San Jose State University
SJSU ScholarWorks

Master's Theses | Master's Theses and Graduate Research

2008

The role of ARE2 cis-acting elements in Hro-twist messenger RNA localization

Jonathan Choi
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Choi, Jonathan, "The role of ARE2 cis-acting elements in Hro-twist messenger RNA localization" (2008). *Master's Theses*. 3508.
DOI: https://doi.org/10.31979/etd.j9uh-b8zq
https://scholarworks.sjsu.edu/etd_theses/3508

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

THE ROLE OF *ARE2 CIS-ACTING* ELEMENTS IN *HRO-TWIST* MESSENGER RNA LOCALIZATION

A Thesis
Presented to
The Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Jonathan Choi
May 2008

UMI Number: 1458162

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 1458162

Copyright 2008 by ProQuest LLC.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 E. Eisenhower Parkway
PO Box 1346
Ann Arbor, MI 48106-1346

© 2008

Jonathan Choi

ALL RIGHTS RESERVED

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. Julio G. Soto

Dr. Robert Fowler

Dr. Brandon White

APPROVED FOR THE UNIVERSITY

Thea I. Williamson 04/30/08

ABSTRACT

THE ROLE OF *ARE2 CIS-ACTING* ELEMENTS IN *HRO-TWIST*

MESSENGER RNA LOCALIZATION

by Jonathan Choi

Twist is an evolutionarily conserved protein hypothesized to have a role in mesoderm formation, which is especially important for embryonic development. *Helobdella robusta* embryos express *Hro-twist* mRNA exclusively at the animal and vegetal teloplasm poles. It is critical that *Hro-twist* mRNA localization be specifically regulated for proper *Helobdella robusta* development. RNA localization is a post-transcriptionally regulated process of determining spatial protein expression, utilizing *cis-acting* elements within mRNA transcripts. To characterize the localization mechanism, sequence elements within the 3′ untranslated region (UTR) of *Hro-twist* mRNA were identified and *AU-rich elements* (*ARE2*) were assessed of their role in mRNA localization. Point mutations of the *ARE2* sites were introduced via site-directed mutagenesis, and mRNA transcripts microinjected into *Helobdella robusta* embryos. Mutation of the sites resulted in abnormalities of localization to the animal and vegetal poles, suggesting a novel role of *ARE2* elements in *Hro-twist* localization.

Acknowledgments

I would like to thank everyone who played a role in supporting me during my research and education, making this thesis possible. First, I would like to thank my mom and dad for their direct support over the course of these past years. I would also like to thank the entire Soto lab for making my time in lab enjoyable and entertaining. With such a great group of people around me, it is easy to have fun while doing the long lab procedures, and to make lab a second home. I thank the numerous individuals who went leech collecting with me, who had to wake up early and endure the tough conditions. I would like to thank Branden Fung for his extended involvement in troubleshooting procedures, leech collecting so frequently that I can no longer count, long nights of embryo injections, and help with my thesis. I want to thank my committee members, Dr. Brandon White and Dr. Robert Fowler, for their input in my project, reviewing my thesis, and their interest in my work. Finally, all of this would not be possible if not for my mentor and friend, Dr. Julio Soto. I want to thank him for the opportunity to work on this research project, for his personal help during procedures, and for all the guidance and direction involved in not only the project, but in my growth as a scientist.

Table of Contents

List of Figures viii

List of Tables ix

1. Introduction 1

2. Literature Review 6

    2.1. Post-transcriptional Regulation: mRNA Stability 7

    2.2. Post-transcriptional Regulation: mRNA Localization 8

    2.3. Mechanisms of RNA Localization 12

    2.4. *Helobdella robusta* Leeches 16

    2.5. Developmental Functions of the Twist Protein 18

    2.6. Conservation of the Twist Gene 21

    2.7. Twist Secondary Structure 23

3. Materials and Methods 27

    3.1. Bioinformatics 27

    3.2. Mutagenic Primer Design 28

    3.3. Site-Directed PCR Mutagenesis 32

    3.4. *Dpn* I Digestion 33

    3.5. Transformation of XL-10 Gold *E. coli* Cells 34

    3.6. DNA Isolation and Sequencing 35

3.7. Template Linearization with *Xho*1 36

3.8. *In-vitro* Transcription of DIG-labeled mRNA 37

3.9. *Helobdella robusta* Zygotes 39

3.10. Microinjection of mRNA Transcripts 40

3.11. Staining and Visualization of Injected Embryos 42

4. Results 44

4.1. Identification of Localization Elements 44

4.2. *ARE2* Elements on the mRNA Secondary Structure 44

4.3. Site-Directed Mutagenesis of *ARE2* Sites 47

4.4. *Xho*1 Digestion and *In-vitro* mRNA Transcription 50

4.5. Injection of *Helobdella robusta* Embryos 54

4.6. Staining and Visualization of Injected Embryos 56

5. Discussion 61

5.1. mRNA Movement along the Microtubule Cytoskeleton 61

5.2. *Cis-acting* Elements in the 3'UTR of mRNAs 62

5.3. Mutations of *Hro-twist ARE2* Elements 64

5.4. Injections of Mutated *Hro-twist* Reveal Functional Role of *ARE2* . . . 66

5.5. Future Studies Pertaining to *Hro-twist* Localization 69

References 71

List of Figures

Figure 1. Diagram of *Hro-twist* transcript secondary structure 46

Figure 2. 1% agarose gel of *ARE2-site1mut* and *ARE2-site6mut* miniprep plasmid DNA for sequencing reaction 48

Figure 3. 1% agarose gel of *ARE2-1-10mut* miniprep plasmid DNA for sequencing reaction 48

Figure 4. Chromatogram file of sequenced *M13F* positive control DNA and *ARE2-site6mut* DNA plasmid 49

Figure 5. Diagram of Sequentially Mutated ARE2 sites 50

Figure 6. 1% agarose gel of *M13F* plasmid, linearized with *Xho*1 51

Figure 7. 1% agarose gel of plasmids w/ sequential mutations, linearized with *Xho*1 51

Figure 8. 1% agarose gel of clean-up/recovered mutants for transcription 52

Figure 9. 1.2% agarose gel of *ARE2-sites9-10mut* mRNA transcript 53

Figure 10. 1.2% agarose gel of control *M13F* mRNA transcript 53

Figure 11. 1.2% agarose gel of mRNA with single and multi-site *ARE2* mutations 54

Figure 12. Equatorial views of embryos injected with exogenous wildtype *M13F* and *ARE2-sites1,2,6-10mut* 57

Figure 13. Axial views of embryos injected with wildtype and mutant 3'UTR *Hro-twist* 58

Figure 14. Axial views of embryos injected with mutant 3'UTR *Hro-twist* 59

List of Tables

Table 1. List of mutagenic primers used for site-directed mutagenesis 31

Table 2. Survival of injected *Helobdella robusta* embryos after microinjection and removal of vitelline membrane 55

Table 3. Percentage of embryos that have altered *Hro-twist* localization when ARE2 sites 1, 2, and 6 through 10 are mutated 60

1. Introduction

The mechanisms and regulation of basic cellular processes are critical in the development of an organism, especially during early embryonic development. The germ layers of the resulting endoderm, ectoderm, and mesoderm eventually give rise to adult tissues and organs, and therefore, cell fate determination must be appropriately determined, beginning in the initial zygotic cell. The specification of future cellular roles is apportioned by the expression of developmental proteins. One form of protein regulation is accomplished through a method of post-transcriptional regulation known as RNA localization (Jansen, 2001).

In *Helobdella robusta*, part of the Glossiphoniidae leech family, embryos divide bilaterally by spiral cleavages, resulting in two asymmetric daughter cells (Weisblat, 2007). These cells further undergo a series of unequal and asynchronous cleavages to produce individual cells with recognizable lineages, regulated by cell type-specific mechanisms (Bissen, 1997). Eventually, the mesoderm gives rise to 32 segmented regions of the adult *Helobdella robusta* body, and a prostomium head (Gleizer and Stent, 1993). It is evident that developmental proteins must be expressed and distributed appropriately in the one-cell stage embryo for later developmental stages in the adult organism.

Because proteins are translated from messenger RNA, cells utilize post-transcriptional regulation of mRNA as a means of protein control. RNA localization is a method of transporting mRNA transcripts to specific locations within a cell for spatial expression of proteins (Jansen, 2001; Kloc et al., 2002). mRNA destined to be localized are transported along cytoskeletal elements, microtubuline and beta-actin, to different areas of a cell (Olynikov and Singer, 1998). Localized mRNA can serve several functions, such as morphegen gradient formation, asymmetric distribution of cell fates, assembly of protein complexes, and long-distance transport of specific mRNAs to coordinate developmental processes between cells (Du et al., 2007). Observed to occur in a multitude of organisms ranging from yeasts, various invertebrates, as well as in vertebrate species such as *Xenopu laevis* and *Drosophia melanogaster*, mRNA localization is found in both embryonic and adult cells (Mohr and Richter, 2001; Bettley et al., 2002). The range of cells displaying mRNA localization includes adult neural cells, such as the axonal localization of *kor* mRNA in mouse dorsal root ganglia (Bi et al., 2006) and *GFAP* mRNA in the oligodentrocytes and astrocytes of rats (Wang et al., 2007), as well as the directional localization of *Ash1* mRNA to budding sites of *Saccharomyces cerevisiae* (Müller et al., 2007; Schmid et al., 2006).

Especially important in developing oocytes, *Drosophila* maternal mRNA transcripts for bicoid (*bcd*), nanos (*nos*), oskar (*osk*) and gurken (*grk*) are found to localize post-transcriptionally (Johnstone and Lasko, 2001). Oskar accumulation is crucial for posterior patterning of the *Drosophila* embryo (Snee et al., 2007). *Bcd* mRNA accumulates to the apical ends of embryos, and *fs(1)K10* and *orb* mRNA localize to the anterior of the oocyte (Snee et al.,2005; Castagnetti and Aphrussi, 2003). In *Xenopus* embryo development, the mRNA *fatvg* is shown to be required for cortical rotation and germ cell formation, where depletion of *fatvg* mRNA actually results in embryos that lack primordial germ cells (Chan et al., 2007). Other mRNAs found to be preferentially localized in developing embryos encode developmental proteins that include, but are not limited to, BicaudalD (*BicD*), Egalitarian (*Egl*), hairy (*h*), and fushi tarazau (*ftz*) (Bullock et al.,2003; Huynh and St Johnston, 2000; Navarro et al., 2004; Mach and Lehmann, 1997). *Krüppel* mRNA, localized to the center of the embryo, is required for the formation of the thoracic segment of the *Drosophila* (Shi et al., 2007). More recently, mRNA for Orthidenticle-1 (*NV-otd1*) has been discovered in wasps (*Nasonia vitripennis*) to be localized to both oocyte poles, resulting in bipolar protein gradients, with the anterior gradient regulating expression of the zygotic head and thoracic gap genes of the localizing mRNAs *bicoid, giant* (*gt*), and

hunchback (*hb*) (Brent et al., 2007). mRNA localization is widespread across all species, and necessary for embryonic development.

Helobdella robusta zygotes exhibt localization of many specific mRNAs. *Nanos* (*Hro-nos*) is concentrated at the animal and vegetal teloplasm of the leech embryo, and *Hro-nos* expressing cells lead to mesoderm segmentation (Kang et al., 2002). *Hro-nos* knockdown leech zygotes actually develop abnormalities of cleavage, abnormal germinal bands, and eventually early embryonic death (Agee et al., 2006). Segmentation is also closely tied with *even-skipped* (*Hro-eve*) and *Hairy and Enhancer of split* mRNA (*Hro-hes*), which peaks during primary blast cell formation (Song et al., 2002; Song et al., 2004). *Le-msx* mRNA is distributed in the cortex of the oocytes and asymmetrically localizes to the poles prior to cleavage (Master et al., 1996). Localization of *dorsal (Hro-dl)* and *snail (Hro-sna)* mRNA appears as segmentally iterated stripes, and may play a role in the diversification of cell types within the *Helobdella robusta* body (Goldstein et al., 2001).

Of particular interest is *Hro-twist* mRNA, which is a homolog of *twist* mRNA found in invertebrates such as the fruitfly, jellyfish, nematode, and lancelet, and vertabrates such as mouse, chicken, frog, and human (Castannon and Baylies, 2002). Twist has a well characterized role in various species in

mesoderm formation, dorso-ventral polarity, and the establishment of dorso-ventral pattern determination (Chen and Behringer, 1995; Thisse, 1987; Sandmann et al., 2007). Twist also plays a role in muscle determination of mesodermal cells to somatic, visceral, pharyngeal muscles, and the dorsal vessel (Sokol and Amros, 2005). *Hro-twist* has been shown to be a maternal mRNA present in *Helobdella robusta* zygotes (Soto et al, 1997), and is shown to localize to the vegetal and animal pole, around the perinuclear region (Soto, 1994).

The purpose of this investigation was to determine possible signal elements within the mRNA that would be involved in the target of *Hro-twist* for the specific mRNA localization to the poles of the *Helobdella robusta* embryo. Specifically, the *ARE2* (*AU-rich element*) sites were determined as a possible *cis-acting* element involved in the process, ascertained by point mutations of the sequence sites and microinjection into stage-one embryos. Staining of the exogenous mRNA revealed significant affects on the localization pattern in the cell.

2. Literature Review

The regulation of protein expression is fundamental in cellular function and development. Proteins are required for all aspects of cellular and molecular biology, from basic structural components such as microfilaments to kinases that phosphorylate other proteins. Involved in activation of complex pathways, they can participate in simple binding interactions with other protein subunits. Proteins serve dual roles, as effectors of other components, as well as functioning as the recipient of particular actions. With such a myriad of roles and behaviors of proteins, it is therefore critical that cells have the capacity to precisely regulate protein expression. Cells use defined mechanisms to control the quantity of proteins, the temporal expression, as well as the specific location in an organized and calculated manner. To complicate this further, the cell must be able to individually regulate hundreds of proteins at once, in a well orchestrated manner that allows for the proper overall function and success of the cell. The inability to control this complex regulation can lead to deleterious effects, ranging from isolated cellular abnormalities to apoptosis.

The central dogma of biology dictates the basic outline of protein expression. All cells have the DNA code that can allow for the formation of any protein. With the two primary steps of transcription and translation, there is the

potential for regulation before, during, and after each individual step to control the downstream expression of proteins. Although cells do regulate proteins after expression, it is not the sole mechanism available, since the cells can regulate the DNA and mRNA as well. It is therefore advantageous for the cell to have options in how it regulates protein expression, such as through mRNA stability and localization.

2.1. Post-transcriptional Regulation: mRNA Stability

Post-transcriptional regulation involves manipulation of the messenger RNA, rather than the protein. One area of post-transcriptional regulation involves the stability of the mRNA. Stability of mRNA prevents degradation, which would otherwise destroy the template necessary for translation. Various mRNA transcripts undergo stability regulation, such as the *utrophin* transcripts in skeletal muscle cells (Gramolini et al., 2001) and *oskar* transcripts in *Drosophila* (Snee et al., 2004). Full length *utrophin* mRNA transcripts are shown to be highly stable, and associate with cytoskeletal-bound polysomes and actin filaments. Although the coding region of the transcript defines the sequence that is translated to the utrophin protein, the 3′ UTR has a distinct role in regulating the stability of the entire mRNA. Deletions of stability elements within the transcript

result in the destabilization of the *utrophin* mRNA, increasing degradation, which ultimately leads to the decline of the utrophin protein.

