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Role of the TLM in the localization of HRO-TWIST mRNA

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ROLE OF THE TLM IN THE LOCALIZATION OF *HRO-TWIST* mRNA

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ABSTRACT

ROLE OF THE TLM IN THE LOCALIZATION OF *HRO-TWIST* mRNA

by Mehrin Farooq

The subcellular localization of mRNA transcripts is critical in sorting cell fate determinants in the developing embryo of the glossiphoniid leech, *Helodella robusta.* Secondary structural motifs are recognized by *trans*-acting factors which aid in the localization of these transported transcripts. Here, the Teloplasm Localization Motif (TLM), a secondary structural motif located in the 3'UTR of *Hro-Twist,* has been defined and characterized. The TLM comprises of a 20 unpaired nucleotide bulge, with one major and one minor stem loop structure projecting from it, and one stem structure in continuum with the rest of the mRNA 3'UTR. Site-directed mutagenesis was used to alter the TLM in four different ways: 1) converting the major stem loop structure into two smaller loops; 2) increasing the size of the major stem loop; 3) inserting a bulge in between the major and minor stem loops, and 4) distorting the structure completely. Three out of the four alterations in the TLM disrupted the polar localization of the *Hro-Twist* transcript indicating the crucial role of the TLM in its localization. Furthermore, structural modeling of other known leech transcripts that localize in the one-cell stage showed that three other mRNAs contain a similar motif (*Lzf2* and *Le-msx*) raising the possibility that this structural element is important in the localization of a class of leech transcripts that localize early in the leech embryo.

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INTRODUCTION

Gene expression is regulated at many levels. The asymmetric distribution of messenger RNA in the cell is an important process in regulating gene expression at the posttranscriptional level. The localization of mRNA to specific sites in a cell generates cell polarity, which is critical in distributing cell fate determinants (Du et al. 2007). Asymmetric mRNA localization is also important for the specification of the germ cell lineage in many organisms including the fruit fly, the nematode worm, zebra fish, and frogs (Zhou and King 2004). Major advantages of localizing mRNA to a discrete site include the controlled synthesis of proteins at targeted locations (Kloc et al. 2002; Paquin and Chartrand 2008) and the production of a morphogen gradient (Du et al. 2007).

mRNA localization is crucial for the development of several organisms and it is widely used across the evolutionary spectrum. In the fruit fly, localization of the *bicoid* mRNA to the anterior and *nanos* mRNA to the posterior of the developing embryo determines its anterior-posterior axis (Kloc et al. 2002). *Krüppel* mRNA, localized to the center of the embryo, is required for the formation of the thoracic segment (Shi et al. 2007). While in the wasp embryo, the anterior localization of the wasp ortholog of *giant* is important for the proper formation of the head and thorax (Brent et al. 2008). In the frog, *vg1* mRNA is localized in the egg vegetal hemisphere (Dale et al. 1993). *Vg1* induces mesodermal fates in cells that would normally become ectodermal if it is mislocalized to the animal hemisphere (Dale et al. 1993). In ascidians, localization of the *posterior end mark (PEM)* mRNA to the posterior region of the embryo is required for unequal cell divisions in the early embryo (Negishi et al. 2007).

Glossiphoniid leech embryos provide an alternative model to study questions of mRNA localization. Zygotes are large, and methods for microinjecting *in-vitro* transcribed mRNAs are easily accomplished. In leeches, maternal mRNAs localize with the teloplasm of zygotes to the cortex prior to the first cleavage (Holton et al. 1994). The teloplasm, which consists of a large number of mitochondria, ribosomes, protein, and mRNA, appears as a clear cytoplasmic area devoid of yolk platelets near the vegetal and animal poles during the first cell cycle (Fernandez et al. 1987; Astrow et al. 1987; Weisblat and Astrow 1989; Holton et al. 1994). Determinants that localize with the teloplasm act in early decision events, such as the assignment of mesodermal and ectodermal fate to cells DM and DNOPQ, respectively. The cell DM is only competent to give rise to mesodermal progeny, because it inherits a determinant(s) localized in teloplasm but not a determinant(s) localized in the animal cortex (Nelson and Weisblat 1992). Early transcripts that localize to the teloplasm have been identified. For instance, *Hro-Nos and Le-msx* transcripts localize in the teloplasm to the polar regions of the oocyte (Kang et al. 2002; Master et al. 1996). *Hro-Nos* is expressed in cells that give rise to the mesoderm (Kang et al. 2002), and *Le-msx*, after multiple cell divisions, becomes concentrated in cells giving rise to the mesoderm and ectoderm (Master et al. 1996).

Soto et al (1997) previously isolated a leech homolog (*Hro-Twist*) to the fruit fly *D-Twist*. In the leech, *Hro-Twist* mRNA is detected in oocytes and during the cleavage stages that lead to the formation of mesoderm (Soto et al. 1997).

In this thesis, an mRNA localization recognition element has been identified in the form of a predicted secondary structural motif called the Teloplasm Localization

Motif (TLM) in *Hro-Twist transcripts*. The TLM consists of a 20 unpaired nucleotide bulge with one major and one minor stem loop structure arising from it, along with a stem structure in continuum with the rest of the transcript. Site-directed mutagenesis was used to alter the TLM structure to determine its role in localizing *Hro-Twist* transcripts in the one-cell stage. Microinjection of the mutated transcripts indicated that three out of the four alterations made localized abnormally in the zygote. Furthermore, it was shown that reducing the unpaired nucleotide bulge from 20 nucleotides to 10 nucleotides resulted in a higher level of abnormal RNA localization than altering only the major stem loop structure. Structural modeling of other leech mRNAs that localize in the one-cell stage showed similar structures in *Le-msx* and *Lzf2*.

LITERATURE REVIEW

Proteins perform a diverse set of functions to regulate many cellular processes. From basic structural components to complex pathways, they are involved in every aspect of cell maintenance and development. It is, therefore essential for the cell to control their spatial and temporal regulation.

Regulation of protein expression starts at the molecular level. The cell controls protein production by regulating gene expression. Genes can be regulated at the pre-transcriptional level by exposing promoter regions and altering nucleosome structure. At the transcriptional level, proteins bind to promoters, enhancers, silencers and other elements to activate or repress the production of mRNA. Genes may also be regulated at the post transcriptional level, by mechanisms like mRNA stability and mRNA localization.

mRNA Stability

mRNA stability is a tightly regulated post transcriptional mechanism which entails prevention of a specific transcript from being degraded, thereby regulating the expression of its translated protein. There are several transcripts that are unstable in non-stimulated cells, but upon stimulation, are stabilized, for example transcripts of proto-oncogenes such as *c-Fos* and *c-Jun* (Shim and Karin, 2002). Several tumors have been identified, such as myeloma and human T cell leukemia, where the *c-myc* transcript was mutated to make it more stable that the non-mutated transcript (Aghib et al. 1990). Other transcripts regulated by mRNA stability include the *utrophin* transcripts in skeletal

muscle cells (Gramolini et al. 2001) and *oskar* transcripts in Drosophila (Snee et al. 2004).

mRNA processing involves addition of m7G(59)ppp(59)N cap structure at the 5' end and poly adenylation (in most transcripts) at the 3' end. The function of the cap and poly (A) tail is to prevent the transcript from being degraded and improve translational efficiency (Shimotohno et al. 1977). Upon direction from the *cis*-elements within the transcripts, the mRNA begins its degradation with 3'to 5' exonucleic deadenylation to remove most or all of the poly (A) tail (Wilson and Treisman 1988) after which, either the cap is removed (Curatola et al. 1995) or the cap may remain and the rest of the transcript is degraded via exonucleolytic degradation (Mukherjee et al. 2002).

The stability of the mRNA is controlled by *cis*-acting elements embedded in the untranslated regions of the transcripts, such as AU rich regions which act as destabilizing elements (Shaw and Kamen 1986) as well as trans-acting factors, such as AUBF (which bind to these regions (Gillis and Malter 1991).

mRNA Localization

mRNA localization is a mechanism where certain mRNAs are transported, localized and locally translated in many eukaryotic cell types (Kindler et al. 2005). It is the movement of mRNA prior to translation along a microtubule to a specific area of a cell where it can then be anchored to the cytoskeleton. This ensures the resultant protein to be expressed in a targeted area (Bashirullah et al. 1998). The transcript can be repeatedly translated at the target location as need for the protein increases, which proves

to be more energy efficient than having the protein made at one location in the cell and then continuously hauled to another subcellular location.

Transport is directed towards the minus ends of the microtubules, and uses the dynein/dynactin motor complex (Wilkie et al. 2001). The same machinery appears to transport several mRNAs into the nascent oocyte from the neighboring, interconnected nurse cells (Bullock et al. 2001). Localization depends on *cis*-acting RNA elements (localization signals) that usually reside in untranslated regions (UTRs) of the transcripts, and which direct association with *trans*-acting protein factors (Bullock et al. 2003). It is a way of storing positional information in an inactive form; it is more energy efficient to localize a relatively small number of mRNAs and translate them many times than to localize a large number of protein molecules (Serano and Cohen 1995).

mRNA localization is particularly critical in development, as mislocalization of any activity, either anterior, posterior, or terminal, has been shown to reorganize the body pattern (Bashirullah et al. 1998). In experiments with *Drosophila* oocytes, when RNA localization is disrupted through mutations, it has been shown to result in the production of misshaped eggs that give rise to headless or other monstrous embryos that die before hatching (Bashirullah et al. 1998).

The mRNAs that localize usually contain one or more *cis*- acting sequence elements that specify how and where the RNA will be localized (Cohen et al. 2005). The pair-rule segmentation genes of *Drosophila melanogaster* localize via signals in their 3'UTRs (Bullock et al. 2003). Localization elements include primary sequences along with secondary structures.

mRNA Localization via Recognition Signals

In order to be localized, the transcripts need to be recognized by the localization machinery, which includes the proteins which bind to the mRNA and transport it to its predestined subcellular locations. Transcripts need to have a localization signal which these proteins can distinguish from a large pool of mRNAs. It has been shown that these signals usually reside in the untranslated regions of the transcripts. These signals can be in the form of nucleotide sequences (zip codes), secondary structures, or a combination of both. *Trans*-acting factors (RNA-binding proteins and non-coding RNAs) bind to these *cis*-acting regions and form tertiary structural motifs.

There are many mRNAs that are recognized via recognition of nucleotide sequences present within the transcript (as reviewed by Jambhekar and Derisi 2007). In many organisms studied, the localization signal primarily resides in the 3' UTR (Jansen 2001).

Sequences in the 3' UTR have been shown to be involved in the apical localization of *even-skipped, fushi tarazu*, and *hairy* pair-rule segmentation genes and the α 1-tubulin and *bicoid* genes (Davis and Ish-Horowicz 1991). The pair-rule genes are expressed as stripes and establish segmental organization in the embryo (Bullock et al 2003). Although most transcripts localize via signals in their 3' UTRs, there are examples where localization occurs via recognition of signals in 5' UTR or coding regions. Fruit fly oocytes (Capri et al. 1997; Thio et al. 2000), yeast (Chartrand et al. 1999; Gonzalez et al. 1999), and nerve cells (Prakash et al. 1997), contain a few mRNAs that have localization signals in the 5' UTR and/or coding region.

A given number of RNA sequences can fold into a secondary structure (Macdonald 1990). The mRNA localization signal which is recognized by the proteins can be sequence of nucleotides embedded within the RNA transcript or a structural motif (Macdonald 1990).

Several RNA secondary structural motifs have been shown to be involved in mRNA localization. Most of these motifs consist of stem loop structures within the 3' UTR of the transcript which are recognized by RNA-binding proteins (Svoboda and Di Cara 2006). There are several transcripts that have been shown to localize via recognition of their secondary structural motifs. For example, the *bcd localization element 1(BLE1)* is a localization signal in the 3'UTR that forms a complex secondary structure containing a stem-loop (Snee et al. 2004). Mutations of *BLE1*, as well as stem-loops III/IV blocks localization of *bcd* (Macdonald and Kerr 1998). Localization of the *gurken* transcript has also been shown to be dictated via a structural motif called the GLS (Van De Bor et al. 2005). The *gurken* transcript is localized to a cresent near the oocyte nucleus. The secondary structural motif has been shown to be involved in controlling the dynein-mediated transport of the *gurken* transcript (Van De Bor et al. 2005).

