes of cytoplasmic staining in the germinal plate at the end of gastrulation. In the earlier stages (1-6), Hro-dorsal staining appears as weak teloplasmic staining. A similar pattern is seen for Hro-snail in the same study, providing evidence that Hro-dorsal activates *Hro-snail.* The results of this experiment do not provide evidence toward the possibility that *H-twi* is a target for dorsal regulation, as H-twi protein was detected before dorsal became nuclear; therefore, it seems that *H-twi* transcription was activated by a gene other than *dorsal.*

The findings by Goldstein *et al.* (2001) implicate a function for *dorsal* and *snail* in cell diversification within segment primordium, as opposed to anteroposterior polarity. Evidence for the role of *H-twi* in development awaits functional studies.

Future investigations

The data in this study present different areas for further investigation. Why is H-twi protein being expressed before gastrulation in the ectodermal precursors when it is expected to be expressed in the mesodermal germinal band, as it was for *Hro-twi* mRNA (Soto, 1994)? Why is H-twi not expressed in the nucleus until mid stage 8 as it is for *Drosophila* in all stages (Thisse *et al.*, 1988)? Why is H-twi protein detected moderately in early stages 1 and 2 in the teloplasm, detected faintly in stages 3 and early stage 4b, then detected strongly from late stage 4b on? How is the transport of H-twi into the nucleus regulated? How is its transcription regulated? Are there dorsal binding sites in the *H-twi* promoter?

Further study of H-twi at the end of gastrulation is needed. If H-twi is found to be expressed in the nuclei during segmentation at this stage, that will provide evidence that *H-twi* is essential to normal segmentation. If it is not found during segmentation, *H-twi* might have another function in leech development altogether. The expression pattern of H-twi in late embryogenesis (stages 9 and 10) needs to be compared to *Hro-twi* mRNA expression. Lineage tracing and sectioning of embryos needs to be completed to pinpoint the location

of H-twi protein. Finally, an embryonic western blot will verify the size of the protein and confirm its presence in the various developmental stages.

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To whom it may concern,

I am giving permission to Christine Nelson to use the leech embryonic figure in her M.S. Thesis. I am the original author of such figure.

Sincerely,

Julio G. Soto, Ph.D.
Assistant Professor of Biology and Science Education