The Tet-Off system has been used to explore the role of the 3'UTR in mRNA stability in chicken embryos, which combines the tetracycline-dependent inducible system with ovo electroporation to moniter mRNA stability in the chick neural tube (Hilgers et al., 2005). The 3' untranslated region of different mRNAs were shown to have varied results in stability. The 3'UTR of *Lunatic Fringe* mRNA strongly destabilizes the transcript, while the 3'UTR of *Fgf8* resulted in the opposite effect, resulting in the increase of stability. From these collective experiments, it is shown that mRNA stability occurs in different species of organisms. Also, more profoundly, there are regulatory components that are incorporated into the mRNA itself, in the untranslated region, that affect stability (Hilgers et al., 2005). This provides a role for the 5' and 3' UTR, since it is already known that they are not incorporated into the protein.

2.2. Post-transcriptional Regulation: mRNA Localization

mRNA localization, also known as trafficking, has been demonstrated in different types of cells from a number of organisms (Bashirullah et al., 1998; Kloc, 2002; Bi et al., 2006; Wang et al., 2007). While mRNA stability allows the

transcript to be stable and long lasting, allowing time for translation, localization takes it a step further, where the transcript is physically transported to a new area for translation. The transcript can be specifically targeted for transportation, physically shuttled to different areas of the cell, where it will be translated into protein. During the process, the mRNA must remain in a state where translation does not occur, until it has reached its final destination. Steady advancements have been made in *Drosophila melanogaster* oocytes and *Xenopus laevis* embryos indicating the localization does in fact occur (Jansen et al., 2005).

Since the discovery of localization in *Drosophila* and *Xenopus* oocytes a decade and a half ago, there have been 100 identified transcripts that display the characteristic of localization (Lipshitz et al., 2000). These transcripts are found in numerous cell types, including oocytes, neurons, glia, fibroblasts, and epithelial cells, spanning organisms from unicellular fungi, to plants and animals. Localization in all of these cells serves several biological functions. First, mRNA localization allows for the cell to direct a high level of production of the encoded region at the specific site of localization. This is strongly shown in the *bicoid* transcript found to be localized to the anterior of the *Drosophila* embryo (Macdonald et al., 1997). High levels of bicoid homeodomain protein at the anterior end is found to be necessary for cells to commit to anterior development.

Another well characterized demonstration of targeted protein production occurs in the budding yeast *Saccharomyces cerevisiae*, where *Ash1* mRNA is localized to the bud and daughter cell upon mitosis, and encodes a repressor that acts to prevent the daughter cell from undergoing mating-type switching(Takizawa, 1997).

Secondly, mRNA localization prevents the production of proteins in an area of the cell that would result in deleterious effects in that region of the cell. *Drosophila's oskar* and *nanos* transcripts are normally localized to the posterior portion of the oocyte and early embryo, where they encode for their respective proteins that dedicate that site to posterior cell fates (Wilhelm, 2005). Mislocalization of these transcripts to the anterior end of the oocyte and embryo results in anterior cells transforming to posterior cells (Wilhelm, 2005). This results in severe developmental abnormalities that do not allow *Drosophila* to continue to develop on to maturity. A similar effect is noted in yeast, with the misexpression of *Ash1* protein in the mother cell resulting in the inappropriate mating-type switching repression (Lipshitz et al., 2000).

There are many other benefits of possessing the ability to localize mRNA. It requires less energy to regulate mRNA because the transcript is smaller than the mass of the amino acids that would make up the fully encoded protein.

Rather than localizing a bulky protein molecule, the lighter mRNA can more easily be shuttled around the cell via microtubules and actin filaments (Jansen, 2001). Also, it is more efficient to localize mRNA because one single transcript provides the template for the translation of numerous proteins. Rather than transporting few massive protein molecules, the cell can therefore localize a greater quantity of smaller mRNAs.

The ability for a cell to localize mRNA allows for the mass accumulation of specific mRNA, and ultimately the congregation of specific proteins in one region of the cell. This allows that region to obtain a specialized function, or cell fate, such as observed by *nanos* protein in the posterior development of the *Drosophila* embryo (Johnstone and Lasko, 2001). The ability to control protein expression also allows for the smooth transition of dense to sparse protein, such as the protein gradient observed in *bicoid* expression in *Drosophila* (Wilhelm, 2005). It is important that a cell has the means to regulate protein expression in specific regions. This allows the cell to control differential development of components within the cell.

2.3. Mechanisms of RNA Localization

There are many individual steps involved in the transport of mRNA along microtubules and micro filaments. First, the specific RNA is targeted and selected from the pool of the many transcripts residing in the nucleus. Once it is identified and isolated, RNA-binding proteins and motor proteins bind to the mRNA and forms a ribonucleoprotein complex (Cote et al., 1999; Deshler et al., 1997). This complex is then anchored to the cytoskeletal track for transport. Transport occurs through interactions with motor proteins along microfilaments and microtubules to specific areas of the cell (Lipshitz et al., 2000). Once arriving to the destination, microfilments anchor the mRNA to subcellular compartments, and translation can proceed (Jansen, 2001).

Before mRNA can be localized, it must first be chosen to undergo the entire process. The nucleus contains numerous RNA that has been transcribed from the DNA. The vast majority of these RNA will not undergo localization. Those RNA will be exported to the cytoplasm where they will be translated to proteins by ribosomes. The non-localized proteins would exist as free floating proteins in the cytoplasm, or destined for export out of the cell for extracellular roles. Proteins that are non-localized must be regulated by post-translational methods, as opposed to post-transcriptionally, as outlined earlier. However,

among the multitude of RNA in the nucleus, RNA that will be localized must be identified and sorted out among the rest of the transcripts (Chartrand et al., 1999; Bi et al., 2006; Jambhekar and DeRisi, 2007).

In the study of *Drosophila*, *Xenopus*, yeast, and mammalian cell culture, localized mRNA contain specific sequences within the transcript that are responsible of its identification (Thio et al., 2000; Snee et al., 2004; Ashwini and DeRisi, 2007). Sequences that reside in the mRNA that play a role in RNA localization are termed *cis-acting* elements. The *K10* gene of *Drosophila* contains potential *cis-acting* elements within the mRNA. It was found that there was a 44 nucleotide RNA sequence element that was required and sufficient for the transport of the *K10* transcript into the anterior region of the cell (Cohen et al., 2005). The specific sequence, similar to sequences in other localizing mRNAs, is universally known as the *TLS*, or *Transport and Localization Sequence* (Serano and Cohen, 1995). The *TLS* in this instance mediates the apical localization of *K10* mRNA ectopically expressed in somatic follicle cells (Karlin-McGinness et al., 1996). The *TLS* has a proposed role in the ability to recruit dynein-containing motor complexes to the RNA (Horne-Badovinac, 2008). It has also been found that the *TLS* activity is not influenced by its location within the mRNA transcript. As long as it is present, the mRNA can be targeted for localization. The *TLS*

structure is made up of a loop and stem. The size of the loop and the length of the stem appear to have an effect on localization, as changing its parameters will adversely diminish localization behavior (Cohen at al., 2005). A vast majority of localized mRNA transcripts, including *Drosophila Orb,* contain the *TLS* signal, which suggests its role in this process (Lantz and Schedl, 1994). However, there are also numerous *Drosophila* genes that do not contain the *TLS* sequence. There is still much research that has to be done in this area, but it appears that all localized mRNA transcripts contain *cis-acting* elements, besides the TLS (Lipshitz et al., 2001). It is the *cis-acting* elements that uniquely identify mRNA so that they can be specifically targeted for different destinations in the cell.

After the mRNA transcript is identified for localization, RNA binding proteins recognize the *cis-acting* elements, forming RNA complexes that will export it out of the nucleus and into the cytoplasm. Ribonucleoproteins bind to the specific sequences of the transcript, usually within the 3'UTR, forming a nuclear core complex assembly (Mohr and Richter, 2001). Proteins that bind to *cis-acting* elements are termed *trans-acting* factors. *Trans-acting* factors combine with mRNA to organize them into large granules or particles, suggesting the existence of a macromolecular transport complex. Research on the *bicoid* mRNA in *Drosophila* has revealed proteins involved in the formation of a *bicoid* localizing

complex (Mohr 2001). It has been shown that at least five proteins play a distinct role in *bicoid* localization: *Exuperantia* (*Exu*), *Exuperantia*-like phenotype (*Ex1*), *Staufen* (*Stau*), *Swallow* (*Swa*), and *Homeless* (Mohr and Richter, 2001). Mutations within any of these proteins resulted in failed localization. When *Exu* mutations are exhibited, the apical localization of bicoid mRNA in nurse cells is lost. Although it is initially transported in the oocyte, it is not sequestered to the anterior pole of the oocyte. Instead, the *bicoid* mRNA is homogeneously distributed within the egg. *Stau* is a trans-acting factor that plays a role in another localized mRNA, *oskar*. *Stau*'s role in the posterior localization of *oskar* further emphasizes the prevailing effect of trans-acting factors in recognizing *cis-acting* elements (Snee et al., 2007). *Swa* has an additional characteristic that is important in the machinery of localization—*Swa* contains a motif that resembles an RNA recognition motif (RRM), further characterizing these trans-acting factors exclusively as RNA binding proteins (Mohr and Richter, 2001; Johnstone and Lasko, 2001).

Not only is localization observed in multicellular organisms, single cell budding yeasts display this process. *Saccharomyces cerevisiae* expresses at least five *SHE* genes (*SHE1-5*). The SHE proteins sort *ASH1* mRNA containing granules to the prospective daughter cells (Chartrand, 1999). Mutations of any of

the five *SHE* genes eliminates *ASH1* mRNA transport to the bud tip. Two of the *SHE* genes encode for proteins that are associated with cytoskeletal proteins. *SHE5* encodes a protein involved in regulating the actin cytoskeleton, and *SHE1* encodes for a unique type of myosin. These two proteins suggest that they may perform as motor proteins that are involved in the active transport of the mRNA along the cytoskeletal tracks of the cell. Once the nuclear core assembly complex is formed and exported into the cytoplasm, they combine with motor proteins, and bind to the cytoskeleton for transport to the final destination of the transcript (Horne-Badovinac and Bilder, 2008). Once the mRNA reaches its destination, it is anchored via microfilaments to the subcellular surface of the cell. Without this anchorage, the mRNA will simply diffuse back into the cytoplasm, negating the localization effect (Jansen et al., 2005).

2.4. *Helobdella robusta* Leeches

Helobdella robusta leeches are invertebrates from the phylum Annelida and subclass Hirudinea. The genus *Helobdella* is part of the Glossiphoniidae family. Leech embryos divide bilaterally and undergo few cell divisions before gastrulation, compared with other organisms of radial symmetry. The adult leech contains 32 segments and a prostomium, or head region (Fernández, 1980,

Gleizer and Stent, 1993; Irvine and Martingdale, 1996;). Glossiphoniidae leeches carry their embryos exteriorly under their dorsoventrally flattened bodies, making it easy to extract embryos for experimental procedures. Upon fertilization, the eggs stay at metaphase in meiosis I until they are moved to the cocoon under the mother (Fernández, 1995). This is useful for researchers because the entire batch of eggs will exit metaphase at the same time, allowing for a large experimental sample that can be averaged and compared with one another. Also, *Helobdella robusta* leech embryos are relatively large in size, allowing for ease of handling, and manipulations via injections.

Numerous proteins are involved in the development of the leech embryo. One such protein is the *Twist* protein, which is shown to isolate to the animal and vegetal pole of Stage 1 embryos (Soto, 1997). *Twist* mRNA undergoes RNA localization, just as the previously described *bicoid, nanos,* and *oskar* transcripts. *Twist* is a zygotically transcribed factor that is required for normal mesoderm formation (Castanon et al., 2001). The mesoderm forms during gastrulation, the layer that lies between the endoderm and the ectoderm. It is also the layer that gives rise to bones, muscles, the circulatory system, connective tissues of the gut and integuments, the gastrointenstinal tract, the reproductive system, and the urinary system (Kang, 2002).

2.5 Developmental Functions of the Twist Protein

The twist gene plays a critical role in the development of the mesoderm of various organisms, specifically in specification and differentiation of cell fates. Twist was first identified in the fly *Drosophila,* and therefore, the majority of research has been done with the model *Drosophila.* Twist has been determined to be a zygotic gene that is necessary for the establishment of dorso-ventral pattern determination (Thisse et al.,1997). Expression of the Twist protein is already present at egg deposition, and is expressed along with ten other genes, collectively know as the "dorsal group", all of which are required for the definition of dorso-ventral polarity (Simpson, 1983; Goldstein, 2001). Twist has been shown to have a synergistic relationship with the maternally-acting gene Dorsal, also part of the "dorsal group" of genes (Shirokawa and Courey, 1997). Genes having this sort of relationship are highly indicative of having related functions, which further suggest that *Drosophila* twist has a role in the establishment of a dorso-ventral pattern in the early embryo. Additionally, among the shared involvement of polarity determination of dorsal group genes, the Twist and Snail genes have further roles for subsequent determination of mesodermal patterns (Ip et al., 1992). *Drosophila* embryos containing mutants of either of these two genes result in morphological abnormalities. The impairment

of development occurs at the onset of gastrulation by the failure of the ventral furrow to form normally. This results in only partial dorsalization, and eventually, failure of full normal mesoderm development downstream (Nüsslein-Volhard, 1984).

In *Drosophila*, twist is initially expressed in the ventral-most cells, located at the bastula stage. At this early stage, Twist is found to be a requirement for the activation of various mesoderm regulators, such as *heartless* (*htl*), a FGF receptor that is necessary for mesodermal migration (Beiman, 1996; Gisselbrecht, 1996; Shishido, 1997), and also *Dmef2*, used in muscle tissue determination (Lilly et al., 1995; Bour et al., 1995; Taylor et al., 1995). Twist is also required to activate Snail, a gene that has a critical role for proper gastrulation (Leptin, 1991; Goldstein, 2001) and maintenance of Twist expression itself (Ip et al., 1992). Gastrulation occurs when cells that express Twist invaginate along the ventral surface of the embryo. The cells then divide two times and expand dorsally, resulting in a monolayer that has close contact with the ectoderm (Leptin and Gunewald, 1990).

Upon completion of the *Drosophila* gastrulation stage, Twist expression begins to modulating into alternating stripes of how and high abundancy levels in each segment of the mesoderm. During this modulation, the mesoderm

divides for a third time, which causes some of the cells to come loose of the ectoderm, and migrate further internally (Bate, 1993). The difference in levels of Twist expression has a direct role in the determinant of mesoderm differentiation. Among the stripes, mesodermal domains that contain high levels of Twist lead to the blockage of formation of tissues such as the visceral mesoderm, and instead lead to the formation of ectopic body muscles (Sokol and Ambros, 2005). On the contrary, domains with low Twist levels result in the inhibition of somatic myogenesis, but allows for the development of other tissue types (Baylies and Bate, 1996). Additionally, when Twist is expressed ectopically in the ectoderm, it represses the epidermal and nervous system differentiation, and instead promotes body muscle formation (Castanon et al., 2002). Therefore, besides initial mesoderm development, Twist has an eventual function in the onset of muscle formation.