In 2005, Cohen et al. identified and characterized a stem loop structure in transcripts of *K10* and *Orb* in the *Drosophila* called the TLS. The TLS was shown to mediate transcript localization regardless of its nucleotide sequence or location within the transcript. It was also shown that altering the size and shape of the structure affected localization. Santos et al. 2008 showed that a stem loop structure in the *wingless* mRNA 3'UTR, called the WLE3, was required for the apical localization of the *wingless*

transcript. The WLE3 motif is conserved among several other apical localizing transcripts. The *c-myc* and *c-fos* transcripts have also been shown to localize via recognition of an AU rich stem loop structure (Chabanon et al. 2005). *C-myc* and *c-fos* transcripts localize to the perinuclear cytoplasm and associate with the cytoskeleton. Also, in bacteria, the *sxy* (tfoX) gene, which is involved in DNA uptake contains a structure in its transcript at the 5' end of the *sxy* mRNA which, when mutated, resulted in lower translational efficiency (Cameron et al. 2008).

Twist

The *Twist* gene encodes a transcription factor harboring the bHLH domain (Thisse et al. 1987) which, *in-vitro* enables Twist to form homodimers and *in-vitro* binds to chromosomes (Thisse et al. 1992). Homologs of Twist have been identified in vertebrates as well as invertebrates including the frog (Hopwood et al. 1989), the chick (Tavares et al. 2001), the nematode (Harfe et al. 1998), the glossophiniid leech (Soto et al. 1997), the mouse (Gitelman 1997) and humans (Chen and Behringer 1995). It was first identified as a zygotic gene, which when mutated resulted in the death of the Drosophila without forming the mesoderm germ layer (Simpson 1983). It was therefore considered to be a mesoderm-determining factor (Castanon and Baylies 2002).

In vertebrates, it is mainly expressed in mesoderm-derived tissues including the head mesenchyme, branchial arches, limb buds and sclerotome (Gitelman 1997; Soto et al. 2002 and Tavares et al. 2001). In the leech, it is maternally inherited and present

throughout development (Soto et al. 1997). It is detected in the oocytes and during the cleavage stages that lead to the formation of mesoderm (Soto et al. 1997).

Development of the Glossophiniid Leech Embryo

Glossiphoniid leech embryos provide an alternative model to study questions of mRNA localization. They are especially useful for the study of mesoderm formation because a single cell division at the fourth cleavage separates precursors for mesoderm and ectoderm (Soto et al. 1997).

In the one cell stage of leech embryos, the teloplasm, which is specialized yolk-free cytoplasm consisting of a large number of mitochondria, ribosomes, protein, and mRNA, segregates towards the vegetal and animal poles during the first cell division (Fernandez et al. 1987; Astrow et al. 1987; Weisblat and Astrow 1989; Holton et al. 1994). The first cleavage is unequal and results in cells AB and CD. The segregated teloplasms go to the larger cell CD (Nelson and Weisblat 1992). Two more cell divisions result in equal sized cells A, B and C and a larger cell D which contains the teloplasms (Nelson and Weisblat 1992). The next cleavage results in quartets of animal micromeres (a′-d′) and vegetal macromeres (A′-D′), where micromere D′ undergoes division to produce an animal daughter cell DNOPQ and vegetal daughter cell DM (Fernandez 1980). Localization of the teloplasm determines the fate of these daughter cells (Nelson and Weisblat 1992). DNOPQ produces ectodermal stem cells and DM produces mesodermal stem cells (Sandig and Dohle 1988; Bissen and Weisblat 1989). Both are precursors of teloblasts (Fernandez 1980). Furthermore, localization of the teloplasm at

or near the animal pole of DNOPQ is required to produce ectodermal instead of mesodermal teloblasts.

MATERIALS AND METHODS

Embryos

Helobdella sp. leeches were collected from a sturgeon farm at Galt, CA, and maintained in the laboratory at 1% ocean water. Embryos were manually removed from the cocoons deposited on the ventral side of the leech immediately after zygote deposition, and transferred to sterile HL saline (4.8 mM NaCl, 8.0 mM $CaCl_2$, 2.0mM $MgCl_2$, 1.2 mM KCl, and 1.0 mM Tris-HCl, pH 6.6) media until they were ready for microinjection.

Bioinformatics

Repfind and UTR BLAST database (http://bighost.ba.itb.cnr.it/BIG/Blast/BlastUTR.html/) were used to map localization sequences (*ACE1*: CAACAAC; *ACE2*: CGACGAC; *ARE1*: ATTTA; *ARE2:* AATAATA; and *CPE*: TTTTTTAT) in transcripts of *Hro-Twist, Le-msx, Hro-Nos, Hro-Hh* and *Hro-Eve.* Predicted secondary structures of transcripts of *Hro-Twist, Le-msx, Hro-Nos, Hro-Hh, Lzf2, Hro-Sna, Hro-Dl* and *Hro-Eve,* along with the 3'UTR of *Hro-Twist* were obtained by submission of mRNA sequences to the Rensselaer bioinformatics web server: (http://mfold.bioinfo.rpi.edu/) with the temperature set to 16°C.

The coding sequence of *Helobdella robusta Hro-Twist* was obtained from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/). The accession number is AF410867.

Primer design

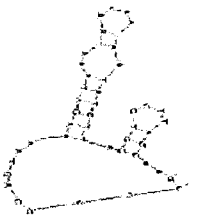
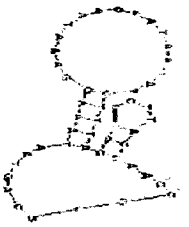
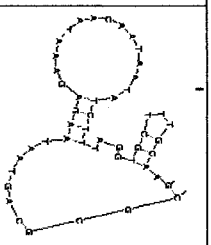
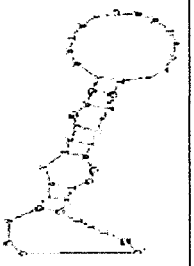
The primers for *Hro-Twist* 3'UTR were designed based on the predicted mfold structures obtained from the Rensselaer bioinformatics web server. Primers were designed to introduce point mutations that brought about various structural alterations in the TLM. Three criteria were taken into consideration when primers were designed: 1) mutations only altered the structure of the TLM and the rest of the 3'UTR structure was not affected, 2) the length of the 3'UTR was not altered, and 3) point mutations did not affect any localization sequences.

Site Directed Mutagenesis

The QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) was used to induce the point mutations. The *Hro-Twist* 3'UTR template used for the mutagenesis reactions was cloned into pBluescript. The plasmid template and primers were diluted to 50 ng/µl and 100 ng/µl respectively in sterilized, Millipore water. Table 1 shows the primers used in this study and the resulting secondary structural changes to the TLM. The reaction mixture for the mutagenesis reaction was as follows: 2.5µl of 10X QuikChange Multi reaction buffer, 50ng of M13F plasmid template, 100 ng of mutagenesis primer, 1µl of dNTP mixture, 1µl of QuickChange Multi enzyme blend (2.5 enzyme units). The reaction was brought to a total volume of 25µl using sterilized Millipore water. The tubes were vortexed and 50µl of mineral oil was added on top of the reaction mixture. The tubes were then inserted in the Thermocycler (Perkin Elmer Cetus DNA Thermocycler 480) with the following settings: one cycle of 95°C at one

minute, along with 30 cycles of one minute at 95°C, one minute at 55°C and 8 minutes at 65°C. Samples were then stored at -20°C. Then, a *Dpn* 1 digest was performed for 3 hr at 37°C, to remove methylated and hemimethylated DNA.

Table 1. List of Mutagenic Primers and Resultant structures. The underlined nucleotides represent mutations.

Mutation Name	**Template Sequence**	**Mutant Primer**	**Structural alteration**
Hrotwi252 A (Mutation A)	5'-TATTATTATAACAG TAATAAGAGAAAT AACAATAATATCTT TAGG-3'	5'-TATTATTATAACAG TAATAAGATAAAT AACAATAATATCTT TAGG-3'	
Hrotwi250 D (Mutation D)	5'-ATTATTATTATAA CAGTAATAAGAGA AATAACAATAATA TCTTTAG-3'	5'-ATTATTATTATAA CAGTAATAAAGGA AATAACAATAATA TCTTTAG-3'	
Hrotwi280 E (Mutation E)	5'-CAATAATATCTTT AGGTGCTTACCAA GAGACAAAAAATG CCTTT-3'	5'-CAATAATATCTTT AGGTGCTTTGCAAG AGACAAAAAATGC CTTT-3'	
Hrotwi276 F (Mutation F)	5'-AATAACAATAATA TCTTTTGGTGCTTA CCAAGAGACAAAA AATGC-3'	5'-AATAACAATAATA TCTTTTGGTACTTA CCAAGAGACAAAA AATGC-3'	

The samples were then transformed into XL10-Gold Ultracompetent *E. coli* cells (Stratagene, cat. #200314). The transformation reaction was prepared as follows: a 14-ml BD Falcon polypropylene round-bottom tube (BD Biosciences, cat. #352059) was first pre-chilled by placing on ice; next, 45µl of freshly thawed cells were added to the tube to which 2µl of XL10-Gold beta-mercaptoethanol mix was added. The tube was then incubated on ice for 10 minutes, after which 5µl of the *Dpn*1 digested sample was added. The resultant mixture was incubated on ice for 30 minutes and then heat pulsed at 42°C for 30 seconds exactly. The samples were then placed on ice for 2 minutes.

The cells were then incubated in 500µl of Luria-Bertani broth, without Ampicillin, for 1 hour at 37 °C, with constant shaking at 225 rpm in a shaker. The cells were then plated on LB agar plates containing Ampicillin (100ug/ml) for 16 hours at 37°C. Colonies were then picked and grown in 3 ml of LB broth with 100µl/ml of Ampicillin for 16 hrs at 37°C with constant shaking at 225 rpm. After cells were grown in culture, plasmid DNA was isolated using Wizard Plus Minipreps DNA Purification System (Promega, cat.# A7510). The isolated DNA was then sequenced at Tocore DNA Sequencing Service. The mutants were sorted out from the non-mutants by analysis of the sequencing results in the program BioEdit Sequence Alignment Editor.

Cultures of cells containing the mutant plasmids were grown as before (streaked on LB agar plates with Ampicillin, 100µl/mg, for 16 hours at 37°C, individual colonies from each plate grown in 100ml of autoclaved LB broth with Ampicillin for 16 hours at 37°C with constant shaking at 225rpm).

After the incubation, 950µl of cells were first obtained from the culture and transferred to cryotubes with 50µl of DMSO for storage of cells containing the mutant DNA at -80°C. The remaining culture was then centrifuged at 2000 x g for 20 minutes in a 50 ml falcon tube (repeated again to pellet the full volume of 100ml of cells). The mutant DNA was then isolated from the cells using the Qiagen HiSpeed Plasmid Midiprep kit (Qiagen, cat. #12643). The concentration of each DNA sample was obtained using the Nanodrop spectrophotometer.

The DNA sample was then linearized with *Xho* I. The linearization sample was prepared in the following way: 10µg of plasmid DNA from the midiprep, 1µl of 100X BSA, 10µl of 10X NEB Buffer #2 (New England Biolabs, cat. #B7002S), 2µl of *Xho* I enzyme (40 enzyme units, New England Biolabs, cat. #R0146S) were added to an eppendorf tube. DEPC-treated water was then added to bring the reaction to a volume of 100µl. The mixture was then incubated at 37 °C for three hours, 65 °C for 20 minutes and finally placed on ice.

Five microliters were run into a 1% agarose gel to verify for linearization of the plasmid (50 minutes of run time: 20 minutes at 70V and 30 minutes at 90V). A band was observed of 3.9kb length.

DNA from the mixture (containing enzyme) was extracted with chloroform in the following way: an equal volume of chloroform was added to the tube and centrifuged at 16,000 *x g* for 5 minutes. The upper aqueous layer (containing the DNA) was then transferred to a new 1.5ml microcentrifuge tube. Two hundred microliters of 100% ethanol (2X the volume of the sample) and 10µl of sodium acetate (0.1X the volume of

the sample) were then added to the tube. The sample was then placed at -20 °C overnight to precipitate out the DNA. The next day, the sample was centrifuged at 12,000 rpm for 15 minutes in the cold (4 °C). The supernatant was discarded and the pellet washed with pre-chilled 70% ethanol (100µl of 70% ethanol added to the mixture followed by 5 minutes of centrifugation at 12,000 rpms at 4°C). The 70% ethanol was then discarded and the pellet was dried using the Rotovac (Savant Speed Vac Plus, SC110A). The pellet was then resuspended in 10µl of DEPC water.