For muscle formation to occur, cells that express high levels of Twist are chosen as "muscle progenitor" cells. These cells divide asymmetrically to give rise to two differing sister cells, each with diminished levels of Twist. The sister cells are known as "founder myoblasts", and both express a unique combination of transcription factors, including Kruppel (Kr), Even-skipped (Eve), and Slouch (S59) (Shi et al., 2007). The information contained within these transcription

factors result in different identities of embryonic muscle, influencing the cells' position, size, epidermis attachment site, and innervation patterns (Castanon, 2002). Finally, the founder cells fuse with neighboring myoblast to form the final pattern of the muscle. Twist is required for the specification of these founder cells, especially seen in interactions with Eve (Halfon et al., 2000; Song, 2002) and S59 (Cox and Baylies, 2001).

2.6. Conservation of the Twist Gene

The twist gene is found across species, and shown to be conserved evolutionarily from simple organisms to *Homo sapiens* (Wang, 1997). In addition to the previously mentioned dorso-ventral formation functions, additional roles have been identified in different organisms. In *Gallus gallus*, or chick, expression of CTwist is involved in the regulation of limb patterning. Twist transcripts are found in developing somites, lateral plate mesoderm, limb mesenchyme, and head mesenchyme. Twist is also found in the proximal region of the feather filaments (Taveres et al., 2001). In *Xenopus* laevis, in addition to the lateral plate mesoderm, Xtwist mRNA is detected in the neural crest and the notochord (Hopwood et al., 1989; Chen and Behringer, 1995). Jellyfish, *Pachymenia cornea* expresses CeTwist in the ectoderm and endoderm of the planula larva and adult

medusa. CeTwist also is found momentarily in the entocodon, which is a mesoderm-like tissue. *Caenorhabdtis elegans* do not express Twist during mesoderm specification of embryogenesis, but is instead first detected in the formation of defecation-associated muscles and in neural cells in the head. It is also expressed in the M blast lineage, and expression declines as body and sex muscles differentiate, and has roles in cell proliferation (Harfe et al.,1998). In *Mus musculus*, the common mouse, MTwi is expressed in presomitic cells, and detected in neural crest mesenchyme, and the branchial arches. Later in development, Twist is expressed in the trunk, specifically in the limb buds and lateral plate mesoderm (Gitelman, 1997). In the mouse, Chen and Behringer (1995) found that embryos with the twist-mutant exhibit a failure of neural tube closure in the cranial region, as well as defects in the head mesenchyme, branchial arches, somites, and limb buds. Humans also express Htwist, and is found in mesoderm-derived cells and tissues. It is also found in the placenta, heart muscle, skeletal muscles, kidneys, and the pancreas. Cell lines developed from normal human tissues are shown to express Twist in mesothelial and endometrial fibroblasts (Wang et al., 1997). Functionally, humans with a mutation in the Twist gene have the autosomal dominant disorder of craniosynostosis, known as Saethre-Chotzen syndrome. Symptoms of this

disease include facial and limb abnormalities in addition to mental retardation (Howard et al., 1997). In the leech, *H. robusta, Hro-twist* mRNA is found to be present early in the oocyte, and throughout early embryonic stages (Soto et al., 1997). Throughout the various organisms, it is evidently clear that Twist has crucial role in determination of cell fate, as well as development of specific locations within an organism.

2.7. Twist Secondary Structure

The protein Twist encodes a basic helix-loop-helix transcription factor, or bHLH (Thisse et al., 1987). The HLH motif was first found in DNA-binding proteins E12 and E47, and the amino acid sequence is homologically similar to proteins that regulate developmental processes such as growth regulation, myogenesis, and neurogenesis (Murre et al., 1989). The HLH consists of two amphipathic helices that is separated by a loop, that varies in size from species to species. This domain is important to maintain the overall tertiary structure, and it has been shown that the HLH region is both necessary and sufficient for protein dimerization (Kadesch, 1993). When comparing the bHLH region of all studied organisms, the protein shares 59%-85% sequence similarity. However, when observing the similarity within vertebrates only, there is an 84%-100%

similarity over the bHLH domain (Castanon, 2002). Saethre-Chotzen syndrome, referred to earlier, is in fact characterized by point mutations within the bHLH domain of human Twist (El Ghouzzi et al., 1997).

Twist also contains a second domain known as the WR motif (Spring et al., 2000). It is highly conserved in vertebrates and jellyfish, but is not, however, identified in the leech. Located between the 20 and 55 amino acid C-terminal to the bHLH region of *Drosophila* Twist, the function of this site is unclear, but a nonsense mutation suggests that the C-terminal domain may be required for Twist activity, stability of mRNA, or protein folding in Saethre-Chotzen patients (Gripp et al., 2000).

In other forms of Twist, such as that from mouse and human, there is a domain that contains an arginine/lysine-rich region and two spans of glutamine/histidine repeats known as CAX, which is N-terminal to the bHLH domain. In the leech, Hro-*twist* contains glycosylation sites and four CAX-rich stretches, three 5′ to the bHLH, and one 3′ of the bHLH (Soto et al., 1997). Stretches that are rich in glutamine are found in various transcription factors and may play a role in gene activation (Dynlacht et al., 1987).

bHLH proteins are shown to bind as dimers to the consesnsus hexanucleotide sequence 5′-CANNTG-3′, known as an E-box (Ephrussi et al.,

1985). E-boxes are DNA sequences that lie upstream of a gene, in a promoter region, and are binding motifs to transcription factors, and can enhance transcription. Twist binds specifically to the E-box, 5'-CATATG-3' (Ip et al., 1992) mainly in the form of a heterodimer (Spicer et al., 1996, Ghouzzi et al., 1997). However, for mouse and zebrafish, it binds as a monomer (Lee et al., 1997; Castanon et al., 2001). Dimerization of Twist occurs at the bHLH domain. Since other proteins are known to also contain the bHLH domain, they may dimerize to either positively or negatively regulate transcription (Castanon et al., 2001). In *Drosophila,* Twist may heterodimerize with the E protein Daugherless (Da), which activates target genes that promote neurogenesis (Campuzano et al., 1985). In some vertebrates, the myogenic transcription factor MyoD can form heterodimers with Twist to activate muscle-specific promoters that contain E-boxes in cell culture (Lassar et al., 1991).

Regions within the N-terminal and C-terminal have been shown to provide sites of further protein-protein interactions. The N-terminal domain of mouse Twist directly binds to histone acetyltranferases (HAT), which is a protein that lacks a bHLH domain (Hamamori et al., 1999). Zebrafish twist has been shown to interact with TAF110, a subunit of the TFIID complex which mediates gene activation (Pham et al., 1999). In *Drosophila* Twist, the N-terminal interacts

with transcriptional regulators such as Dorsal, which synergizes with twist to regulate snail (Ip et al., 1992; Shirokawa and Courey, 1997). The C-terminal domain of mouse Twist is found to be necessary for the repression of Mef2 activity in tissue culture (Spicer et al., 1996). Altogether, various regions of the Twist protein provide interaction sites for proteins, leading to either homodimer or heterodimer bHLH combinations. This will subsequently affect DNA binding affinity and preferential target gene selection.

3. Materials and Methods

3.1. Bioinformatics

The full coding sequence (CDS) of the leech species *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. Although the project focused on the 3′ untranslated region of the gene, the FASTA output includes the entire 1,891 nucleotide sequence, beginning with the 5′ untranslated region, and the coding region. The 3′ UTR start nucleotide is at position 983.

REPFIND, a program that employs a bioinformatics database to find clustered, exact repeats in nucleotide sequences, was used to identify potential localization elements. The P-value cutoff was set to 0.0001. These possible localization elements were confirmed in the UTR Blast database from UTB BLAST (http://bighost.ba.itb.cnr.it/BIG/Blast/BlastUTR.html/). In addition to the resulting elements, more *ARE2* elements, AATAATA, were located by a simple word search with Microsoft Word 2003. The program was also used to annotate the entire *Helobdella robusta Hro-Twist* gene sequence.

The folding of the mRNA secondary structure was determined by submission of the RNA query to the Burnet Institute Mfold Server (http://mfold.burnet.edu.au/). Adobe Photoshop CS2 was used to locate *ARE2* elements present on stem loops and other regions of the *Hro-twist* mRNA.

3.2. Mutagenic Primer Design

Primers were designed to introduce point mutations among the various localization elements, mutating the *ARE2* elements, AATAATA, to AGGAATA. The goal was to produce plasmids containing single *ARE2* mutations, as well as plasmids containing multiple *ARE2*s mutations. Since six *ARE2* elements share common regions, special consideration was taken to include surrounding altered nucleotides in addition to the focal mutation target, to prevent undesired mutations.

The 3′ UTR of *Hro-twist* is particularly complex for conventional PCR reactions because of its abundancy of adenosine and thymidine nucleotides. In addition to the weaker hydrogen boding of the A and T bases, primers introducing two point mutations result in mismatches that do not allow for proper annealing. Taken together, the design requirements are more stringent for effective annealing to the template.

Primers are recommended to be a minimum of 40% guanine/cytosine content. *ARE2* elements, however, are mostly found within regions high in adenine and thymine, so the aforementioned requirement was not met. To compensate, primers were extended beyond typical base lengths. Maximum primer lengths were to be no more than 55 nucleotides, diminishing secondary structure formation. Minimum melting temperatures were desired to be 75 °C. It was preferential to terminate the 3'-end with one or two C or G bases for increased elongation success of the polymerase. In addition, because the template is circular, it is ideal for the 5'-end to also be capped with a G or C base, for ease of ligation of nicks for a closed product. Finally, the location of the primer was such that the mutation was exactly located in the center of the primer. If there was a need to include a guanine or cytosine cap, the primer could be shifted by only one nucleotide over.

Primer design was partially accomplished using the Primer3 program (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 1998). Mutagenic primers were also determined from Stratagene's web-based QuickChange Primer Design Program, which allowed for the option of setting mismatches (http://www. stratagene. com/qcprimerdesign).

The previous programs resulted in potential primers, but were significantly deficient in fulfilling the stricter requirements. Manual primer examination was accomplished on Microsoft Word 2003, and melting temperature and GC% content were individually confirmed with Primer Check (http://www.iit-biotech.de/service-primer-check.shtml), and University of Washington's Oligonucleotide Primer Check (http://depts.washington.edu/ bakerpg/ primertemp/primermelttemp.html), which employs an alternative melting temperature algorithm.

Oligonucleotide high-throughput synthesis was performed by Operon Biotechnologies, Inc (https://www.operon.com/products_main.php) (Table 1). Though a *beta*-cyanoethyl phosphoramidite method was applied to produce salt-free primers, longer primers were requested to be purified by subjection to polyacrylamide gel-electrophoresis (PAGE), which removes incomplete truncated oligonucleotides and free nucleotide bases.

Lyophilized oligonucleotide were resuspended by vortexing with 1.0 mL of 10mM Tris-Cl, 1.0 mM EDTA buffer, pH 8.0, and stored in -20 °C.

Table 1. List of mutagenic primers used for site-directed mutagenesis.

Primer Name	Mutates Sites	Primer Sequence
Primer1 A	1	5′-GGCAGATGAATAAAAGTGAACTTA**GGAATA** ACGGATGTTACTTACGTAAC -3′
Prime2 A	2	5′-CATATGTGTGTATGATAAAA**GGAATA**GT**AAT AATA**ATAATAT -3′
Primer5 A-end	5	5′-TGATAAA**AATAATA**GT**AATAATAATAGGA**TT TATTATTATTATTATTATAACAG -3′
Primer 6A	6	5′-CAGTAATAAGAGAAATAAC**AGGAATA**TCTTT AGGTGCTTACC -3′
Primer7 A	7	5′-AACAGCAACAACAACAACAAC**AGGAATAAT** **A**ATAAAATTATGGTGG -3′
Primer8 A	8	5′-AACAACAACAACAACAAT**AGGAATAATA**AA ATTATGGTGG -3′
Primer 9A-end	9	5′-CAACAACAACAAC**AATAATA**ATAGGAAAAT TATGGTGGTATTA**T**AAAC -3′
Primer 10A	10	5′-AATGTTATTTTATATCGTCCT**AGGAATA**ACAC ACACACACACACTCAC -3′
Primer 7-9-9end	7, 8, 9	5′-AACAGCAACAACAACAACAAC**AGGAATAG GAGGA**AAATTATGGTGG -3′
Primer 9-9end	8,9	5′-CAACAACAACAAC**AATAATAAGGAGGA**AAA TTATGGTGGTATT -3′

Bolded nucleotides represent *ARE2* elements. Underlined nucleotides represent mutations. Primer 7-9-9end and Primer 9-9end were required for sequential deletions because multiple *ARE2* sites were close together.

3.3. Site-Directed PCR Mutagenesis

Reagents for the PCR reaction were obtained from Stratagene's QuikChange Multi Site-Directed Mutagenesis Kit (http://stratagene.com, Cataglog #200514).

Template DNA for the reaction was obtained from a previously cloned plasmid containing the *Hro-Twist* 3'UTR insert. The insert was previously PCR amplified from the cDNA clone p1.2, which includes the 3'UTR as well as 120 bases upstream that code for the final 60 *Hro-twist* amino acids (Soto et al., 1997). Amplification with the M13F universal forward primer introduced the *Xho*1 restriction site, and a reverse primer added a linker for an *Eco*R1 restriction site. The insert was subcloned into the vector pBluescript SK+ (Stratagene, pBSK+), a 2958 bp phagemid that contains the Ampicillin-resistance gene. The insert is a 957 base pair fragment containing the entire 3' UTR, inclusive of the final 48 bases of *Hro-twist* coding region. Ligation was done with *Eco*RI restriction digest at the 5' end of the insert, and *Xho* I restriction digest at the 3' end for directional cloning, resulting in a final template for mutagenesis of 3,915 bp in length.

Mutagenesis primer and M13F plasmid template concentrations were quantified by a NanoDrop ND-1000 Spectrophotometer. Primers were diluted to 100 ng/μl and M13F template to 50 ng/μl by Millipore H_2O. Reaction mixture

consisted of 2.5 μl of 10X QuikChange Multi reaction buffer, 50 ng of M13F plasmid template, 100 ng of appropriate mutagenesis primer, 1 μl of dNTP mixture, 1 μl of QuickChange Multi enzyme blend (2.5 enzyme units), and appropriate volume of Millipore H2O to bring the total volume to 25 μl. The mixture was gently vortexed in a 0.5 ml Eppendorf PCR tubes (http://www.eppendorf.com, Cat. #952020057) and overlayed with 50 μl of mineral oil. Thermocycler (Perkin Elmer Cetus DNA Thermocycler 480) input settings were set to one cycle of 95 °C at 1 min, with 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 8 mins at 65 °C (2 mins/1 kb of plasmid length). Soak setting was fixed at 4 °C overnight, and then stored at -20 °C.

3.4. *Dpn* I Digestion

Upon thawing of PCR reaction, 20 μl of amplification products was transferred to a 1.5ml Eppendorf tube, with careful attention to avoid mineral oil carryover. One μl (10 enzyme units) of *Dpn* I restriction enzyme was added directly to the amplification product and mixed thoroughly by pipetting. Incubation for 3 hrs was performed on a 37 °C heat block (Thermodyne Typ17600) to digest methylated and hemimethylated DNA, resulting in only the predominant mutated product.

3.5. Transformation of XL-10 Gold *E. coli* Cells

XL10-Gold Ultracompetent *E. coli* cells were obtained from Stratagene (Cat. #200314) and stored at -80 °C until just prior to use. For each transformation reaction, 2 µl of XL10-Gold *beta*-mercaptoethanol mix was added to 45 µl of freshly thawed cells in a prechilled 14-ml BD Falcon polypropylene round-bottom tube (BD Biosciences Cat. #352059). Following 10 mins of incubation on ice, with gentle stirring every 2 mins, 5 µl of the *Dpn*1-digested PCR mutagenesis reaction was introduced to the mix. Equilibrization of the DNA-*E.coli* blend was achieved on ice for 30 mins prior to a heat pulse of 30 seconds in a 42 °C water bath, followed by a 2 min cold shock on ice. If transformation efficiency is low, 10ul of PCR reaction was used instead. To allow time to confer expression of Ampicillin resistance, the cells were allowed to incubate in 500 µl of Luria-Bertani broth, without Ampicillin, for 1 hour at 37 °C, with constant shaking at 225 rpm. White colonies containing mutated M13F plasmid were allowed to develop for 16 hrs overnight by spreading 200 µl of the cells on LB agar plates containing ampicillin (100ug/ml), in a 37 °C incubator.

3.6. DNA Isolation and Sequencing

Colonies were picked with inoculating loops from the plates and allowed to grow in 3 ml of LB broth with 100 μl/ml of ampicillin in glass test tubes for 16 hrs overnight in a 37 °C shaker, with constant shaking at 225 rpm. 1.5 ml of the cells was spun down in a 1.5 ml Eppendorf tube for 2 mins at 16,000 X G in a table top microcentrifuge. After vacuum aspiration of the supernatant, the mutated M13F DNA was isolated from the pellet from one of two mini-purification kits: Wizard Plus Minipreps DNA Purification System (Promega Cat.# A7510) and Rapid Plasmid DNA Daily Mini-Prep (V-Gene Biotechnology, Cat.# 110110-25). The final M13F mutant plasmid was eluted with 20 μl of TE.

To confirm positive point mutation of the plasmid, 5 μl of samples were submitted to Tocore DNA Sequencing Service (http://www.nucleics.com/) or Sequetech Corp. (http://www.nucleics.com/). Chromatogram files were analyzed by the program BioEdit Sequence Alignment Editor to evaluate sequencing accuracy and to identify which cell culture contains cells with mutated plasmids.

Cultures containing mutant plasmids were streaked across LB agar plates containing 100 μl/mg of ampicillin, and allowed to grow colonies over 16 hrs at 37 °C. An individual colony from the streak was inoculated into 100 ml of pre-

autoclaved LB broth with 100 µl/mg of ampicillin in a 500 ml glass flask, and allowed to incubate overnight for at 37 °C, at 225 rpm.

In a 50 ml BD falcon tube, the culture was pelleted, 50 ml at a time, with two centrifugations at 2,000 X G for 20. The mutated DNA was extracted from the pellet using the Qiagen HiSpeed Plasmid Midiprep kit (http://www1.qiagen.com/, Cat. #12643), and eventually eluted in 1.0 ml of Millipore H_20. Concentration was determined by Nanodrop spectrophotometer readings. Five µl of sample DNA was run on a 1.0% agarose gel for 45 mins at 90 volts to determine purity and proper length of mutated Hro-*twist* 3'UTR.

3.7. Template Linearization with *Xho*1

The plasmid was linearized by *Xho*1, just downstream of the Hro-twist 3'UTR, to allow for 3' run-off *in vitro* transcription, for the RNA polymerase to make the correct sized mRNA, in the proper sense orientation. The digest mixture comprised of 10 µg of plasmid DNA from the midiprep, 1 µl of 100X BSA, 10 µl of 10X NEB Buffer #2 (New England Biolabs, Cat. #B7002S), 2 µl of *Xho*1 enzyme (40 enzyme units, New England Biolabs, Cat. #R0146S), and appropriate volume of H_20 to bring the total volume to 100 µl. Digestion occurred for 3 hrs on a 37 °C heat block, followed by 20 mins at 65 °C, and 5 mins

on ice. Complete digestion was confirmed by running 4 µl of the digest reaction on a 1% agarose gel for 1 hour at 90 volts, to reveal a discrete band of 3.9k bp.

DNA extraction was performed by simple addition of 100 µl of chloroform (Sigma Aldrich), and gentle vortexing. Upon centrifugation at 16,000 X G for 5 mins, the DNA containing top layer was transferred to a new 1.5 micro centrifuge tube. The linearized plasmid was precipitated with 200 ul of 100% ethanol, 10 µl of sodium acetate, and 2 µl of the visualization marker RNase-free GlycoBlue (Ambion, Cat. #AM9515). After an overnight precipitation at 4 °C, the DNA was pelleted for 25 mins at 12,000 rpm in a 4 °C cold room. The pellet was washed with 1 ml of -20 °C ethanol, and centrifuged again for 10 mins. The pellet was then allowed to dry for 10 mins under spinning in a vacuum (Savant Speed Vac Plus, SC110A), set to a medium drying rate, and under vacuum. The linearized M13F plasmid was resuspended in 10 µl of Millipore H_20 treated with 0.1% diethylpyrocarbonate (DEPC).

3.8. *In-vitro* Transcription of DIG-labeled mRNA

mRNA of the 3'untranslated region of *Hro-twist* were transcribed via run-off transcription with T3 RNA polymerase (Promega, Cat. #P402A). 5' 7-metheyl guanosine nucleotide (m7G(5')ppp(5')G), a guanosine cap structure, was

incorporated into the synthesized RNA to mimic the natural capped structure of *in vivo* mRNA (Promega, Cat. # P171B). The resulting single-stranded RNA transcript was digoxygenin-labeled by the amalgamation of DIG-11-UTP into every 20-25th nucleotide (Roche Diagnostics, Cat. #11093274910). The *in vitro* transcription reaction consisted of 2.5 μl of mutated M13F DNA (approximately 1ug of DNA), 4 μl of Transcription Optimized 5X Buffer (Promega, Cat. #P118B), 5 μl of Rib m7G Cap Analog diluted to 5mM in Millipore H_2O, 2 μl of 10X DIG RNA Labeling Mix, 2 μl of 100mM dithiothreitol (DTT, Promega, Cat. #P117B), 2 μl of RNasin Plus RNAase Inhibitor (80 enzyme units, Promega, Cat. #N261A), and 1 μl of T3 RNA polymerase (80 enzyme units). Prior to reagent handling, the work bench was thoroughly treated with RNaseZap (Ambion, Cat # 9780). RNA transcription occurred for 3 hrs at 37 °C, upon which 2 μl of RQ RNase-free DNase (2 enzyme units, Promega, Cat#M6101) and 2 μl of 10X reaction Buffer (Promega, M198A) was added to digest away the original DNA template. DNase activity was halted after 30 mins at 37 °C by the addition of 0.8 μl of RNase-free 0.5M EDTA, pH 8.0, and 10 mins of heat inactivation for 10 mins at 65 °C. The RNA was snapped cooled on ice for 2 mins.

RNA precipitation was obtained by a lithium chloride method, which neutralizes the negative phosphate backbone charge to precipitate out large

mRNA effectively. The lithium chloride is not co-precipitated. To the reaction tube, 2.5 µl of 4 M LiCl and 75 µl of cold 100% ethanol was added. Two µl of 15 mg/ml GlycolBlue was added to visualize the RNA. After precipitation for 30 mins at -20 °C, recovery was accomplished by centrifugation at 4 °C under 10,000 x g for 15 mins. The RNA pellet was washed with 200 µl of cold 100% ethanol, and centrifuged again, before drying in a Rotovac without heat for 5 mins. The RNA transcript was resuspended in DEPC-treated H_2O for 30 mins at room temperature, minimally mixed, and immediately placed in -80 °C. The quality of the RNA, purity, and presence of contaminating DNA was determined by gel electrophoresis with a 1.2% agarose gel, with 1X TBE running buffer. The gel was allowed to run for 30 mins under low voltage. RNA concentration was determined by a Nanodrop spectrophotometer reading.

3.9. *Helobdella robusta* Zygotes

Helobdella robusta leeches were collected in run-off ponds located at a sturgeon farm in Galt, CA. The leeches have preferences for darkness, and are sparsely found under water leaves and intermingled deeply among *Caulerpa sp.* seaweed. Small pond snails, *Physa sp.*, were also collected as a food source for the leeches. The organisms were maintained in the laboratory in 1% seawater:

sterile Millipore H_2O, with freshwater aquarium salt (Aquarium Pharmaceuticals, Inc, #106C), NovAqua-plus water conditioner (Kordon-Novalek, Inc., #33156) , and the nitrate-ammonia removing Amquel-plus (Kordon-Novalek, Inc., #33456), according to packaging conditions.

Helobdella robusta leeches are hermaphroditic, and each individual can therefore internally produce fertilized eggs. Pre-embryonic tissue appears as a cohesive white strip along the midline of the leech. Prior to deposition, it can be observed that the zygotes disassociate from one another, and develop as individualized pink cells. Eggs are arrested at metaphase I until deposition out of the leech (Fernández and Olea, 1987; Wedeen et al., 1990). Immediately upon deposition, with the aid of blunt-end forceps, the clutch of approximately 25-50 embryos was separated from the leech and amniotic sac in a petri dish containing sterile HL saline (4.8 mM NaCl, 8.0 nM CaCl2, 2.0 mM MgCl2, 1.2 mM KCl, and 1.0 mM Tris-HCl, pH 6.6) (Weisblat and Huang, 2001).

3.10. Microinjection of mRNA Transcripts

Upon deposition of embryos, 1st polar bodies develop within 20 minutes. Therefore, injection must take place during this critical period. Prior to injections, microcapillary needles were made by pulling capillary tubing –

Borosil 1.0mm OD X 0.75 mm ID (Frederick Haer & Co., Cat. #30-30-0) with a Flaming/Brown Micropipette Puller (Sutter Instrument Co., Model P-87) and the tip opened by jewler forceps under a dissecting microscope. Settings of the micropipette puller were set to: pressure = 200, heat = 320, pull = 100, velocity = 10, and time = 80.

Mutated and control M13F mRNA was diluted to 25 ng/μl, in a 0.2 N KCl, 0.1% fast green solution (Fast Green FCF, Sigma, Cat.#F-7252). Fast green stock solution (1% in 0.2 N KCl) was passed through a 20 ml syringe with a 0.2 μm Acrodisc filter (Gelman Sciences, Cat.# 4192) to prevent clogs in the needle. The loaded needle was mounted on a FemtoJet microinjector (Eppendorf, Cat.# 5247-000.013) under a Nikon Type 102 Dissecting Microscope (Makroskop, M420). To normalize the volume of injections, settings were adjusted for the InjectMan Nl-2 micromanipular (Eppendorf, Cat. #5181-000.017) such that the pressure and length of injection would ensure the same sized spherical droplet in mineral oil. The diameter of the sphere was normalized to be 100 μm, measured on a micrometer, which resulted in a constant injection of 12.5 pg of mRNA.

Upon normalization of injection variables, harvested embryos were lined along a stage consisting of 1% agarose in Millipore H_20 in a plastic petri dish. The stage comprised of a trough resulting from the embedding of stacked

microscope slides in the agarose. Injection occurred rapidly within the 20 minute injection window, and the embryos were allowed to develop in HL saline until teloplasm formation, 3-4 hrs later. Zygote development was halted by fixation with 4% paraformaldehyde (Electron Microscope Sciences, Cat. #15710), 0.25X PBS, and 100 mM cacodylic acid in Millipore H20 for 1 hour at room temperature, with constant rocking. After several PBS washes, embryos were dehydrated with 50% Methanol-PBS, and 100% methanol, and stored at 4 °C overnight.

3.11. Staining and Visualization of Injected Embryos

Embryos were rehydrated by washes of 60% methanol:PBS, and 30%. Vitelline membranes were carefully removed under dissecting microscope in a petri dish with 000 insect pins (http://www.finesscience.com/), and incubated for 3 hrs in 0.22 μm filtered blocking solution: 10% goat serum (Pierce, Cat. #PI-31873), 25% bovine serum albumin Promega, Cat. #W384A) in PBS with 0.1% Tween-20. Embryos were allowed to incubate overnight in anti-DIG Fab (Roche Diagnostics, Cat. #11093274910) at a concentration of 1:200. After thorough washing with PBT for 4 hrs, the cells were washed with Coloration Buffer (100mM Tris-HCl, 1.0mM NaCl, and 0.1% Tween-20, pH 9.5). Embryos were

then transferred to 9-well staging Pyrex glass dishes (Corning Inc., Cat. #7220-85), and NBT/BCIP reagent (Roche Diagnostics, Cat. #1697471) was added for staining of the DIG-labeled mRNA. After thorough washing with PBS, the embryos were dehydrated through an increasing concentration of ethanol washes.

Clearing of the embryo was achieved by methyl salicylate (Sigma, Cat. #M2047), which allows light to more easily pass through the embryo, while retaining the staining of mRNA. Photographs were taken of the animal and vegetal view as whole mounts, as viewed with a brightfield under a Zeiss Axiophot microscope. Embryos were stored in 80% glycerol at 4 °C. Images were viewed in Adobe Photoshop CS2.

4. Results

4.1. Identification of Localization Elements

The *Hro-twist* full CDS (accession AF410867) was entered into REPFIND and UTR Blast to yield possible localization elements. Various elements were found that included *ACE1* (CAACAAC), *ACE2* (CGACGAC), *ARE1* (ATTTA), *ARE2* (AATAATA), and *CPE* (TTTTTTAT). *ACE1* was found readily in the *Hro-twist* gene, with 10 in the coding region, and 5′ in the 3′UTR. It was found that three *ACE2* sites were located in the coding region, but devoid in the 5′ or 3′ UTR. *ARE1* sites were also located in both regions, with two in the coding region, and five in the 3′UTR. The CPE element was found close to the 3′-terminal of the gene. Of particular interest were the *ARE2 cis-acting* elements. All 10 of the elements were found within the 3′UTR. It was for that reason that injection of mRNA was only of the 3′UTR. Also, the 5′UTR lacked any possible localization elements.

4.2. *ARE2* Elements on the mRNA Secondary Structure

Mfold was used to predict possible foldings of secondary structures (Figure 1). Of the most likely structure, a search was conducted of the 10 *ARE2*

sites. Sites were labeled 1 through 10 starting from the 5′ end of the 3′UTR. From herein forth, transcripts will be identified with the nomenclature *ARE2*-site#mut, with # signify the site that contains the mutation. It was found that Sites 3, 4, and 5 were clustered within the same region, as well as Sites 7, 8, and 9. These six sites are overlapping sites, and both regions appear on stem-loop structures. This may indicate more substantial roles for these particular sites. Site 1 and Site 10 are both small loop structures. Sites 6 makes up half of a larger loop of a stem-loop. Site 2 is part of the main body of the transcript, but found within one nucleotide of the 3-4-5 region. The location of the sites on the secondary structure may affect how the site is presented to any possible trans-factor proteins. Also, it is possible that they have various degrees of function to localization of the entire mRNA transcript.