In-vitro Transcription of DIG-labeled mRNA

The linearized DNA was then transcribed to produce DIG-labeled RNA. The *in-vitro* reaction was prepared as follows: 1ug of DNA, 4µl of Transcription Optimized 5X Buffer (Promega, cat. #P118B), 5µl of Ribo m7G Cap Analog (Promega, cat. # P171B) diluted to 5mM in Millipore H20, 2µl of 10X DIG RNA Labeling Mix (Roche Diagnostics, cat. #11093274910), 2µl of 100mM dithiothreitol (DTT, Promega, cat. #P117B), and 2µl of RNasin Plus RNAase Inhibitor (80 enzyme units, Promega, cat. #N261A). DEPC water was added to bring the volume to 19µl and 1µl of T3 RNA polymerase (80 enzyme units, Promega, cat. #P402A) added. The reaction sample was incubated at 37°C for 3 hours, after which 0.8 µl of EDTA was added and incubated at 65°C for 10 minutes. The sample was then kept on ice.

The DIG-labeled and capped *Hro-Twist* mRNA was precipitated by adding 2.5 µl of 4 M LiCl and 75µl of cold 100% ethanol, followed by an incubation at -20°C for 30 minutes. After precipitation, the sample was centrifuged for 15 minutes at 10,000 *x g* in

the cold (4 °C). The RNA pellet was washed with 200µl of cold 100% ethanol and dried in a Rotovac without heat for 5 minutes. The RNA was then resuspended in 10 µl of DEPC water at 37°C for 30 minutes. Using the Nanodrop spectrophotometer to obtain the concentration of the RNA, aliquots of RNA at 25 ng/µl, in a 0.2 N KCl, 0.1% fast green solution (fast green FCF, Sigma, cat.#F-7252) were prepared and stored at -80 °C.

Microinjection of Mutant mRNA Transcripts in *Helobdella sp.* Zygotes

Gravid leeches (having a white line along the abdomen) were constantly watched until the embryos started to deposit. During the deposition process the leech twisted around itself and released embryos two at time. The embryos were then separated from the leech using blunt end forceps and lined up in an agarose gel (agarose in 1% sea water) channel, set in a petri dish.

The prepared RNA was loaded through capillary action in capillary tube needles. The needles were prepared in the following way: capillary tubing, Borosil 1.0mm OD X 0.75 mm ID (Frederick Haer & Co., cat. #30-30-0) was flamed and pulled with a Micropipette Puller (Sutter Instrument Co., Model P-87) with the following settings: pressure = 200, heat = 320, pull = 100, velocity = 10, and time = 80. The tips of the resulting needles were then cut with jewelers forceps.

After the RNA was loaded in the capillary needle, the needle was then inserted into the microinjector apparatus which consisted of a FemtoJet microinjector (Eppendorf, cat.# 5247-000.013) and a Nikon Type 102 Dissecting Microscope (Makroskop, M420).

The needle was calibrated before injecting into embryos. Three parameters were calibrated on the FemtoJet microinjector: Pc (pressure needed to prevent leaking), Pi (injection pressure), and Ti (injection time). All three settings were optimized to ensure that the resultant drop was at a diameter of 100 um (in mineral oil). This resulted in a volume of 5×10^{-4} µl released in each injection. This was calculated in the following way: The diameter of the drop was 100µm, therefore the radius was 50µm. This was plugged into the equation for the volume of sphere= $(4/3)\pi \times (\text{radius})^3$, to calculate the volume of the injected drop which was 523333 μm^3 or 5 x 10-4 µl (1 $\mu m^3 = 1.0 \times 10\text{-}9\mu l$).

With the concentration of the RNA at 25 ng/µl and the volume of the injected sample being 5 x 10-4 µl, each injection was normalized and each embryo was injected with 12.5pg of RNA. The embryos were injected within 20 minutes of deposition and before formation of the first polar body.

After all the embryos were injected, they were transferred into a petri dish containing HL saline where they were allowed to develop for 3-4 hours until the teloplasm was formed. Upon teloplasm formation the embryos were fixed with 4% paraformaldehyde (Electron Microscope Sciences, cat. #15710), 0.25 X PBS, and 100 mM Cacodylic acid in sterilized Millipore water, for 1 hour at room temperature, with constant rocking. They were then washed with PBS and dehydrated with a wash of 50% methanol in PBS and finally with 100% methanol. They were then stored overnight at 4°C.

The next day, the embryos were gradually rehydrated with washes with increasing concentrations of PBS (first with a methanol:PBS ratio at 60:40, then 30:70 and finally

with 100% PBS). They were then devitellinized with 000 insect pins under a dissecting microscope. The embryos were then incubated for 3 hours at room temperature in blocking solution prepared in the following way: 10% goat serum (Pierce, cat. #PI-31873), 25% bovine serum albumin (Promega, cat. #W384A) in PBS with 0.1% Tween-20 and filtered through a 0.22um filter. After the 3 hour incubation time, anti-DIG Fab (Roche Diagnostics, cat. #11093274910) was added at a concentration of 1:200 and the mixture was incubated overnight (8 hours) at 4°C, and then 4 hours at room temperature. The embryos then went through three 15 minute, followed by three 1-hour-long PBT washes after which coloration buffer was added to them. The Coloration buffer was prepared in the following way: 100mM Tris-HCl, 1.0mM NaCl, and 0.1% Tween-20, pH 9.5. The embryos were washed twice (with 5 minute incubations at room temperature with constant rocking) in the Coloration buffer after which they were transferred to a 9-well staging dish (Corning Inc., cat. #7220-85) with minimal transfer of Coloration buffer.

NBT/BCIP reagent (Roche Diagnostics, cat. #1697471) was added to the well with the embryos and the embryos were incubated in the dark for 10-15 minutes. After staining was visible, the embryos were washed with PBS and slowly dehydrated with increasing concentrations of ethanol. The embryos were then cleared with methyl salicylate (Sigma, cat. #M2047) and viewed as whole mounts using a Zeiss Axiophot 2 microscope.

RESULTS

Identification of Localization Elements in *Hro-Twist, Hro-Eve, Le-msx, Hro-Hes, Hro-Nos* and *Hro-Hh*

mRNA sequences of *Hro-Twist* (accession AF410867), *Hro-Eve* (accession AF409098), *Le-msx* (accession U61846), *Hro-Hes* (accession AY144625), *Hro-Nos* (accession U85192)and *Hro-Hh* (accession AF517943) were entered into Repfind and UTR Blast because they are expressed in the early embryo. *Hro-Twist, Hro-Hes, Hro-Nos* and *Le-msx* are expressed in the one cell stage where as *Hro-Eve* and *Hro-Hh* are expressed in stage 7 (Song et al. 2002) and stage 8 (Kang et al. 2003) respectively. The following localization elements were found: *ACE1* (CAACAAC), *ACE2* (CGACGAC), *ARE1* (ATTTA), *ARE2* (AATAATA), and *CPE* (TTTTTTAT). These elements were mapped in the above mentioned transcripts to look for a conserved pattern (Figure 1). Mapping them showed that there was no conserved pattern of any of the localization sequences in their presence or location within the transcripts. For example, the 3'UTR of *Hro-Twist* had five *ARE2* sites where as the 3'UTR of *Le-msx, Hro-Hes, Hro-Eve* and *Hro-Hh* had none.

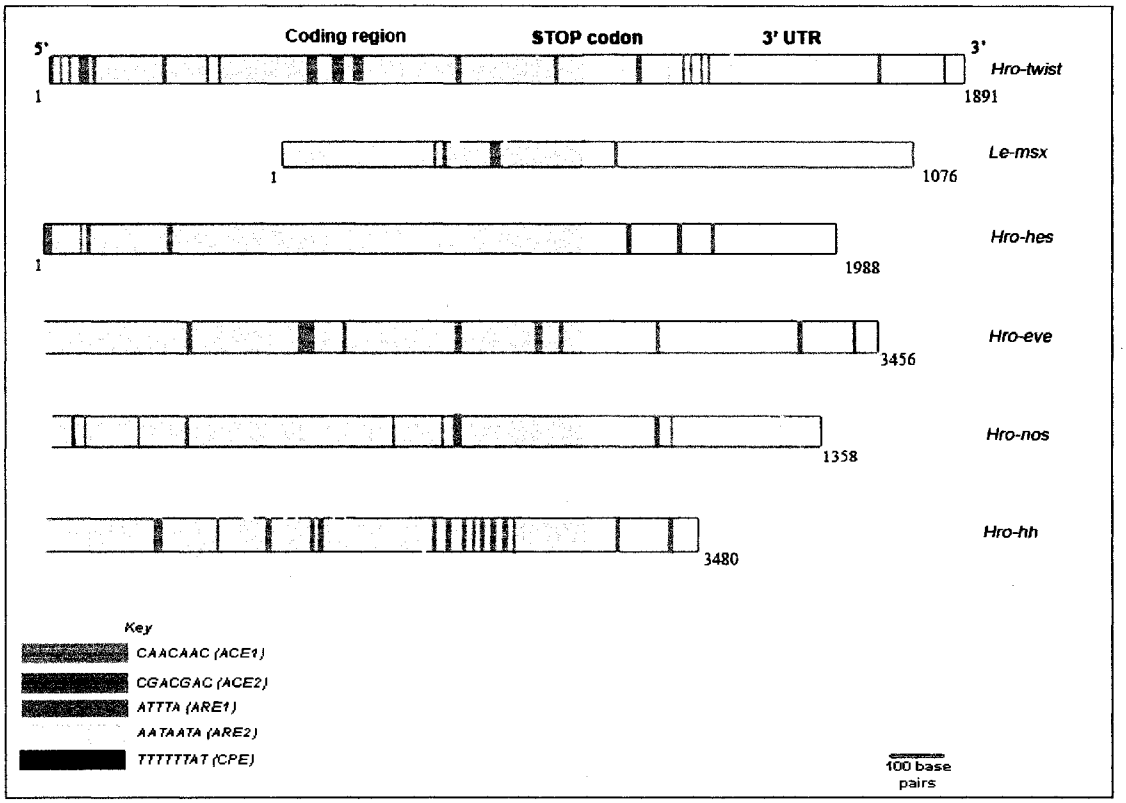
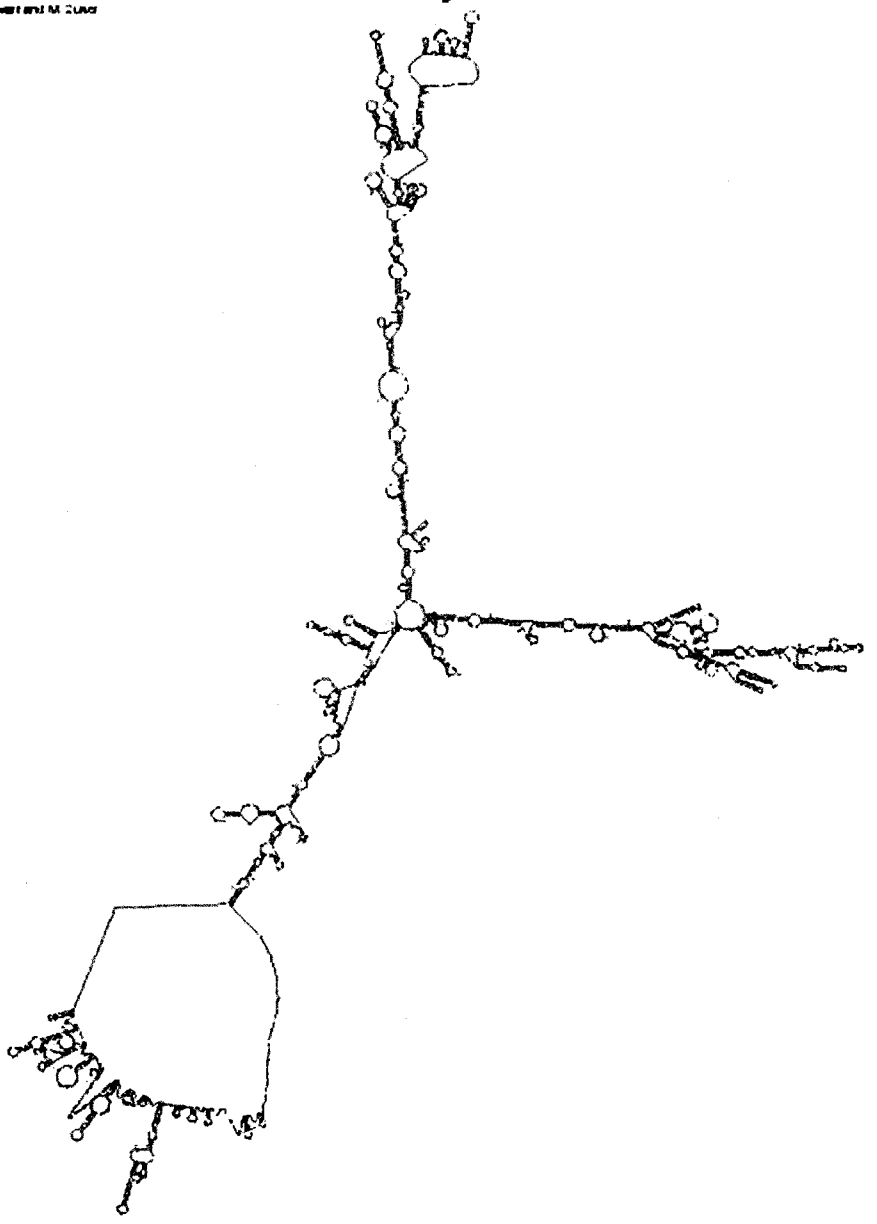


Figure 1. Localization sequences mapped out in transcripts of *Hro-Twist, Le-msx, Hro-Hes, Hro-Eve, Hro-Nos* and *Hro-Hh* determined by UTR BLAST

Identification of a Conserved Structure in Transcripts of *Hro-Twist, Hro-Eve, Le-msx, Hro-Snail, Hro-Dl, Lzf2, Hro-Hes, Hro-Nos* and *Hro-Hh*

Having found no pattern of conservation of localization elements in the transcripts, the mRNA sequences of the above mentioned genes were then entered into Mfold (http://mfold.bioinfo.rpi.edu/) to look for a conserved structure (Figure 2).

Predicted Secondary structure of *Hro-Twist*



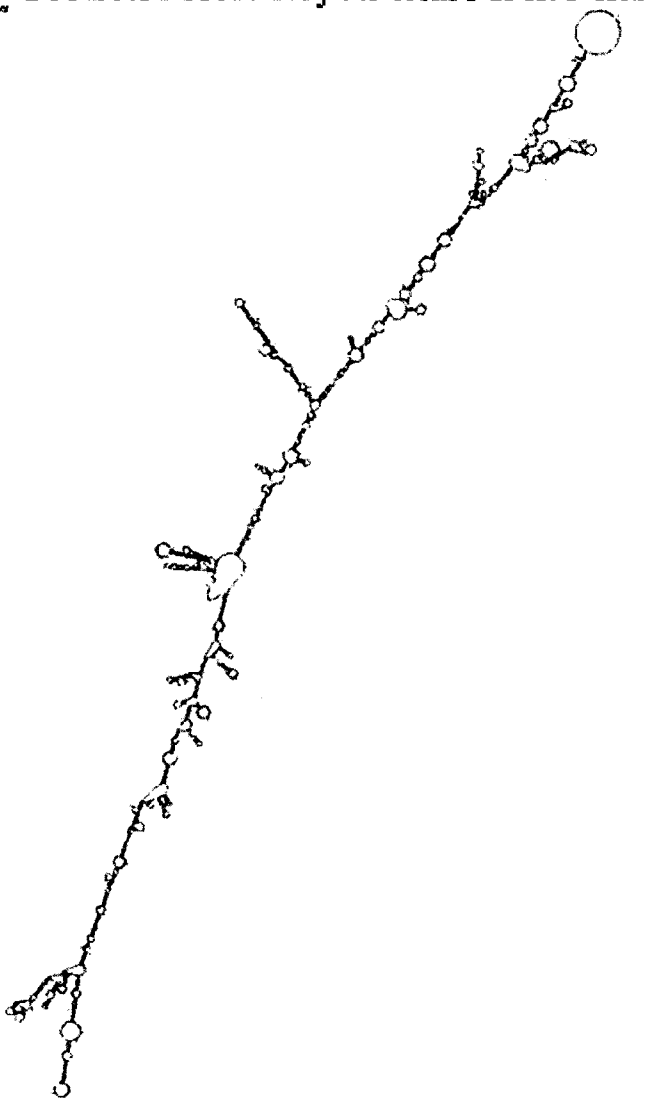
dG = -409.05 complete twist

a.

b.

c.

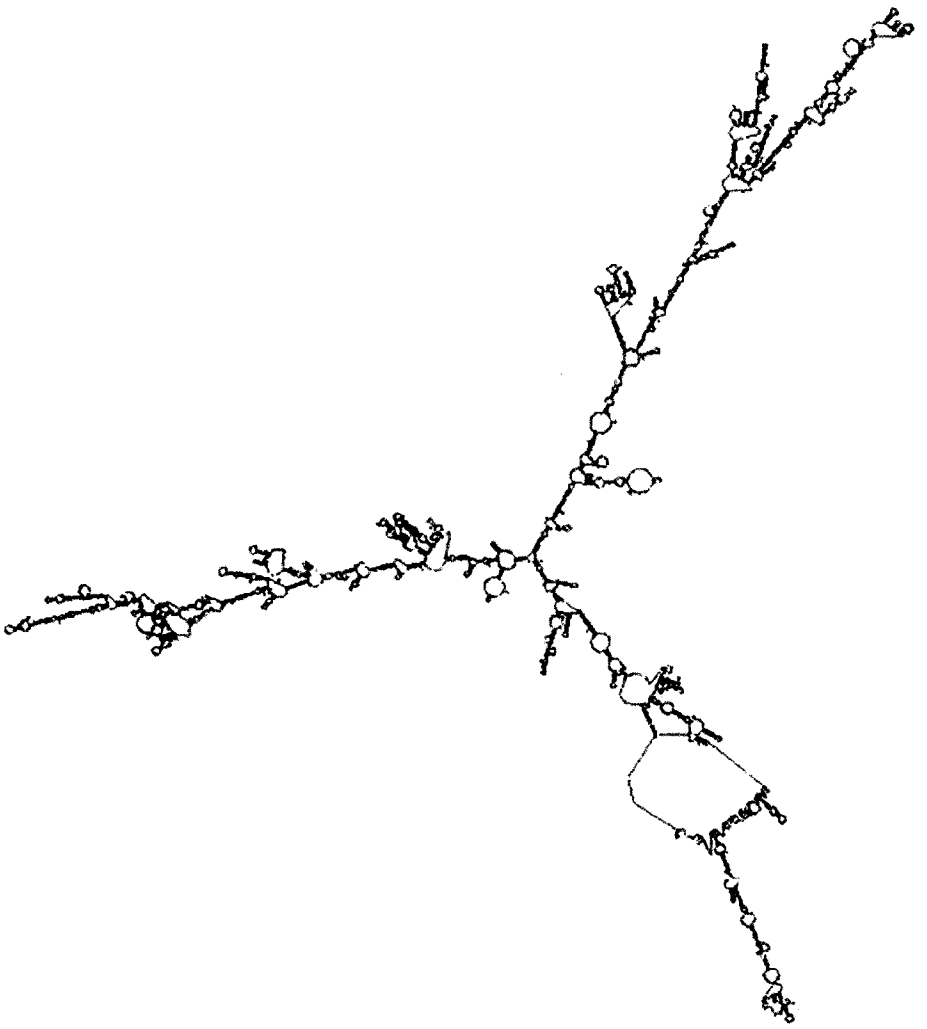
Predicted Secondary structure of *Hro-Hes*



dG = -346.65 hes

Output of ss_graph
by D. Stewart and M. Zuker

Predicted Secondary structure of *Hro-Hh*

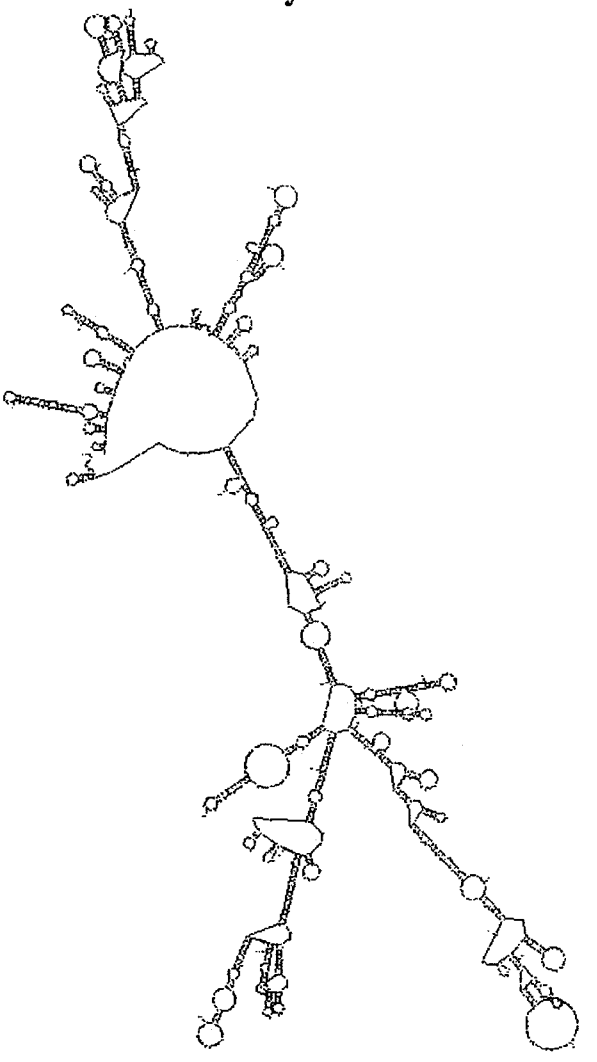


dG = -688.44 hh

d.

Output of ss_graph
by D. Stewart and M. Zuker

Predicted Secondary structure of *Hro-Nos*

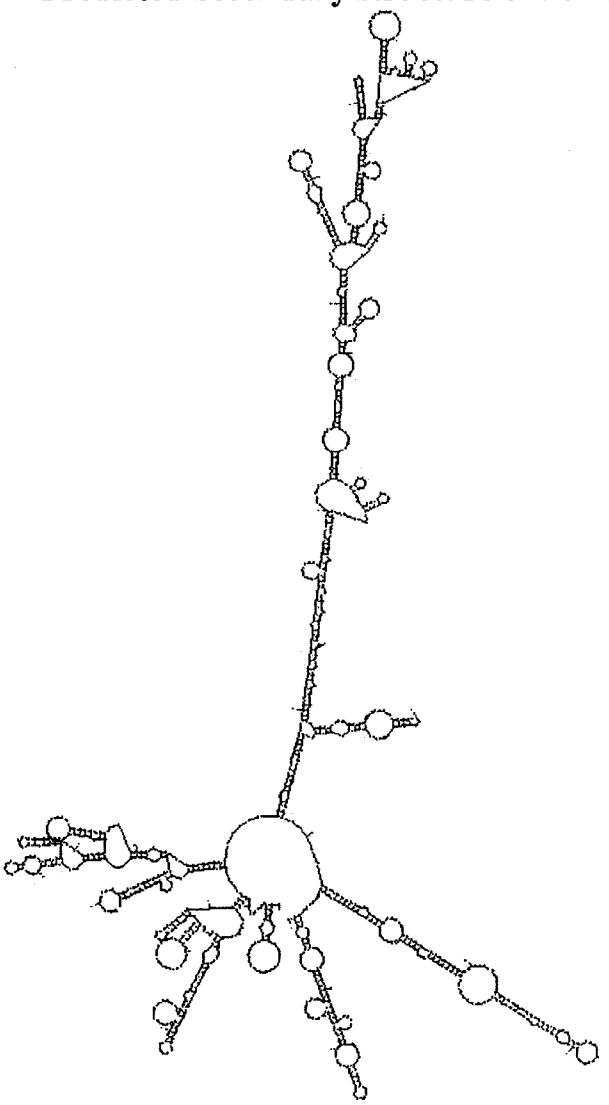


dG = -290.38 nanos

e.