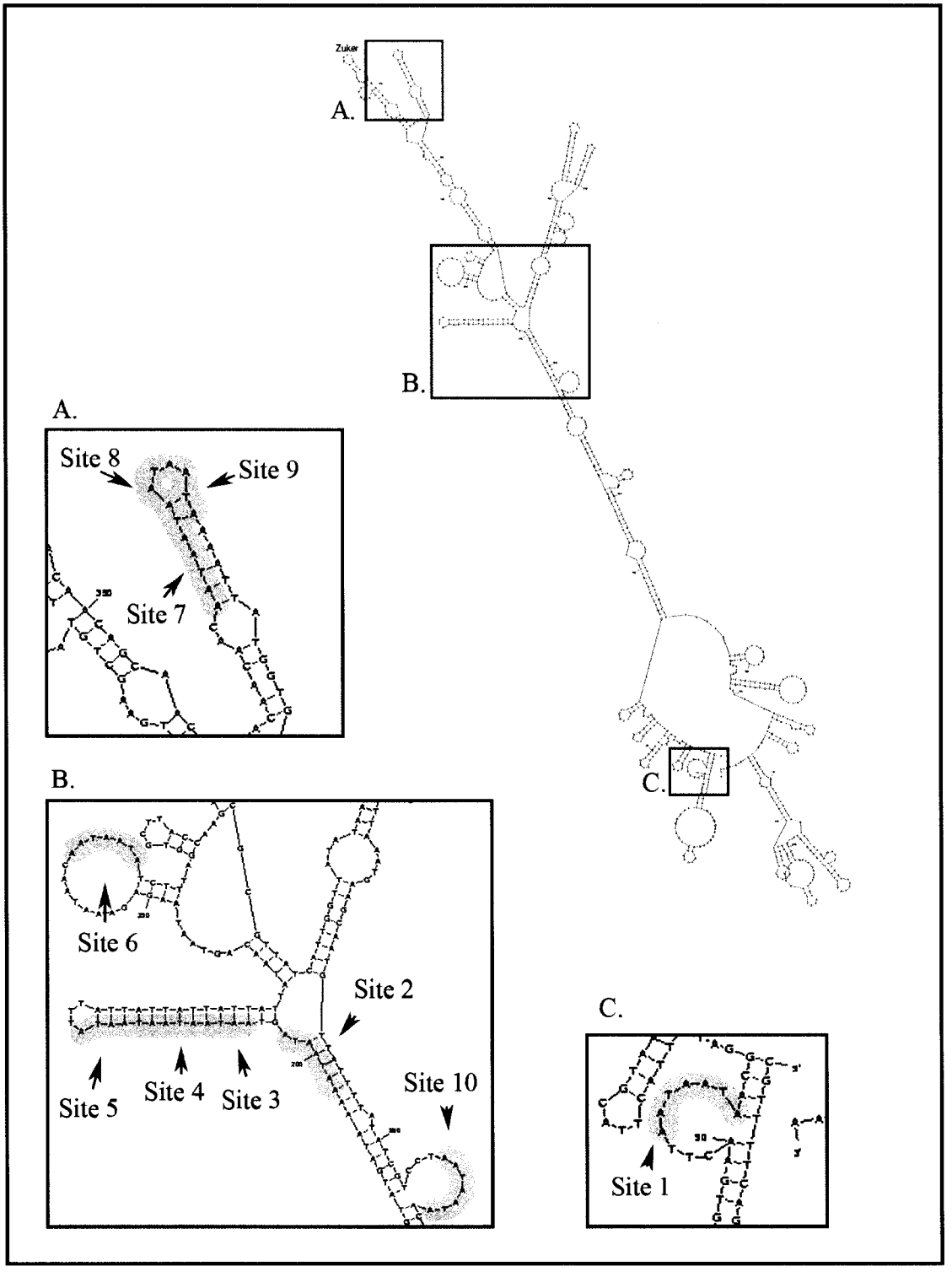


Figure 1. Diagram of *Hro-twist* transcript secondary structure. Folding determined by Mfold. The position of ten *ARE2* elements are identified

4.3. Site-Directed Mutagenesis of *ARE2* Sites

Various primers were used to introduce point mutations into *ARE2* sites. Upon transformation of XL10-Gold *E. coli* cells, plasmid DNA was purified and sequenced for confirmation of mutated *Hro-twist* DNA (Figure 2 and Figure 3). There were two phases of mutagenic reactions. The first was to introduce single site mutations of each of the ten *ARE2* elements. Successful mutations were attained of seven of the *ARE2* sites (Figure 4). The exceptions were Sites 3, 4, and 5. Although different primers, and PCR conditions were attempted, Sites 3, 4, and 5 were unable to incorporate the mutated bases, despite successful transformation and DNA purification.

The second phase of the mutagenic reactions involved accumulating mutations within the same transcripts. Mutations started at the 3' end of the 3'UTR, starting with Site 10. Progressively, Site 9 was then mutated, and further sites continually mutated upstream along the UTR. Mutation of Sites 3, 4, and 5 were skipped over. Site 1 was then mutated in the sample *ARE2*-sites6-10mut, which contains mutations of Sites 6 through Sites 10. Finally, Site 2 was mutated, producing the *Hro-twist* 3' UTR that contains the maximum number of *ARE2* mutations, *ARE2-sites1,2,6-10mut* (Figure 5).

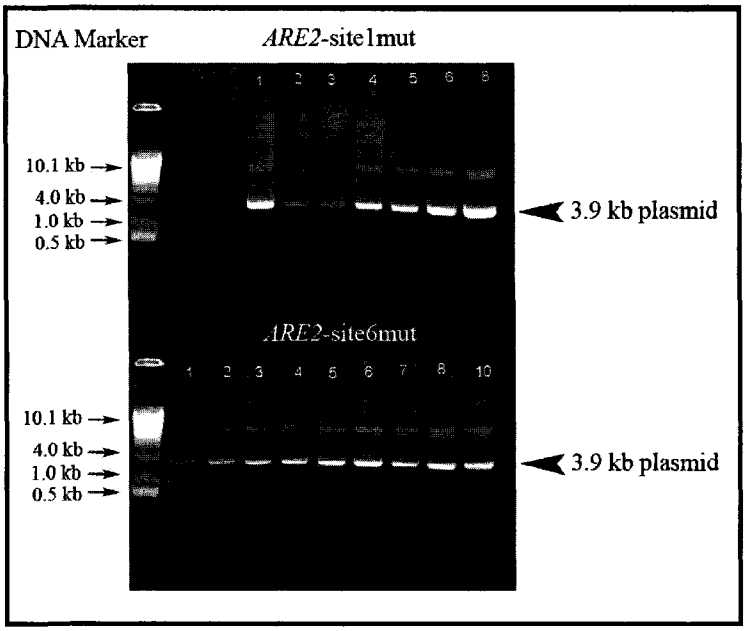


Figure 2. 1% agarose gel of *ARE2-site1mut* and *ARE2-site6mut* miniprep plasmid DNA for sequencing reaction. Lanes contain isolations from various colonies. 1.0kb DNA ladder (Roche).

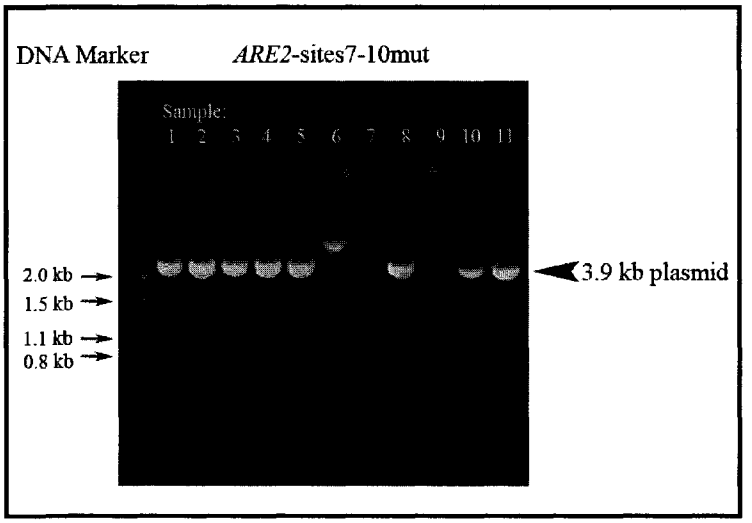


Figure 3. 1% agarose gel of *ARE2-1-10mut* miniprep plasmid DNA for sequencing reaction. Lanes 6, 7, and 9 were not used for sequencing reactions. 1.0kb DNA ladder (Fisher).

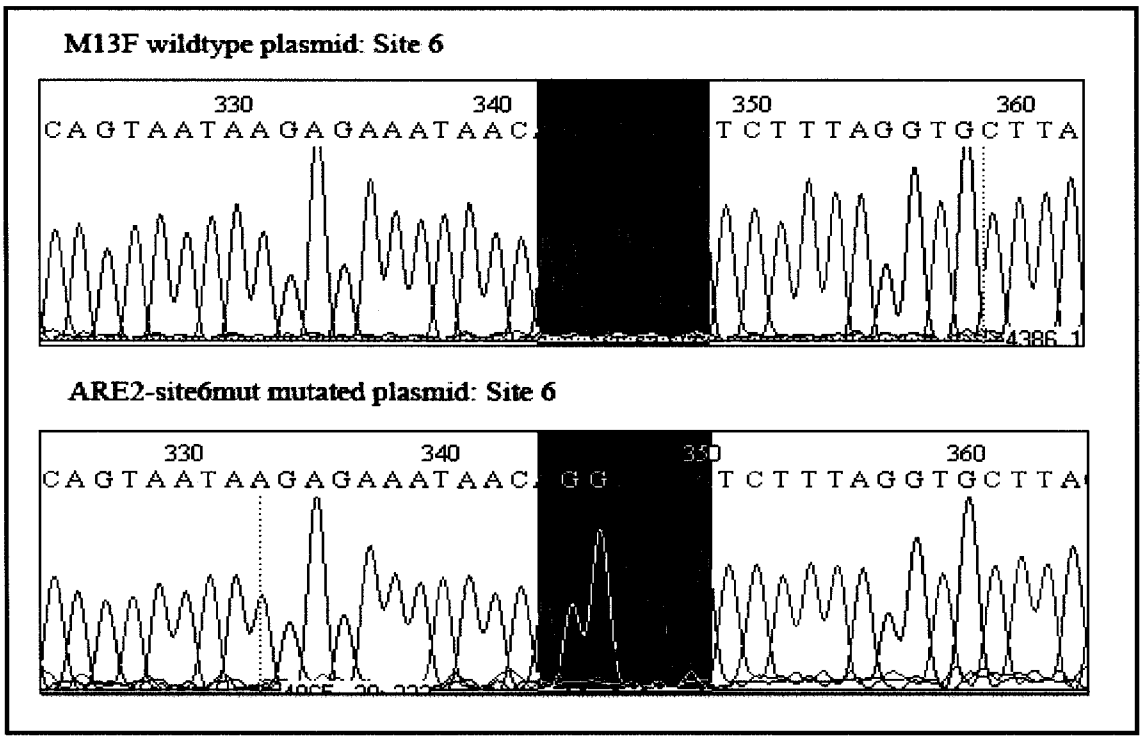


Figure 4. Chromatogram file of sequenced *M13F* positive control DNA (top) and *ARE2*-site6mut DNA plasmid (bottom). Viewed with Bioedit Sequence Alignement Editor. Sequencing shows sharp bands, indicating pure DNA. Area highlighted showed the original *ARE2* Site 6, and confirmed the mutation. AATAATA has been mutated to AGGAATA. Sequencing was obtained from Tocore, LLC.

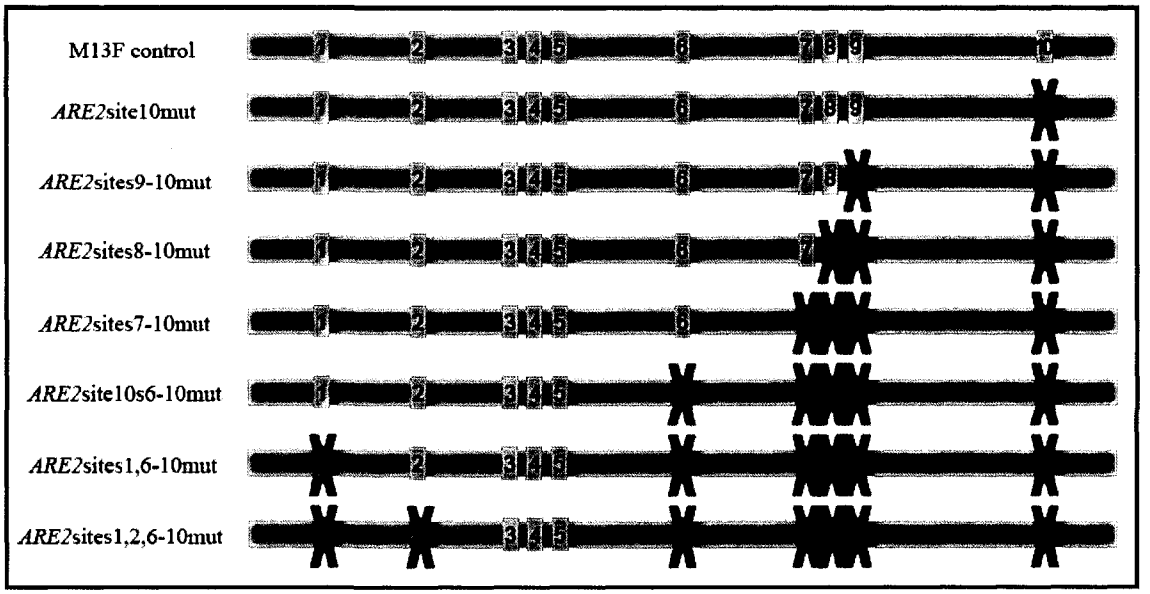


Figure 5. Diagram of Sequentially Mutated ARE2 sites. Sites 3, 4, and 5 were A/T rich and did not incorporate the mutations

4.4. *Xho*1 Digestion and *In-vitro* mRNA Transcription

Prior to the *in-vitro* transcription reaction, midipreps of the successfully mutated plasmids were linearized with *Xho*1 enzyme to produce a linear template for transcription. Clean-up was attained by a chloroform DNA extraction method, and precipitated with 100% EtOH, as outlined in the protocols. Gel electrophoresis showed bright clean bands at 3.9 kb, the size of the linearized plasmid + 3'UTR (Figure 6, Figure 7, and Figure 8). Since the transcripts are the same length, any noticeable effects can be attributed to the point mutations, rather than transcript lengths.

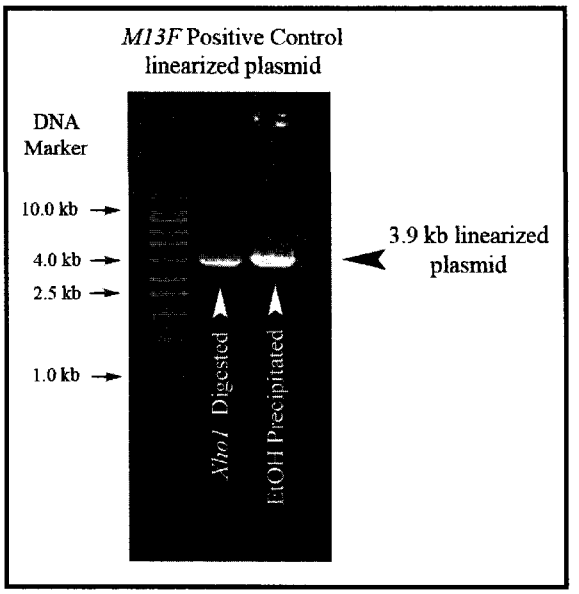


Figure 6: 1% agarose gel of *M13F* plasmid, linearized with *Xho*1. Lane 1: *M13F* positive control plasmid, linearized with *Xho*1 digestion (40 unites for 3 hrs). Lane 2: Ethanol clean-up of linearization with EtOH precipitation. 1.0 kb DNA ladder (AllStar).