Output of ss_graph
by D. Stewart and M. Zuker

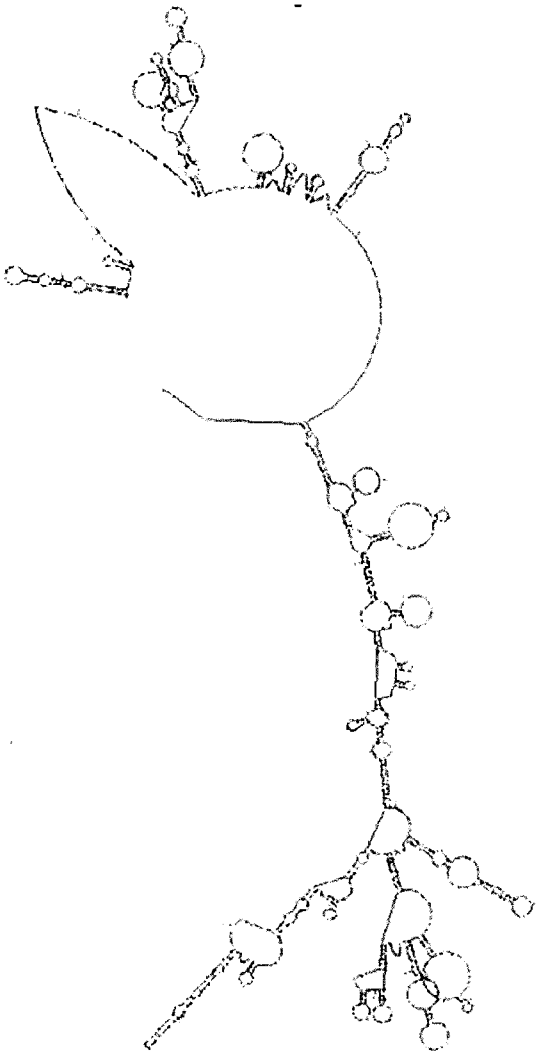
Predicted Secondary structure of *Le-msx*



dG = -204.44 Le-msx

f.

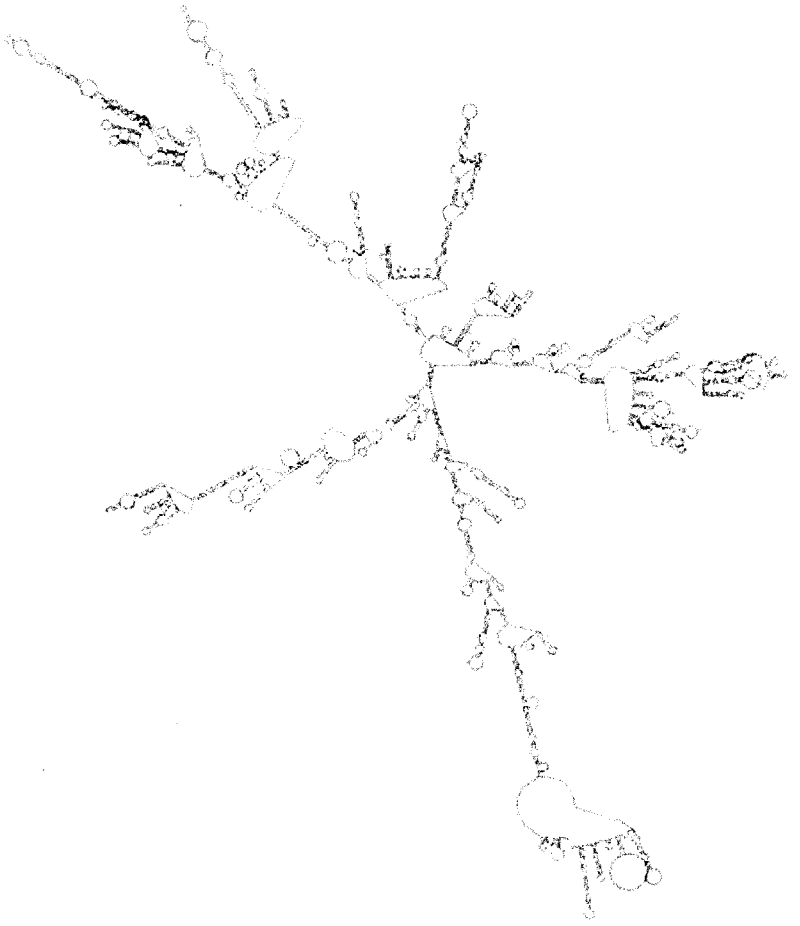
Predicted Secondary structure of *Hro-Sna1*



dG = -186.12 sna1

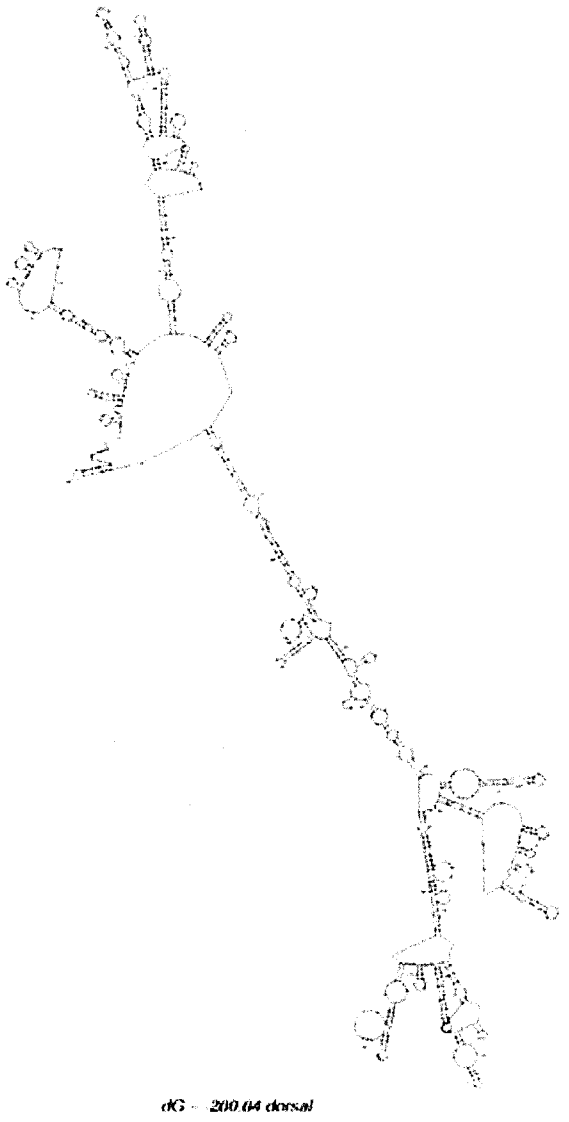
g.

Predicted Secondary structure of *Lzf2*



h. dG = -575.97 Lf2 complete

Predicted Secondary structure of *Hro-Dl*



i.

Figure 2. Predicted secondary structures and respective measures of free energy(ΔG) of a. *Hro-Twist*, b. *Hro-Eve*, c. *Hro-Hes*, d. *Hro-Hh*, e. *Hro-Nos,* f. *Le-msx,* g. *Hro-Sna1*, h. *Lzf2* and i. *Hro-Dl* determined by Mfold.

In the *Hro-Twist* transcript, a structure was found in the 3'UTR region which was similar to a structure in *Le-msx,* and two structures in *Lzf2* (Figure 3). In *Hro-Twist* this structure was named TLM, or Teloplasm Localization Motif.

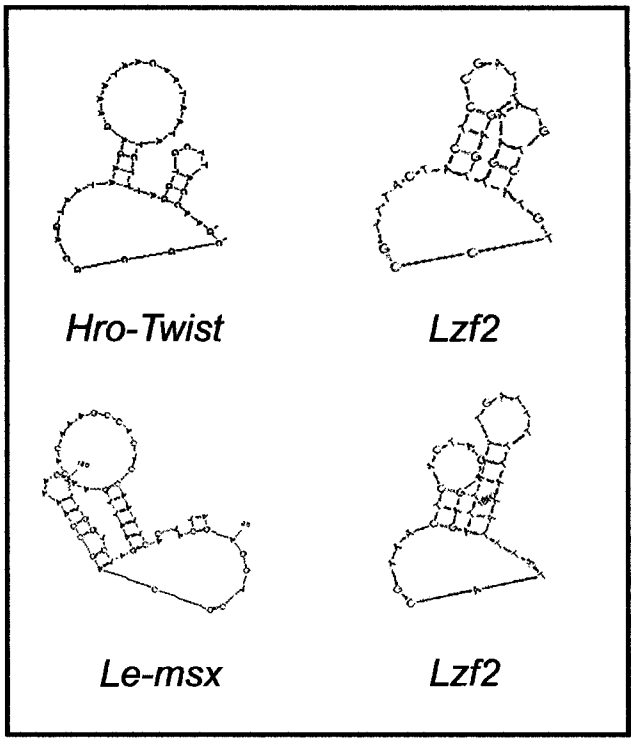


Figure 3. Similar predicted secondary structural motifs found in *Hro-Twist, Lzf2, and Le-msx.*

Alteration of the TLM in the 3'UTR of *Hro-Twist*

In this experiment, only the 3'UTR was to be injected into the embryos so that the injected mRNA would not saturate the localization machinery. Therefore, before designing mutations in the TLM, only the 3'UTR of *Hro-Twist* was entered into Mfold to check for the presence of the TLM (Figure 4).

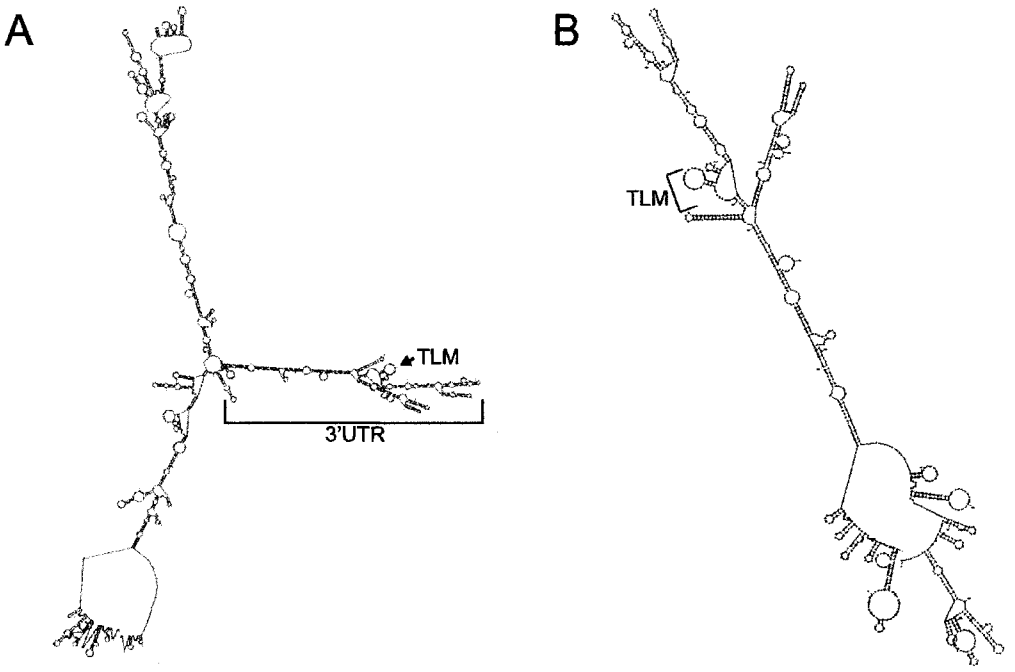


Figure 4. Predicted secondary structure of *Hro-Twist.* A. Predicted secondary structure of *Hro-Twist* with 3'UTR region and TLM indicated. B. Predicted secondary structure of *Hro-Twist* 3'UTR only with TLM indicated.

The predicted structure (Figure 4) of the 3'UTR folded alone contained the TLM. Also, the 3'UTR alone folded in the same manner as when in continuum with the rest of the transcript.

Point mutations, designed using Mfold, were then made in the TLM of the 3'UTR of *Hro-Twist*. Four alterations were made: 1) converting the major stem loop structure into two smaller loops; 2) increasing the size of the major stem loop; 3) inserting a bulge in between the major and minor stem loops, and 4) distorting the structure completely

(Figure 5). The alterations in the TLM did not affect the structure of the rest of the 3'UTR and affect the localization elements.

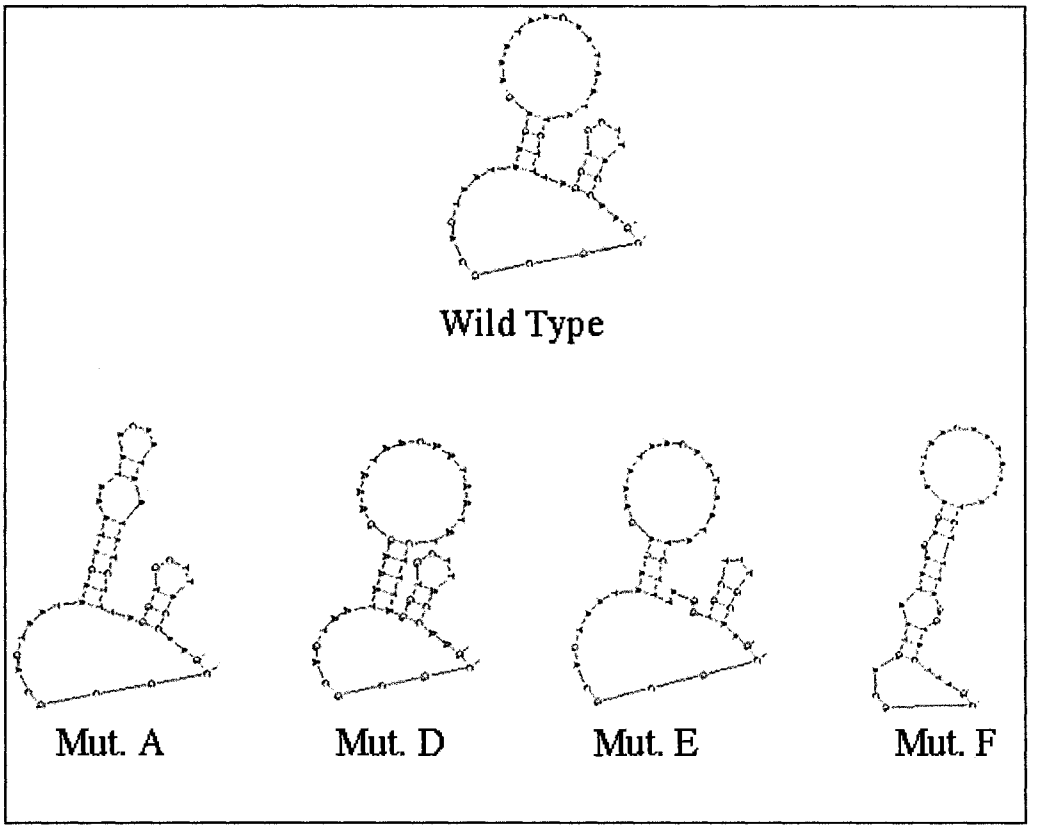


Figure 5. Mfold predictions of four TLM alterations. Only the TLM is shown. The rest of the 3'UTR structure was identical in all the four mutations and wild type.

Site-Directed Mutagenesis to Alter the TLM

The primers designed with Mfold were used to insert point mutations in the TLM, thereby altering its structure. To ensure that the site was successfully mutated, the DNA, obtained from minipreps was sequenced. The sequencing results indicated successful substitution mutations (Figure 6). All four alterations were successfully made.

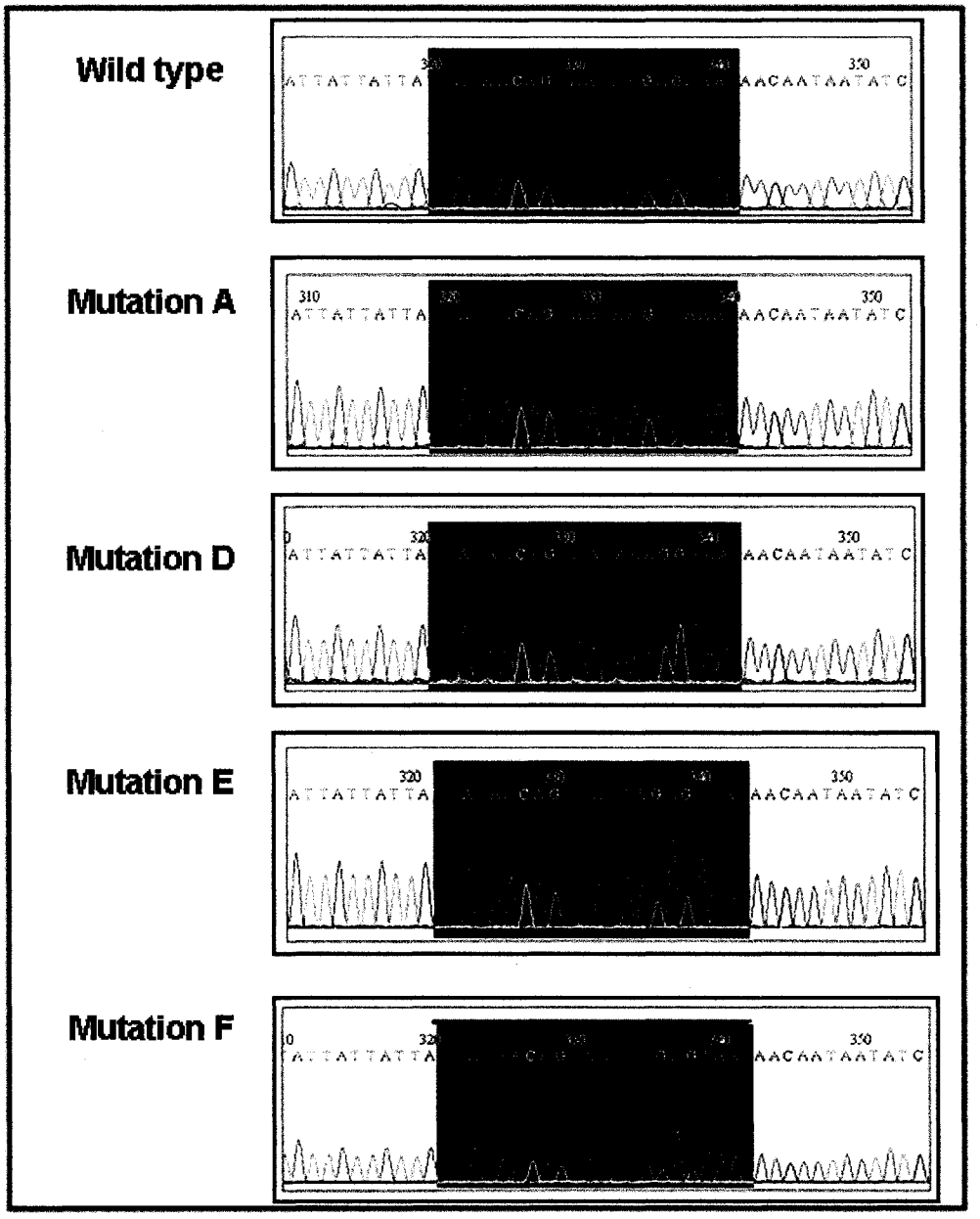
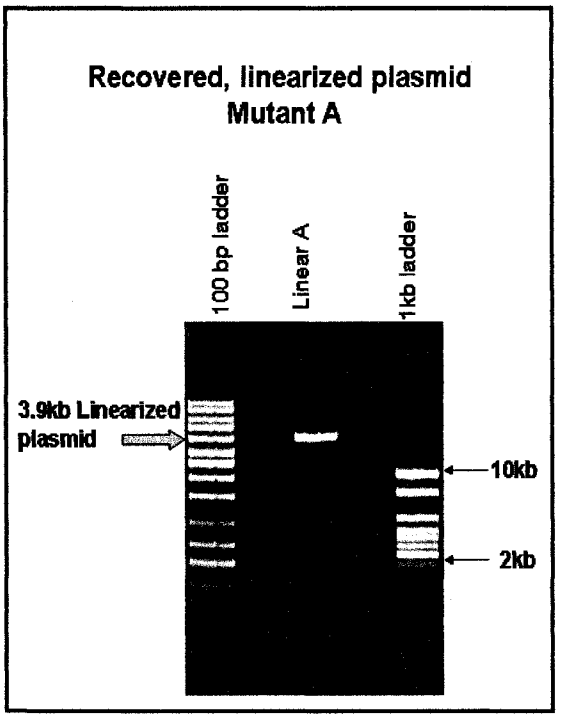


Figure 6. Chromatogram file of sequenced wild type and mutant DNA. Viewed with Bioedit Sequence Alignment Editor. Area highlighted showed the region of the DNA that was mutated.

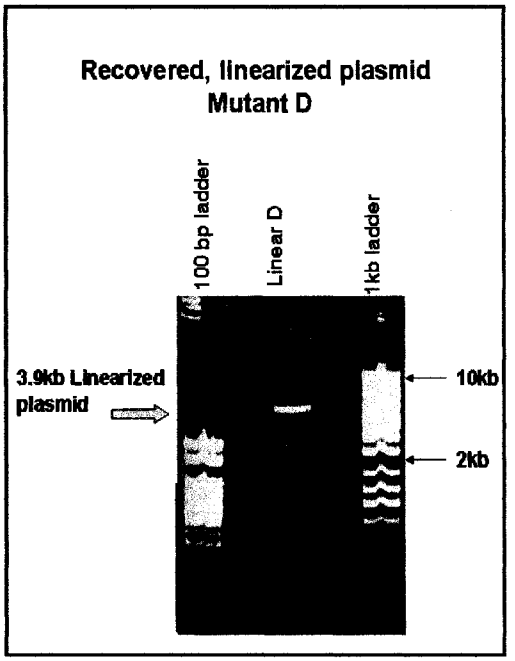
Xho I Digestion of Mutated Plasmid

The mutated DNA was then cut with *Xho1* to linearize it for *in-vitro* transcription. The circular plasmid needed to be linearized so that the RNA polymerase would be able to produce individual transcripts. The plasmids containing the altered 3'UTR were successfully linearized and recovered from solution via precipitation (as mentioned in protocol) (Figure 7)



a.

b.



c.

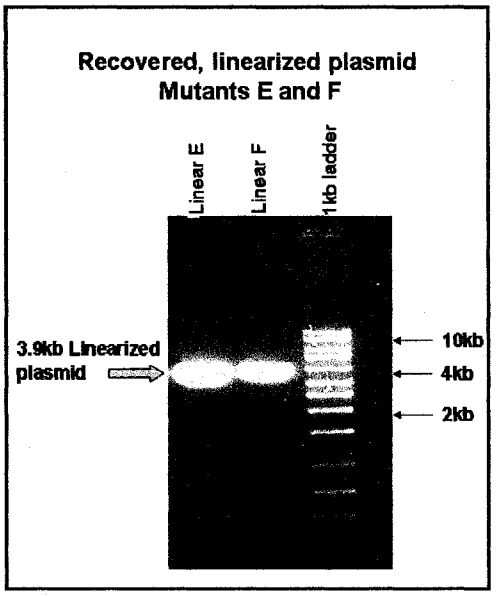
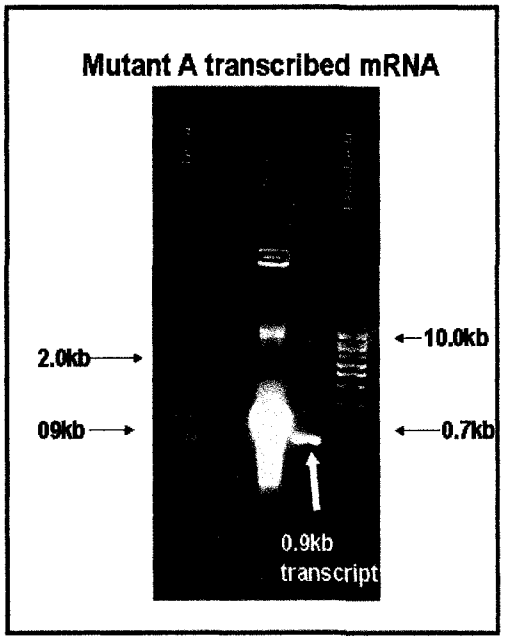


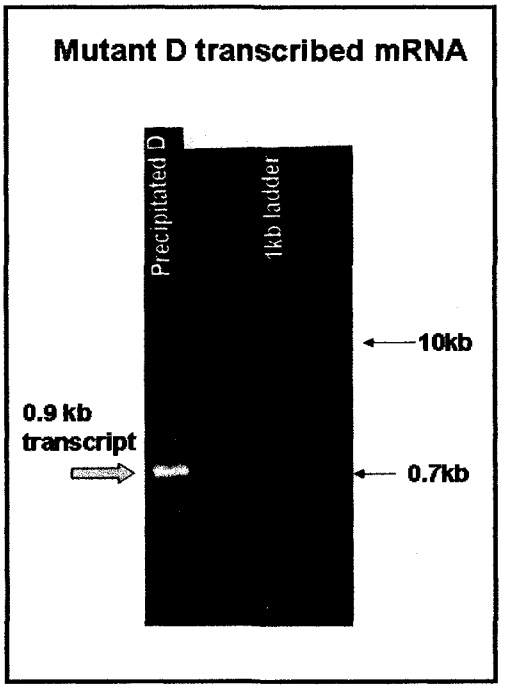
Figure 7. Gel picture of linearized plasmids. Gel Electrophoresis: 1% agarose gel of mutated plasmids linearized with Xho1 digestion. a. linearized mutant A, b. linearized mutant D, c. linearized mutant E and F.