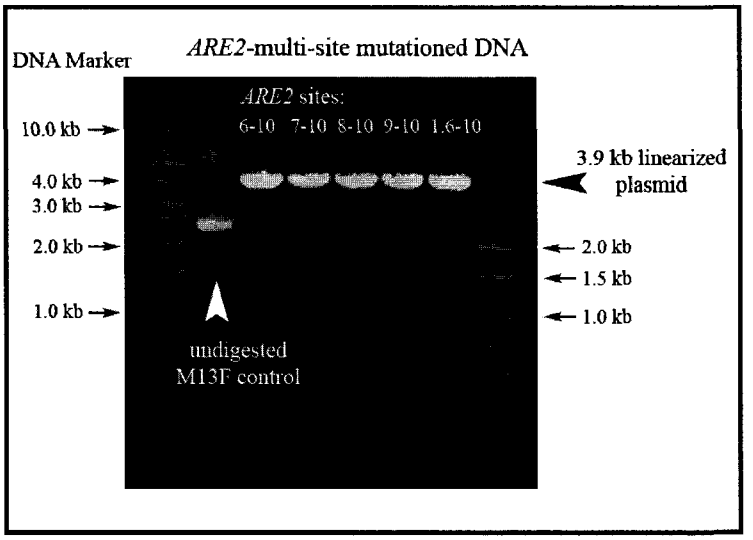


Figure 7: 1% agarose gel of plasmids w/ sequential mutations, linearized with *Xho*1. Linearization with 40 units of *Xho*1 for 3 hrs. These samples contain the sequential mutations of the *ARE2* sites. Lane 1 contains the *M13F* control, undigested, to ensure digestion of the other plasmids 1.0 kb DNA ladder (AllStar).

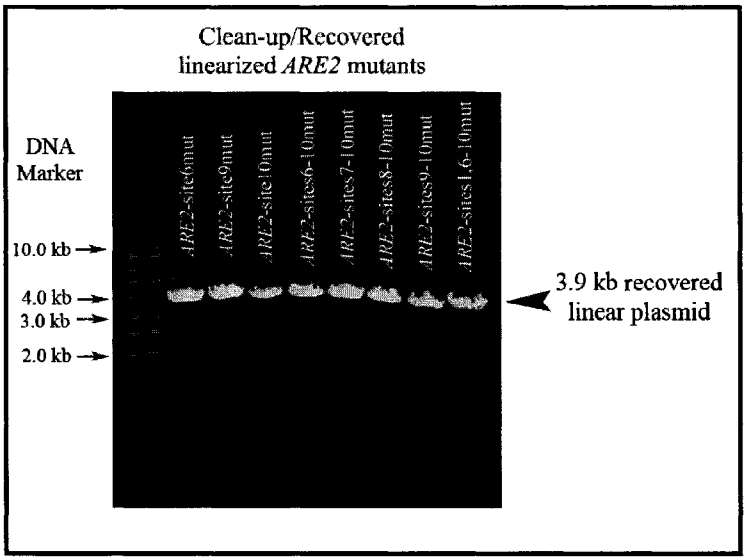


Figure 8: 1% agarose gel of clean-up/recovered mutants for transcription. Clean-up with chloroform and precipitated in EtOH. Gel includes samples with single site mutations, as well as samples with sequential mutations (*ARE2-sites1,6-10mut*) 1.0 kb DNA ladder (AllStar).

In-vitro transcription reactions LiCl precipitated and DNase treated to remove short incomplete transcripts. The electrophoresis gels showed that the reactions were successful in producing clear RNA bands at 0.9 kb, the size of the *Hro-twist* 3'UTR RNA transcript, minus the pBluescript SK+ phagemid (Figure 9, Figure 10, and Figure 11). Of particular success was the extremely high concentration of the *M13F* positive control. These RNA transcript were co-transcriptionally labeled with DIG-11-UTP, and were immediately ready for injection into *Helobdella robusta* zygotes.

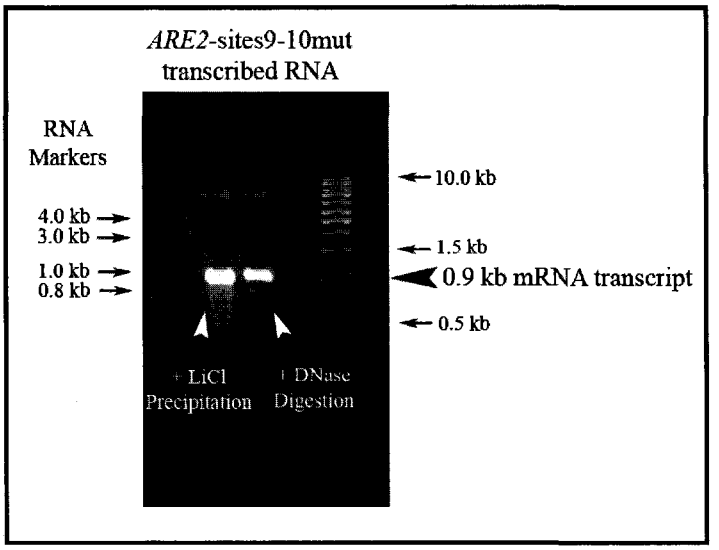


Figure 9: 1.2% agarose gel of *ARE2-sites9-10mut* mRNA transcript. Lane 1: LiCl precipitation of in-vitro reaction. Lane 2: DNase treatment to remove any remaining DNA. Bright band at 0.9kb indicates clean RNA transcript. 1.0 kb RNA ribladder (Fisher).

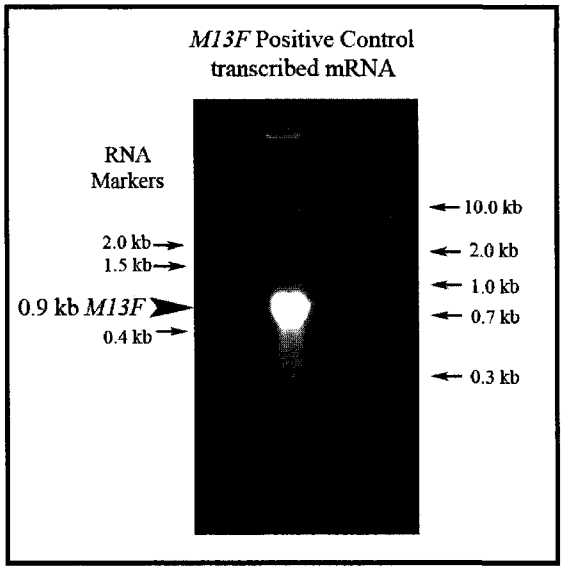


Figure 10: 1.2% agarose gel of control *M13F* mRNA transcript. Sample was DNase treated and LiCl precipitated. Bright band at 0.9kb indicates clean RNA transcript. 1.0 kb RNA ribladder (Fisher).

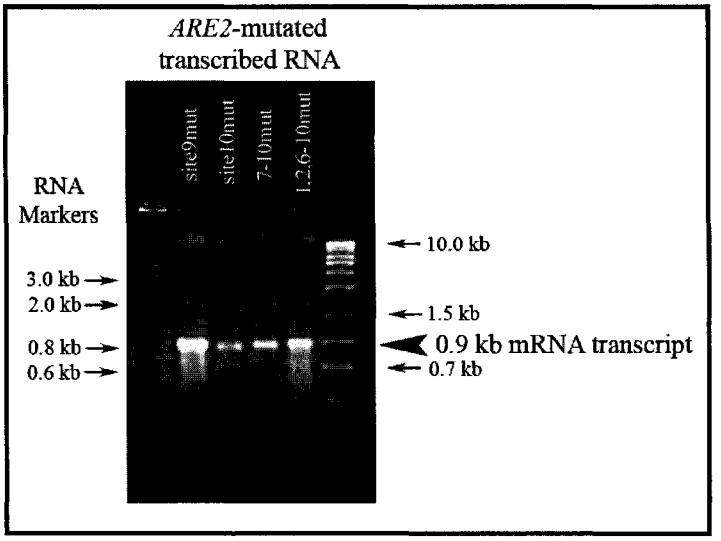


Figure 11: 1.2% agarose gel of mRNA with single and multi-site *ARE2* mutations. Lanes 1 and 2 contain single site *ARE2* mutations. Lanes 3 and 4 contain sequential *ARE2* mutations. Samples were DNase treated and LiCl precipitated. Bright band at 0.9 kb indicates clean RNA transcript. 1.0kb RNA ribladder (Fisher).

4.5. Injection of *Helobdella robusta* Embryos

Upon deposition of embryos, the clutch of 20-50 embryos were divided in half, one set for injection with *M13F* unmutated control mRNA, and the other with *ARE2-sites1,2,6-10mut* mRNA, which contained the maximum achieved total mutations of Sites 1, 2, and 6 through 10. Injections were made as rapid as possible within 20 mins, before 1st polar body formation, and the cells were allowed to incubate until teloplasm formation, about 3-4 hrs later.

This injection period subjected the embryos to rough conditions. Additionally, the embryos further underwent harsh handling during the removal

of the vitelline membrane by 000 sized insect needles. Therefore, roughly more than half the embryos survived to the staining stage (Table 2).

Table 2. Survival of injected *Helobdella robusta* embryos after microinjection and removal of vitelline membrane.

Experiment number	Total Number of embryos injected	Number of Embryos injected w/ control	Number of surviving M13F embryos	Number of Embryos injected w/ mutated 3'UTR	Number of Surviving mutation embryos
1	35	19	13	16	12
2	33	16	10	17	8
3	40	20	14	20	13
4	54	27	15	27	18
5	82	40	18	42	22
6	50	25	13	25	11
7	26	13	6	13	7
8	20	10	3	10	2
9	30	18	7	12	5
10	24	12	4	12	5
11	23	11	5	12	7
12	75	35	13	40	17
13	24	12	7	12	8
14	35	17	13	18	14
15	40	20	16	20	18
16	39	20	18	19	17
17	30	15	14	15	15

Clutches were injected with both *M13F* control mRNA and *ARE2-sites1,2,6-10mut*.

4.6. Staining and Visualization of Injected Embryos

Staining of the DIG-labeled RNA was achieved by anti-Fab fragment incubation, along with coloration with NBT/BCIP reagent. Staining resulted in purple coloration to directly identify exogenous *M13F* and *ARE2-sites1,2,6-10mut* mRNA localization. Clearing of the embryo was achieved by methyl salicylate, which resulted in a translucent light-brown color. Equatorial views of the embryos indicate that both exogenous wildtype and mutated mRNA localized specifically to the animal and vegetal poles (Figure 12). The yolk was devoid of exogenous *Hro-twist* 3'UTR mRNA. There were no noticeable differences in localization pattern between the control and mutated samples from the equatorial view.

However, differences between wildtype and mutated RNA were apparent from the animal views. Side by side comparison of embryos with wildtype *M13F* and mutated 3'UTR clearly show the distinction (Figure 13). The wildtype *M13F* control was concentrated to the center of the teloplasm in both poles. Staining appeared in one distinct and intact area. Exogenous mRNA was clearly absent from the periphery of the cell. In contrast, exogenous *ARE2-site1,2,6-10mut* mRNA aggregated into two distinct areas within poles. Though the majority of the mutated RNA amassed in one large location, a small subset localized as a

separate circular region (Figure 14). When *ARE2* sites 1, 2, and 6 through 10 are mutated, localization is altered such that the mRNA do not get transported to the same regions of the poles as in the *M13F* wildtype control.

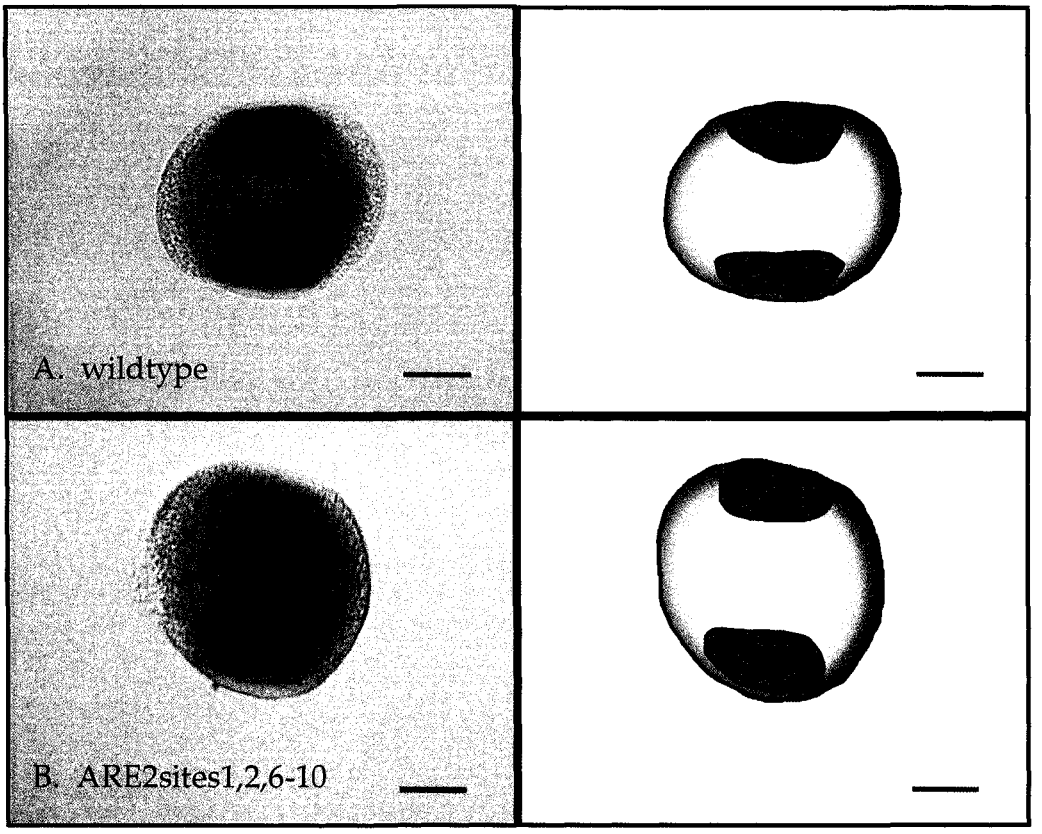


Figure 12: Equatorial views of embryos injected with exogenous wildtype *M13F* and *ARE2-sites1,2,6-10mut.* A: Injection of 12.5 pg of *M13F* wildtype mRNA. B: Injection of 12.5 pg of mutated *ARE2-site1,2,6-10mut* mutated mRNA. Images showed staining of DIG-labeled RNA. Staining by NBT/BCIP incubation with anti-DIG Fab fragments. Overlay images on the right showed that wildtype and mutated *Hro-twist* 3'UTR both localized to the animal and vegetal poles of *Helobdella robusta* cell-1 embryos. Scale bar = 100 μm.