In-vitro Transcription

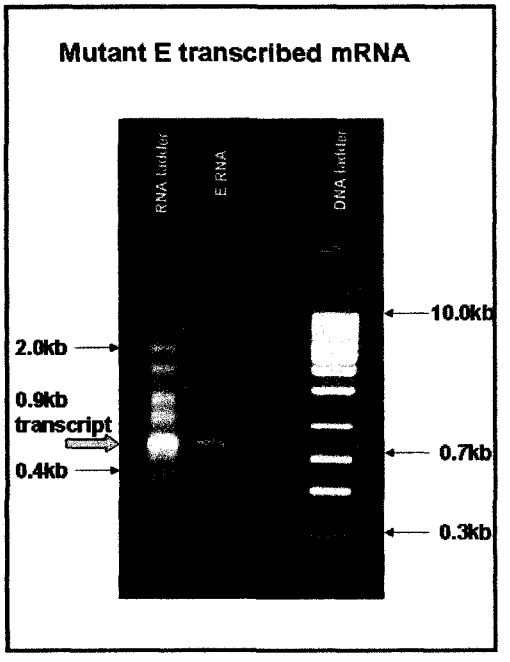
The linearized DNA served as a template for the T3 RNA polymerase to produce mutated transcripts. The transcripts were capped and Digoxygenin labeled. The size of the linearized plasmid was 3.9kb.



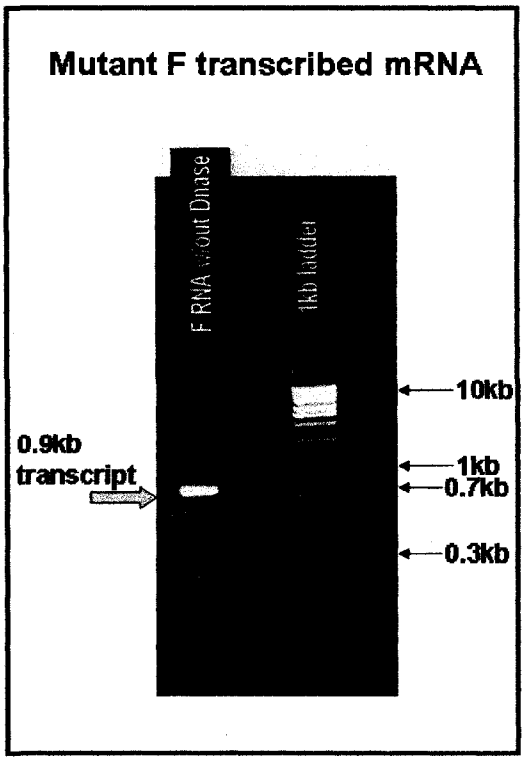
a.


Mutant D transcribed mRNA
Precipitated D
1kb ladder
10kb
0.9 kb
transcript
0.7kb

b.


Mutant E transcribed mRNA
RNA ladder
E RNA
DNA ladder
10.0kb
2.0kb
0.9kb
transcript
0.4kb
0.7kb
0.3kb

c.



d.

Fig 8: Gel picture of *in-vitro* transcription. Gel Electrophoresis: 1.2% agarose gel, 1X TBE, of transcribed mutated RNA transcript. a. Mutant A transcribed mRNA, b. Mutant D transcribed mRNA, c. Mutant E transcribed mRNA and d. Mutant F transcribed mRNA.

Microinjection of Mutated Transcripts in *Helobdella robusta* Embryos

As soon as the last embryo was deposited from the leech, the embryos were harvested and transferred to a gel trough for injections, as mentioned in the protocol. After injections the embryos were incubated in HL saline for 3-4 hours until development of the teloplasm. After 3-4 hours, the white teloplasm was noticeably visible at the polar

regions of the otherwise pink embryo. The embryos were then fixed according to the mentioned protocol.

For visualization of the mutated transcripts, the DIG-labeled mRNA was incubated with anti-Fab fragment and stained with NBT/BCIP reagent. As indicated in Table 2, only a small number of embryos, from a large pool of injected embryos were able to attain the coloration. Staining of the embryos varied from 10-30 minutes and resulted in a dark purple coloration of the injected mRNA. After the embryos were stained, they were cleared with methyl salicylate which reduced the dark purple intensity of the coloration and minimized background staining.

The embryos were then viewed under a Zeiss Axiophot 2 microscope.

Table 2. Statistics for Injected Embryos

Mutation Injected	Number of embryos injected	Number of embryos stained	Number of embryos with a phenotype	Number of embryos wild type	Number of embryos over stained	Number of embryos with faint staining
Mutation A	83	18	11	7	0	0
Mutation D	153	16	9	7	0	0
Mutation E	156	10	1	9	0	0
Mutation F	76	20	18	0	0	2

Wild Type Localization

Figure 9 shows an embryo with the wild type localization pattern. Figure 9A shows the wild type TLM structure and Figures 9B and 9C show the corresponding axial and equatorial views respectively. The axial view shows staining in the center of the embryo and the equatorial view shows discrete localization of the transcripts to the animal and vegetal poles.

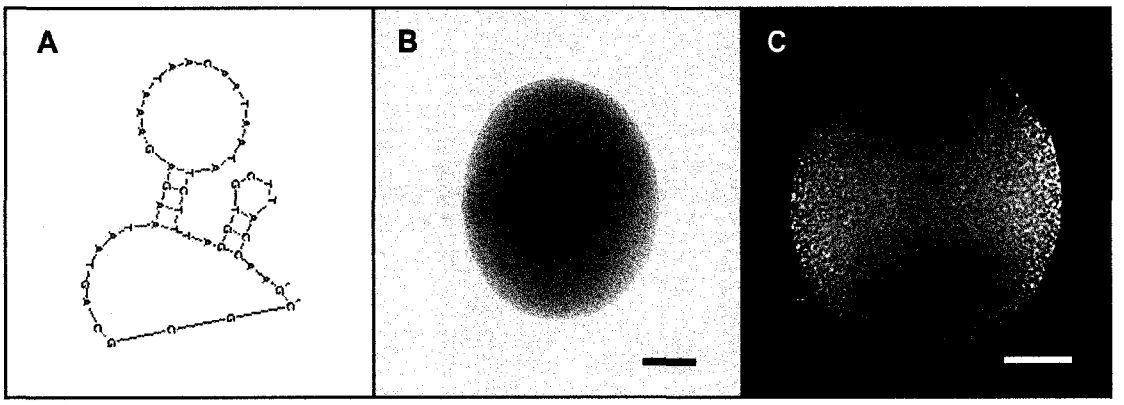


Figure 9. Images of embryos with wild type mRNA localization patterns. A. Secondary structure of TLM with no mutations, B. Axial view of RNA localization pattern, C. Equatorial view of RNA localization pattern.

Mutation A: (conversion of major loop into two smaller loops)

Eighty three embryos were injected with the Mutation A transcripts, out of which only 18 were able to stain (Table 2). Seven out of the 18 embryos showed wild type localization pattern of the RNA. The rest of the 18 embryos showed abnormal localization in the axial views as well as the equatorial views of the embryo (Figure 10).

Although the localization pattern was abnormal, it was not consistent; a mosaic pattern was observed. RNA was localized to various sites in the different embryos. The embryo shown in Figure 10 shows localization of RNA at only the animal pole; the mutation A prevents the transcripts from localizing to the vegetal pole.

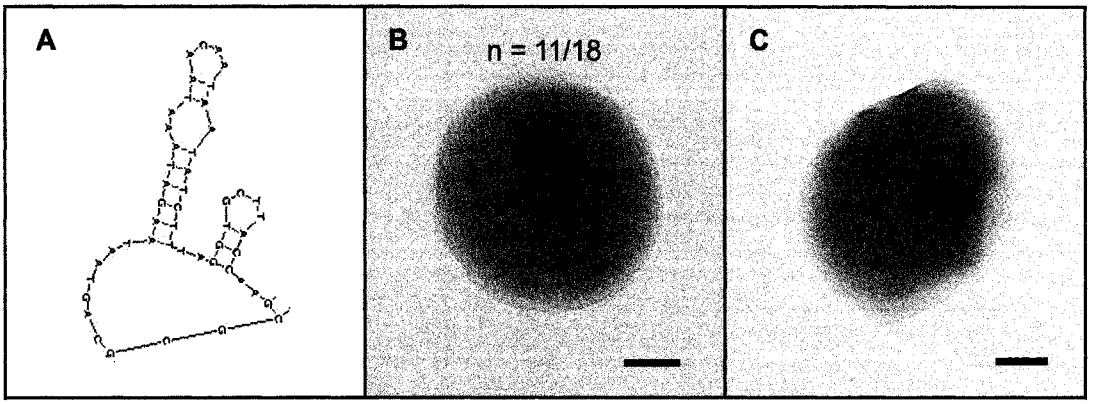


Figure10. Images of embryos with Mutant A transcripts showing an abnormal phenotype. A. Secondary structure of TLM with mutation A, B. Axial view of RNA localization pattern, C. Equatorial view of RNA localization pattern. Images show an abnormal localization pattern in the axial as well as the equatorial view.

Mutation D: (increase in size of the major stem loop)

Out of 153 embryos injected with the Mutant D transcripts, 16 attained staining (Table 2). This was a more subtle alteration in the structure than mutation A. Seven embryos showed wild type localization patterns (polar localization) where as 9 showed abnormal localization of the transcripts. These embryos also displayed an inconsistent, mosaic pattern of localization. Figure 11 shows an image of an embryo with an abnormal

localization pattern. In the axial view (Figure 11 B), three staining areas are observed, some of which is detected in a small area of the teloplasm, instead of a centralized staining, as was observed in the wild type axial view (Figure 9 B). In the equatorial view, staining in the center is observed, which could be the site of injection. Again, there is localization to only the animal pole as was seen in the equatorial image of the Mutant A embryo (Figure 10 C). There is also staining at the side of the embryo which is also abnormal.

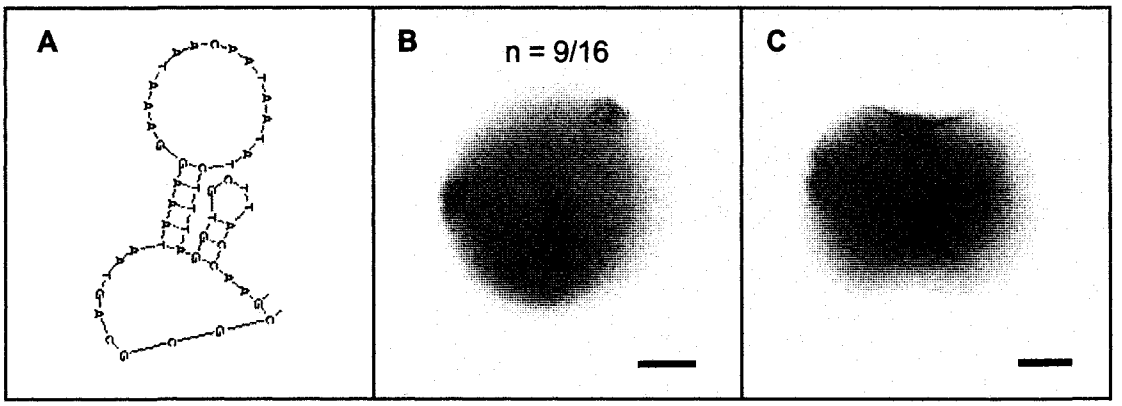


Figure 11. Images of embryos with Mutant D transcripts showing an abnormal phenotype. A. Secondary structure of TLM with mutation D, B. Axial view of RNA localization pattern, C. Equatorial view of RNA localization pattern. Images show an abnormal localization pattern in the axial as well as the equatorial view.