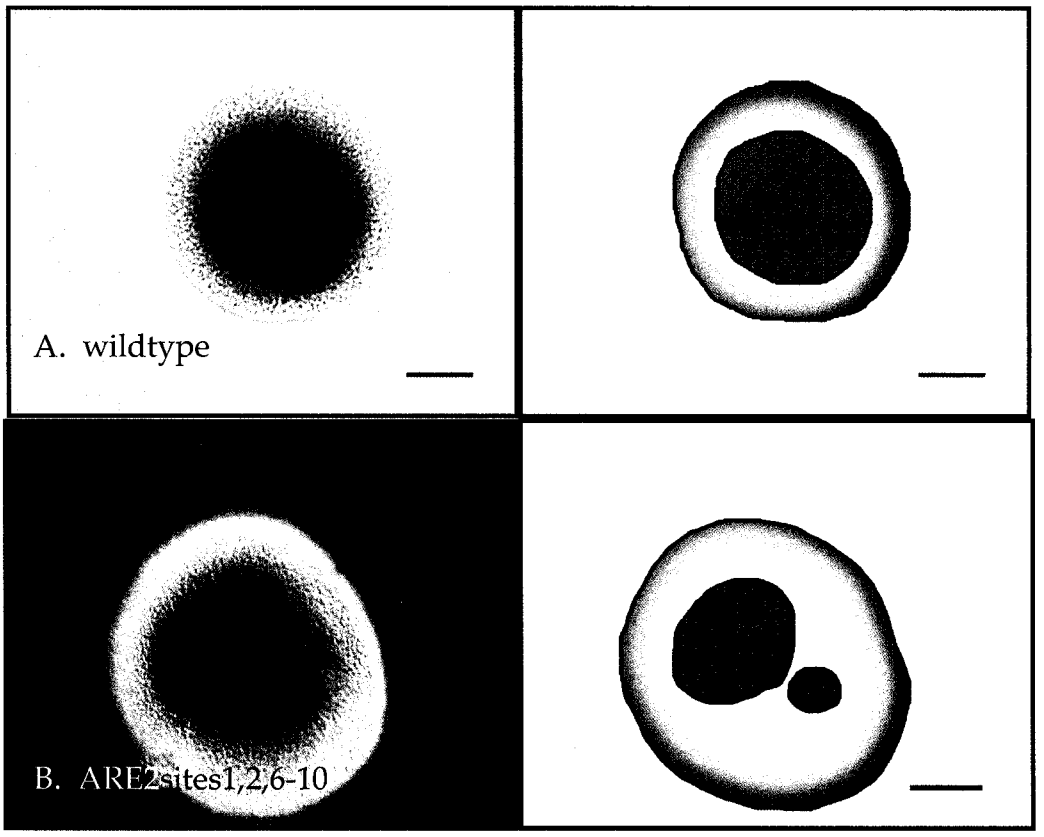


Figure 13: Axial views of embryos injected with wildtype and mutant 3'UTR *Hro-twist*. A: Injection of 12.5 pg of *M13F* wildtype mRNA. B: Injection of 12.5 pg of mutated *ARE2-site1,2,6-10mut* mutated mRNA. Images showed staining of DIG-labeled RNA. Staining by NBT/BCIP incubation with anti-DIG Fab fragments. Overlay images on the right showed that wildtype *Hro-twist* 3'UTR localized to the center of the pole of the cells. *Hro-twist* 3'UTR mRNA with mutations of Sites 1,2,6-10 showed localization to two distinct pools, one of which was larger than the other. Scale bar = 100 μm.

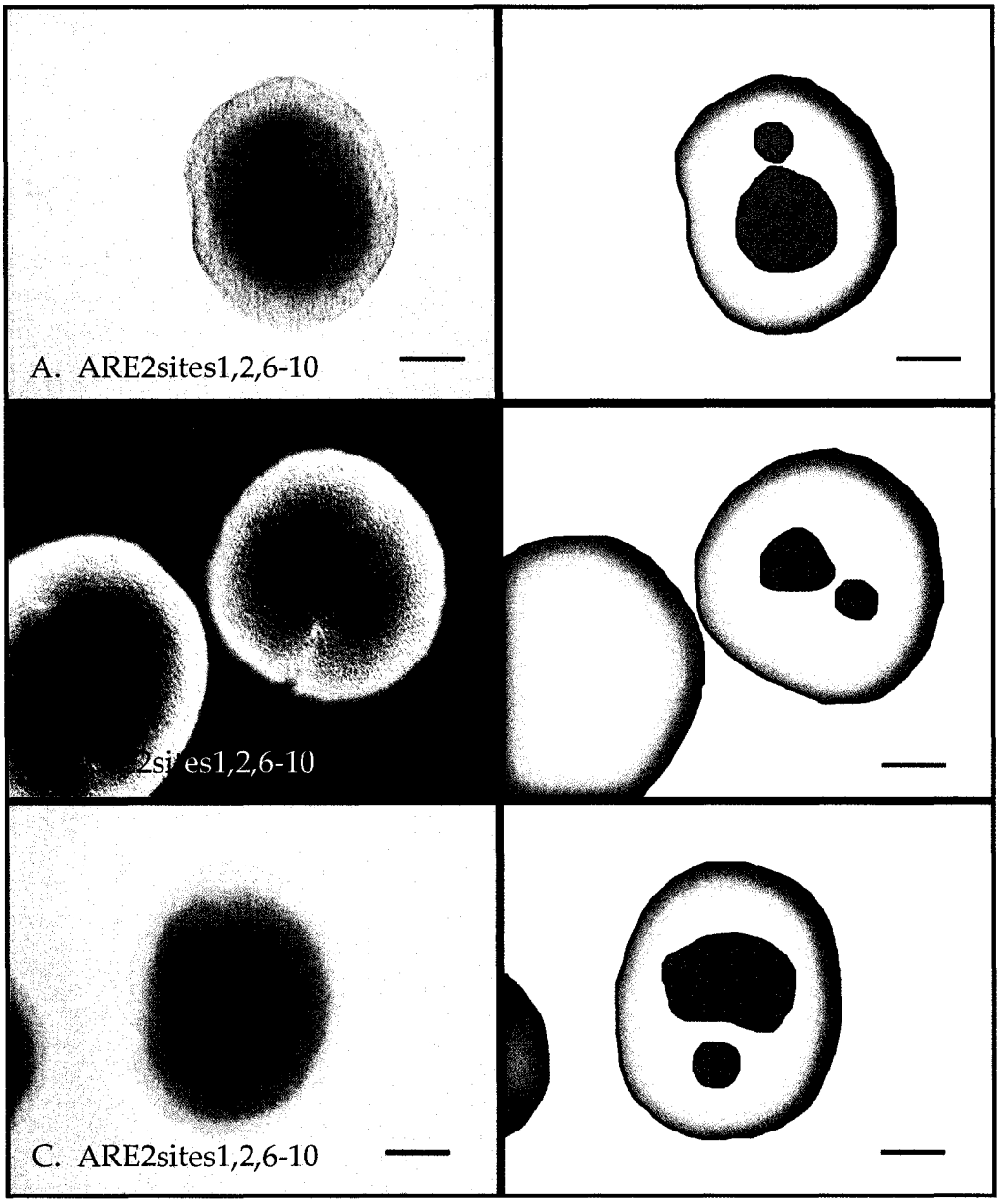


Figure 14: Axial views of embryos injected with mutant 3'UTR *Hro-twist*. Injection of 12.5 pg of *ARE2-site1,2,6-10mut*. Images showed staining of DIG-labeled RNA. Staining by NBT/BCIP incubation of anti-DIG Fab fragments. Overlay images on the right provided additional evidence that mutations of *ARE2* sites resulted in the localization of *Hro-twist* 3'UTR mRNA to two distinct pools, one of which was larger than the other. Scale bar = 100 μm.

Table 3. Percentage of injected embryos that have altered *Hro-twist* localization when *ARE2* sites 1, 2, and 6 through 10 are mutated.

Experiment number	Embryos displaying abnormal localization pattern	Embryos displaying wildtype localization pattern	Embryos overstained inconclusive pattern	Percentage displaying abnormal localization
3	4	4	0	100%
9	4	1	2	80.0%
10	5	1	1	83.33%
15	1	1	4	50.0%

Results show that a significant percentage of one-cell stage *Helobdella robusta* embryos display abnormal localization to the animal and vegetal poles of when injected with *Hro-twist* 3'UTR containing mutated *ARE2* elements. Control injections showed 0% of the observed irregular patterns.

5. Discussion

5.1. mRNA Movement along the Microtubule Cytoskeleton

mRNA localization has been observed in various organisms to transport mRNA for proteins associated to development and cell fate determination. *Xenopus* and *Drosophila* embryos both transport mRNA, such as *bicoid, oskar, nanos, gfap, kor, hairy,* and *gurke* mRNA, which have direct roles in mesoderm formation, cell polarity determination, segmentation of tissue patterns, and other developmental processes of the growing organism(Snee et al., 2004; Nakamura et al., 2004; Wang et al., 2007). It is the organized localization of these mRNAs that allow for the proper spatial expression of necessary proteins at specific sites.

mRNA are transported via the cytoskeletal system, by association with molecular motors that move directionally along microtubulin tracts (Vale and Milligan, 2000). The major families of motor proteins are minus end-directed dyneins and plus end-directed kinesins (Fischer, 2000). Myosins are plus end-directed, and also involves the actin cytoskeleton. For example, in *Drosophila* oogenesis, mRNA transcripts are localized along the microtubule cytoskeleton such that a microtubule organizing center (MTOC) and minus ends are situated at the posterior end of the oocyte, while the plus ends extend to the anterior.

This organization correlates to the behaviors of BicD and egalitarian (Theurkauf et al., 1993; Theurkauf et al., 1998). Also, the minus-end directed kinesin-related protein Nod-β-gal localizes to the anterior, while plus-end motor Khc-β-gal localizes in the opposite direction, supporting that microtubules can be used as localization tracks (Christensen et al., 1987; Christensen et al., 1994). Further evidence indicate that microtubule depolymerizing drugs disrupt localization of *bcd* and *osk* mRNA (Pokrywka et al., 1991; Clark et al., 1994).

5.2. *Cis-acting* Elements in the 3'UTR of mRNAs

Various *trans-acting* factors have been determined to bind to various mRNA. These proteins can recognize *cis-acting* elements within the mRNA and associate with motor proteins for transport (Johnstone and Lasko, 2001; Ashwini and DeRisi, 2007). *Bicoid* mRNA of *Drosophila* embryos contain a localization signal in the 3'UTR that forms a complex secondary structure containing a stem-loop V, known as *the bcd localization element 1*(*BLE1*) (Snee et al., 2004). Mutations of *BLE1*, as well as stem-loops III/IV blocks localization of *bcd* (Macdonald and Kerr, 1998). The protein Exuperantia (Exu) is a *trans*-factor that dimerizes with the *BLE1* region, as well as Swa protein, and Staufen (Stau), which is used in the latter stages of mRNA localization (Meng and Stephenson, 2002; Schnorrer and

Nusslein-Volhard, 2000; Riechmann and Ephrussi, 2004). Associated with *Oskar* mRNA regulation, the proteins Bru, Apontic, p50, and Bicaudal-C have been shown to bind to the mRNA at both the 5′ and 3′ end of the *osk* mRNA (Webster et al., 1997; Gunkel et al.,1998; Nakamura et al., 2004). *Cis-acting* elements of *oskar* include the binding sites *Bru response elements* (*BRE*), located on the 3'UTR (Kim-Ha, et.al, 1995; Rongo, et. al 1995). *Gurken* contains *cis-acting* elements known as *grk localization element 1 and 2* (*GLE1/2*), and the *anterior cortical ring* (*ACR*) element, and include *trans-acting* factors such as cappuccino, spire, and maelstrom (Clegg et al., 1997; Saunders and Cohen, 1999; Thio et al., 2000; Quinlan et al., 2005; Quinlan et al., 2007).

Since *Hro-twist* displays localization in *Helobdella robusta* leeches, it is likely that the transcript contains *cis-acting* elements, as found in other localizing mRNAs. Proteins would therefore dimerize with the *Hro-twist* mRNA, to allow for the recognition and binding to the microtubule cytoskeletal tracks. *Cis-acting* elements are normally found within the UTR regions of the mRNA, primarily in the 3'UTR, as shown in various mRNAs like *bcd* (Snee et al., 2004). These elements appear as short, nontandem repeats, such as the CAC-containing motifs of *Vg1* and *Vg2*, as well as the CAC-rich regions of 3′ UTR of *Xcat-2*, known as the *mitochondrial cloud localization element* (*MCLE*) of *Xenopus* oocytes (Zhou and

King, 1996; Bettley et al., 2002). Other short repeating and conserved clusters include the *Vg1 LS*, the *E2* motif, and the *VM1* motif (Kwon et al., 2002; Lewis et al., 2004; Czaplinksi and Mattaj, 2005). In *Xenopus* oocytes, consensus RNA signals direct germ layer determinants to the vegetal cortex, and suggest that there may be a combination of *cis-acting* elements, clustering of elements, as well as possibly mRNA secondary structure in the characterization of mRNA localization (Bubunenko et al.,2002). Similarily, a REPFIND bioinformatics database search of *Hro-twist* revealed many short repeating clusters within the 3' UTR.

5.3. Mutations of *Hro-twist ARE2* Elements

Fragments of the *Hro-twist* 3' UTR were deleted via PCR, progressively eliminating discovered *cis-acting* elements. Upon microinjection of the transcribed mRNA into *Helobdella robusta* embryos, it was found that localization of the transcripts to the animal and vegetal poles were impaired to a greater extent for shorter fragments that contained less elements, thus suggesting a role of those *cis-acting* elements for mRNA localization. *AU-rich elements* (*ARE2*), containing the adenine and uracil motif AAUAAUA, were specifically targeted for mutation to directly characterize its role in *Hro-twist* mRNA localization.

Sited-directed mutagenesis was used to introduce point mutations within *ARE2* sites, mutating them from AAUAAUA to AGGAAUA, using mutagenic primers (Table 1). It was desired to create transcripts for single site mutations for all ten *ARE2* sites, as well, as well as consecutive mutations from the 5′ end (Figure 5). As shown from the resulting gels and sequencing chromatograms (Figures 2-4), plasmid DNA were successfully mutated and cleanly purified for individual *ARE2* sites 1, 2, 6, 7, 8, 9, and 10. *ARE2* sites 3, 4, and 5 are located within an adenine and thymine rich region, with primer GC% content as low as 8.89%, far lower than the recommended GC% content of 40%. Because adenine and thymine bases only form two hydrogen bonds, annealing strength is diminished. Also, since the primers were longer (up to 56 bases), there was an increased likelihood of primer secondary structure formation, also disrupting annealing to the template. Various trials with altered annealing temperatures and a myriad of primer compositions were designed, but point mutations were unable to be introduced at those particular sites. Since traditional PCR approaches were insufficient to mutate Sites 3, 4, and 5, a new method must be utilized to attain full mutation of the entire *ARE2* sites. Therefore, the transcript containing the multiple site mutations, *ARE2sites1,2,6-10mut*, still retain the 3-4-5 region, which is notably located on a stem-loop structure (Figure 1).

mRNA was successfully *in-vitro* transcribed of all the mutated transcripts, capped with a 5' 7-methyl guanosine nucleotide cap structure, and digoxygenin-labeled. Purified transcripts were free of DNA and short incomplete transcripts, ensuring the RNA samples were all the same length. This is critical because it allows the attribution of any observed effects solely to the mutation of specific *ARE2* sites, rather than deletions of regions, which would unduly remove other possible *cis-acting* elements or substantially alter mRNA structure. In addition to mRNA from the unmutated wildtype control (*M13F*) and the total mutation (*ARE2sites1,2,6-10mut*), a library of mRNA was transcribed to include single site mutations (*ARE2site1mut, ARE2site2mut, ARE2site6mut, ARE2site7mut, ARE2site8mut, ARE2site9mut,* and *ARE2site10mut*), and sequentially mutated transcripts (*ARE2sites9-10mut, ARE2sites8-10mut, ARE2sites7-10mut, ARE2sites6-10mut,* and *ARE2sites1,6-10mut*), ready for future injection studies (Figure 5).