Mutation E: (insertion of a bulge between the major and minor stem loops)

Out of all the alterations made to the TLM structure by the various mutations, Mutation E was the most subtle one. Neither of the stem loop structures were altered; a

small bulge of two nucleotides was inserted between them. Ten out of the 156 embryos that were injected, were able to stain (Table 2). Nine out of the 10 showed normal wild type localization and only one embryo showed an abnormal localization pattern. Figure 12 shows the equatorial and axial views of an embryo displaying the wild type localization pattern. The staining in the center of the embryo in the equatorial view is indicative of the site of injection.

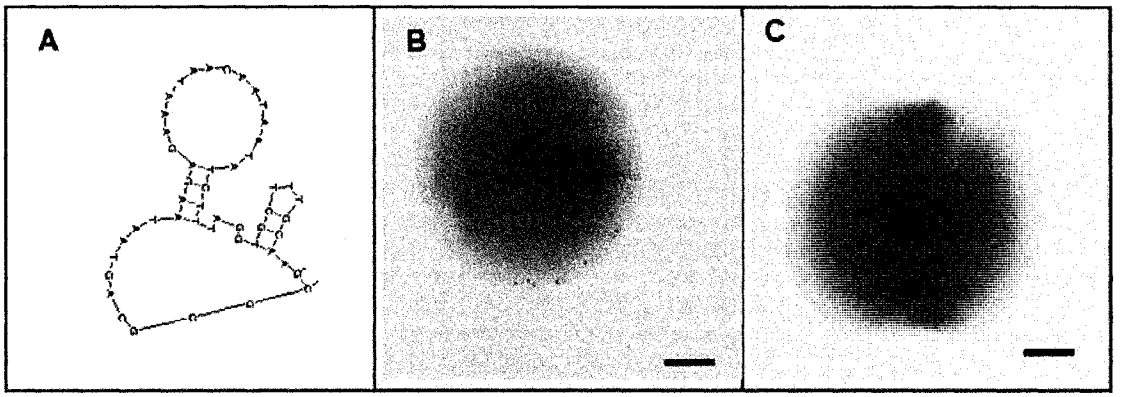


Figure 12. Images of embryos with Mutant E transcripts showing a wild type phenotype. A. Secondary structure of TLM with mutation E, B. Axial view of RNA localization pattern, C. Equatorial view of RNA localization pattern. Images show a normal localization pattern in the axial as well as the equatorial view.

Mutation F: (distortion of the TLM with the exception of the major stem loop structure)

Mutation F showed the highest degree of alteration to the TLM structure relative to the other mutations. Seventy six embryos were injected with the Mutant F mRNA out

of which 20 were able to stain. Out of the 20 embryos that were stained, 18 showed abnormal localization patterns. The other two were also abnormal in their localization, but had very faint staining. Figure 13B shows the axial view of an embryo displaying abnormal localization. Instead of having staining in the center like the wild type axial image (Figure 9B), it has staining around the edges. Figure 13C is not an exact equatorial view; it is slightly rotated to show that only one side of the embryo was stained. The equatorial view (which is not shown) showed no staining. In the slightly rotated view, which is an almost equatorial view, the transcripts are localized outside of the teloplasm of both poles (Figure 13).

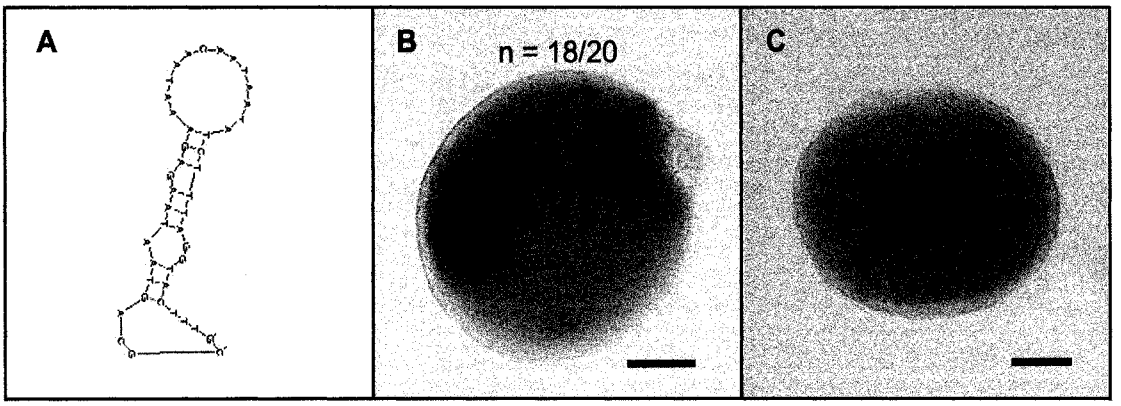


Figure 13. Images of embryos with Mutant F transcripts showing an abnormal phenotype. A. Secondary structure of TLM with Mutation F, B. Axial view of RNA localization pattern, C. Slightly rotated, equatorial view of RNA localization pattern. Images show an abnormal localization pattern.

DISCUSSION

mRNA Localization in the Embryos

In order to distribute cell fate determinants in a developing embryo, several RNAs are localized to specific sites in the one-cell zygote. These maternal mRNAs present in the early zygote may encode developmental proteins, which become restricted only to the cell that received the transcript after mitosis (Etkin and Lipshitz 1999). Transcripts that localize in the early stages of embryonic development are crucial in germ cell specification, migration, and differentiation (Horvay et al. 2006).

In this thesis, the predicted folding of three mRNA transcripts that localize in the one-cell stage of the leech zygote, *Hro-Twist, Le-msx and Lzf2,* was analyzed. All three undergo predetermined localization in the single cell zygote, and each plays a different role in the development of the embryo. It is imperative that they are properly distributed in the one-cell stage so that they can assume their individual specific roles later on in development. Transcripts of *Lzf2* are localized in the embryo along the length of the segmented trunk in both the ectodermal and mesodermal tissues (Savage and Shankland 1996). *Le-msx* is expressed in the dividing precursors of the mesodermal and ectodermal lineages, including the central nervous system and epidermis (Master et al. 1996). Although the specific role of *Hro-Twist* has not been determined, there are implications for a role in mesoderm determination.

Although all of the mentioned transcripts assume different roles in the development of the embryo, they have identical roles in the one-cell stage, which is

localizing to the animal and vegetal poles. In an attempt to find a conserved structural motif, secondary structures of each of the mentioned transcripts were analyzed.

Mfold predictions showed a similar structural motif in *Lzf2, Hro-Twist, and Le-msx*. Here we have analyzed and characterized the motif in *Hro-Twist* mRNA, the TLM.

Recognition of Signals Involved in mRNA Localization

In order for the RNA to be localized in an organized fashion, it must be recognized by the cellular transport machinery, including RNA-binding proteins. These proteins need to find these RNAs out of a large pool of mRNAs and bind to them so that they can guide them to their prescribed destination. There are signals that reside within the untranslated regions of the transcript that these proteins recognize; for example, the pair-rule segmentation genes of *Drosophila melanogaster* localize via signals in their 3'UTRs (Bullock et al. 2003). These signals can be in the form of nucleotide sequences, secondary structures, or a combination of both.

There are several mRNAs that are recognized via recognition of nucleotide sequences present within the transcript. For example, a region containing a CAC repeat has been shown to localize β-actin mRNA (Ross et al. 1997). Another example is the localization of myelin mRNA, which is recognized by a 21-nucleotide region (Ainger et al. 1997). However, recognition elements are not exclusively defined by their nucleotide sequences; secondary structural motifs have also been shown to be involved.

RNA sequences can fold into a secondary structure (Macdonald 1990). These secondary structures contain motifs, many of which have been shown to be involved in

mRNA localization. These motifs consist of stem loop structures that are recognized by RNA-binding proteins, and are usually found in the 3'UTR of localized transcripts. Several studies have shown the role of these motifs in the RNA localization. For instance the secondary structure of *bicoid,* consisting of multiple stem loops is involved in its recognition and transport in the fruit fly ovary and embryo (Snee et al. 2005). Other examples include the GLS, a stem loop structure involved in *gurken* mRNA localization (Van De Bor et al. 2005), and the TLS, a stem loop structure found in the transcripts of *K10* and *Orb.* The TLS is sufficient for proper RNA localization regardless of its sequence or position within the *K10* and *Orb* transcript (Cohen et al. 2005). In addition, Santos et al. 2008, showed that a stem loop structure in the *wingless* mRNA 3'UTR, called the WLE3, was required for the apical localization of the *wingless* transcript. The WLE3 motif is conserved among several other apical localizing transcripts. The c-*myc* and c-*fos* transcripts have also been shown to localize via recognition of an AU rich stem loop structure (Chabanon et al. 2005). c-*myc* and c-*fos* transcripts localize to the perinuclear cytoplasm and associate with the cytoskeleton.

Identifying a single recognition element becomes further complicated when both the nucleotide sequences as well as the secondary structure play a role in the localization of mRNA as in the case of the *Xenopus* oocytes, where a combination of nucleotide sequences and structural motifs are recognized for localizing the mRNA (Bubunenko et al. 2002).

In this study, however, I have specifically looked at the role of a secondary structural motif in *Hro-Twist* mRNA, without interfering with conserved functional localization sequences.

TLM Activity is Affected by Alterations in the Motif

The results indicate different levels of TLM activity caused by different levels of alteration in its structure. For example, mutations which had brought about subtle changes in the TLM (Mutation A and D) showed a lower level of change in TLM activity as opposed to creating a drastic alteration in the motif (Mutation F) which significantly affected the localization of the of the *Hro-Twist* mRNA. Furthermore, the data indicate the functional role of each individual component of the motif in localization of the transcript. For example, mutations A and D had affected only the major stem loop structure causing a reduction in the level of RNA localization. Mutation F had affected the minor stem loop structure, the major stem loop structure as well and the unpaired bulge from where the stem loop structures had emerged and this caused a greater reduction. The role of the individual stem loop structures are further supported by results of mutation E, where the size of the major stem loop structure was not altered and the minor stem loop structure was only altered by one nucleotide. With no significant alterations to the stem loop structures there was no significant aberration seen in the localizing transcripts.

The data also support the role of the overall structure to be of greater significance than the nucleotides with the structure. This is shown in mutation E, where insertion of

the bulge between the two stem loop structures caused a shift in the nucleotides resulting in a minor stem loop structure composed of nucleotides entirely different than the nucleotides in the wild type minor stem loop structure. As long as the structural integrity was maintained, having a different set of nucleotides did not seem to affect localization.

Predicted secondary structures of early leech transcripts were analyzed in order to examine if a common secondary structural motif is used by all of the mRNAs that localize to the teloplasm in the one-celled embryo. Of the structures examined, *Hro-Sna1* and *Hro-Dl* mRNA expression patterns have not been reported but their proteins are detected in the teloplasm before the first cell division (Goldstein et al. 2001). Furthermore, the other mRNAs folded for this study have a maternal component of expression and localize to the teloplasm of uncleaved zygotes (*Hro-Twist,* Soto et al. 1997; and this study; *Lzf2,* Savage and Shankland 1996; *Le-msx,* Master et al. 1996; and *Hro-Nos*, Kang et al. 2002). Based on these conserved expression patterns, it is possible that *Hro-Twist, Lzf2, Le-msx,* and *Hro-Nos* mRNAs use similar mRNA secondary structural motifs to achieve teloplasm localization. Moreover, structural analysis showed that three of the four maternal mRNAs (*Hro-Twist, Lzf2,* and *Le-msx*) share a predicted, secondary structural motif. Perhaps, these mRNAs belong to a class of leech maternal transcripts that use a common mechanism of RNA localization. However, it remains to be determined if the similar motifs (in predicted *Lzf2* and *Le-msx* secondary structures) have a conserved function of mRNA localization in the leech zygote. Finally, it would be interesting to examine which *cis*-acting sequences are involved in the localization of maternal leech transcripts to the teloplasm. In summary, it can be concluded that the

TLM is recognized in the zygote and plays a pivotal role in the localization of the *Hro-Twist* mRNA transcripts to the animal and vegetal teloplasm.

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