5.4. Mutated *Hro-twist* Reveal Functional Role of *ARE2*

M13F and *ARE2sites1,2,6-10mut* mRNA were successfully injected into *Helobdella robusta* embryos at 1-cell stage, prior to first polar body formation. Staining of the DIG-labeled RNA was achieved by anti-DIG Fab fragments, and incubation with NBT/BCIP coloration reagent. Various factors influence the

quality of mRNA detection, including the microinjection process. *Helobdella robusta* embryos have a 20 minute injection period, so injection of *Hro-twist* after first polar body formation may alter the transportation machinery function, involving the motor protein-cytoskeletal complex or impaired dimerization to *trans-acting* factors. Also, embryo penetration of the needle may physically damage cell integrity, as it was observed that embryos may expel their cytoplamic fluid, and do not mature into the two-cell stage. The removal of the vitelline membrane subjected the embryos through harsh conditions that may damage the cell membrane. Breaks in the membrane could lead to unequal incorporation of anti-DIG antibodies, as well as unequal distribution of the coloration reagent, resulting in misleading staining. Finally, incubation time with the NBT/BCIP and the clearing reagent methyl salicylate could result in under-staining, and more commonly observed, over-staining. Overstaining of several embryos overwhelm the cell such that differences in localization are undifferentiable between the wildtype and mutated *Hro-twist* mRNA.

Equatorial views of successfully injected and stained embryos show that *Hro-twist* 3'UTR does in fact localize to the animal and vegetal poles by teloplasm formation of 1-cell stage embryos (Figure 12). Localization appears slightly asymmetric, in agreement with other mRNAs that also localize asymmetrically to

embryo poles, such as *Bcd, orb,* and *oskar* (Chartrand et al., 2001; Johnstone and Lasko, 2001; Castagnetti and Aphrussi,k 2003; Snee, 2007). Localization indicates that the 3'UTR of *Hro-twist* may contain functional *cis-acting* elements, and bioinformatically predicted to be *ARE1, ARE2, ACE1,* and *ACE2.* The localization of the full length *Hro-twist* transcript is most likely due to elements found only in the 3'UTR. The 3'UTR of other localizing mRNA transcripts have been determined to also contain localization elements (Betley et al., 2002; Kwon et al., 2002; Lewis et al., 2004; Czaplinksi and Mattaj, 2005). *ARE2* sites 1, 2, and 6 through 10 do not appear to have a strong role in the axial transport directly from the yolk and towards the poles because exogenous mRNA was able to migrate to the poles (Figure 12).

However, some equatorial staining images indicate that slight differences may occur at the distribution of exogenous mRNA at the vegetal and animal poles. Upon further analysis, axial images from animal and vegetal poles present a stark contrast between exogenous wildtype and mutated *Hro-twist* 3'UTR mRNA (Figures 13 and Figure 14). In the exogenous wildtype, *Hro-twist* 3'UTR aggregated in a centralized area, with the periphery clear of exogenous mRNA. *Hro-twist* 3'UTR localized as previously observed in leech embryos (Soto, 1997). However, injection of mutated *Hro-twist* with mutated *ARE2* sites resulted in

abnormal localization at the poles. The majority of the transcript localized to one main area, but appeared off-centered when compared to the *M13F* wildtype. The localization machinery was sufficient to localize the mRNA generally to the poles, however, the specific destination was altered. Additionally, a second lesser pool of mutated exogenous *Hro-twist* mRNA localized to a significantly smaller region adjacent to the main area. The target destination of these transcripts were severely altered, and were distinct from the rest of the localized mRNA. This may indicate that *ARE2* elements are utilized at the onset of the binding to correct motor proteins, altering the path along the microtubule cytoskeleton, ultimately leading to a different destination (Fischer, 2000; Vale and Milligan, 2000). Since Twist protein plays roles in mesoderm formation, segmentation, and ventral-dorsal polarity determination (Sandmann, 2007) and *Helobdella robusta* embryos express Hro-twist protein (Nelson, 2004), the uncharacteristic localization *Hro-twist* would lead to aberrations in development that would cause abnormal cellular fates, and possibly death.

5.5. Future Studies of *Hro-twist* Localization

Future goals of *Hro-twist* research would involve determining which specific *ARE2* sites have stronger functions, using the previously transcribed

mRNA of single and sequential mutations of 3' *Hro-twist*. Also, it should be determined if clusters of *ARE2* twist play a stronger role than individual sites, as suggested in *Vg1* localization in *Xenopus* (Lewis et al., 2004). Of interest is the position of *ARE2* elements among the secondary mRNA structure which also has roles in mRNA localization (Bubunenko et al. ,2002; Snee et al., 2004). Site 3, 4, and 5, though unable to be mutated by site-directed mutagenesis, occupy the same stem-loop branch, and may be deleted to ascertain possible roles in localization. Also, the 3'UTR can be co-transcribed with the coding region, and possibly other transcripts to further strengthen its role in mRNA localization. Finally, it should be determined what proteins recognize and bind to the *ARE2* sites, and function as *trans-acting* factors.

mRNA localization is a commonly utilized means of regulating the location of proteins expression. It is critical in designating developmental proteins to sites of cell type determination, necessary for organismal development. *Hro-twist* is crucial in proper mesoderm formation in the *Helobdella robusta* embryo, and *cis-acting* elements, such as *ARE2*, and *trans-acting* factors are tightly orchestrated to achieve proper development.

References

Agee, S.J., Lyons, D.C., Weisblat, D.A., 2006. Maternal expression of a NANOS homolog is required for early development of the leech *Helobdella robusta*. *Dev Biol* 298 (1), 1-11.

Bashirullah, A, Cooperstock, RL, Lipshitz, HD, 1998. RNA localization in development. *Annu Rev Biochem* 67, 335-94.

Bassell, G., Kelic, S.,2004. Binding proteins for mRNA localization and local translation, and their dysfunction in genetic neurological disease. *Cur. Opin Neur* 14, 574-581.

Bate, M., 1993. The development of *Drosophila melanogaster. Cold Spring Harbor Laboratory Press* 1, 1013–1090.

Baylies, M.K., Bate, M., 1996. Twist, a myogenic switch in *Drosophila. Science* 272, 1481–1484.

Beiman, M., Shilo, B.Z., Volk, T., 1996. Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev* 10, 2993–3002.

Bettley, J.N., Frith, M.C., Graber, J.H., Choo, S., Deshler, J.O., 2002. A ubiquitous and conserved signal for RNA localization in chordates. *Curr Biology* 2 (20), 1756-1761.

Bi, J., Tsai, N-P., Lin, Y-P., Loh, H.H., Wei, L-N, 2006. Axonal mRNA transport and localized translational regulation of κ-opioid receptor in primary neurons of dorsal root ganglia. *Proc Natl Acad Sci* 103 (52), 19919–19924.

Bissen, S.T., 1997. Developmental control of cell division in leech embryos. *Bioessays* 3, 201-7.

Bour, B., O'Brien, M.A., Lockwood, W., Goldstein, E.S., Bodmer, R., Taghert, P., Abmayr, S.M., Nguyen, H.T., 1995. Drosophila MEF2, a transcription factor that is essential for myogenesis. *Genes Dev* 4, 1322–1331.

Brent, A.E., Yucel, G., Small, S., Desplan, C., 2007. Permissive and instructive anterior pattern rely on mRNA localization in the wasp embryo. *Science* 315 (5820), 1841-3.

Bubunenko, M., Kress, T.L., Vempati, U.D., Mowry, K.L., King, M.L., 2002. A Consensus RNA Signal That Directs Germ Layer Determinants to the Vegetal Cortex of Xenopus Oocytes. *Developmental Biol* 248 (1), 82-92.

Bullock, S. L., D. Zicha, and D. Ish-Horowicz. 2003. The Drosophila hairy RNA localization signal modulates the kinetics of cytoplasmic mRNA transport. *EMBO J* 22, 2484–2494.

Campuzano, S., Carramolino, L., Cabrera, C., Ruiz-Gomez, M., Villares, R., Boronat, A., Modolell, J., 1985. Molecular genetics of the achaetescute gene complex of *Drosophila melanogaster*. *Cell* 40, 327–338.

Carson, J.H., Kwan, S., Barbarese, E., 1998. RNA trafficking in myelinating cells. *Curr. Opin. Neuro.* 8, 607-612.

Castagnetti, S., Ephrussi, A., 2003. Orb and a long poly(A) tail are required for efficient oskar translation at the posterior pole of the *Drosophila oocyte*. *Development* 130 (5), 835-43.

Castanon, I., Von Stetina, S., Kass, J., Baylies, M.K., 2001. Dimerization partners determine the activity of the Twist bHLH protein during *Drosophila* mesoderm development. *Development* 128, 3145–3159.

Chan A.P., Kloc M., Larabell, C.A., LeGros, M., Etkin, L.D., 2007. The maternally localized RNA fatvg is required for cortical rotation and germ cell formation. *Mech Dev* 124 (5), 350-63.

Chartrand P., X-H., Meng, R.H. Singer and R.M. Long, 1999. Structural elements required for the localization of *ASH1* mRNA and of a green fluorescent protein reporter particle in vivo. *Elsevier Science Ltd* 9, 333-336.

Chen, Z.F., Behringer, R.R., 1995, twist is required in head mesenchyme for cranial neural tube mophogenesis. *Genes Dev* 9 (6), 686-699.

Christensen, AK, Kahn, LE, Bourne, CM, 1987. Circular polysomes predominate on the rough endoplasmic reticulum of somatotropes and mammotropes in the rat anterior pituitary. *Am. J. Anat.* 178, 1–10.

Christenson, LB, McKearin, DM, 1994. orb is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. *Genes Dev* 8, 614–28.

Clark I, Giniger E, Ruohola-Baker H, Jan LY, Jan YN, 1994. Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol.* 4, 289–300.

Clegg NJ, Frost DM, Larkin MK, Subrahmanyan L, Bryant Z, Ruohola-Baker H, 1997. Maelstrom is required for an early step in the establishment of *Drosophila* oocyte polarity: posterior localization of *grk* mRNA. *Development* 124 (22), 4661-71.

Cohen, RS, Zhang, S, Dollar, GL, 2005. The positional, structural, and sequence requirements of the *Drosophila TLS* RNA Localization element. *RNA* 11 (7), 1017-29.

Cote, C.A., Gautreau, D., Terry, N.A., Mowry, K.L., 1999. A *Xenopus* protein related to hnRNP I has an essential role in cytoplasmic RNA localization. *Mol. Cell* 4, 431–437.

Cox, V., Baylies, M.K., 2001. Investigating somatic muscle specification through analysis of the twist promoter. *A. Dros. Res. Conf.* 42, 673.

Czaplinski, K., Mattaj, I.W., 2006. LoVe interacts with Vg1RBP/Vera and hnRNP I in binding the Vg1-localization element. *RNA* 12 (2), 213-22.

Davis, I., and D. Ish-Horowicz. 1991. Apical localization of pair-rule transcripts requires 3′ sequences and limits protein diffusion in the *Drosophila* bastoderm embryo. *Cell* 67, 927–940.

Deshler, J.O., Highett, M.I., Abramson, T., Schnapp, B.J., 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. *Curr. Biol.* 8, 489–496.

Deshler, J.O., Highett, M.I., Schnapp, B.J., 1997. Localization of *Xenopus* Vg1 mRNA by vera protein and the endoplasmic reticulum. *Science* 276, 1128–1131.

Du, T., Schmid, M., Jansen, R.P., 2007. Why cells move messages: The biological functions of mRNA localization. *Semin Cell Dev Biol* 2, 171-7.

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

El Ghouzzi, V., Le Merrer, M., Perrin-Schmitt, F., Lajeunie, E., Benir, P., Renier, D., Bourgeois, P., Bolcato-Bellemin, A., Munnich, A., Bonaventure, J., 1997. Mutations of the TWIST gene in the Saethre-Chotzen syndrome. *Nat. Genet.* 15, 42–46.

Emmons S, Phan H, Calley J, Chen W, James B, Manseau L, 1995. cappuccino, a *Drosophila* maternal effect gene required for polarity of the egg and embryo, is related to the vertebrate limb deformity locus. *Genes Dev* 9, 2482–94

Ephrussi, A., Church, G., Tonegawa, S., Gilber, W., 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. *Science* 250, 1104–1111.

Fernández, J., 1980. Embryonic development of the Glossiphoniid Leech Theromyzon rude: Characterization of developmental stages. *Dev. Biol.* 76, 245-263.

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

Fischer, JA. 2000. Molecular motors and developmental asymmetry. *Curr. Opin. Genet. Dev.* 10, 489–96.

Gisselbrecht, S., Skeath, J., Doe, C., Michelson, A., 1996. Heartless encodes a fibroblast growth factor receptor involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev* 10, 3003–3017.

Gitelman, I., 1997. Twist protein in mouse embryogenesis. *Dev. Biol.* 189, 205–214.

Gleizer, L. Stent, G.S., 1993. Developmental origin of segmental identity in the leech mesoderm. *Development* 117, 177-189.

Goldstein B., Leviten, M.W., Weisblat, D.A., 2001. Dorsal and snail homologs in leech development. *Dev Genes Evol* 211 (7), 329-37.

Gramolini, A., Belanger, Jasmin, B., 2001. Distinct regions in the 3' untranslated region are responsible for targeting and stabiling utrophin transcripts in skeletal muscle cells. *J of Cell Biol* 154, 1173-1183.

Gripp, K.K., Zackai, E.H., Stolle, C.A., 2000. Mutations in the human TWIST gene. *Hum. Mutat.* 15, 150–155.

Gunkel N, Yano T, Markussen FH, Olsen LC, Ephrussi A, 1998. Localization dependent translation requires a functional interaction between the 5′ and 3′ ends of *oskar* mRNA. *Genes Dev* 12, 1652–64

Halfon, M.S., Carmena, A., Gisselbrech, S., Sackerson, C.M., Jimenez, F., Baylies, M.K., Michelson, A.M., 2000. Ras pathway specificity is determined by the integration of multiple signal-activated and tissue restricted transcription factors. *Cell* 103 (1), 63–74.

Hamamori, Y., Sartorelli, V., Ogryzko, V., Puri, P.L., Wu, H.Y., Wang, J.Y., Nakatani, Y., Kedes, L., 1999. Regulation of histone acetyltransferase p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* 96 (3), 405–413.

Harfe, B.D., Fire, A., 1998. Muscle and nerve-specific regulation of a novel NK-2 class homeodomain factor in *Caenorhabditis elegans*. *Development* 125 (3), 421–429.

Hilgers, V., Pourquie, O., and Dubrulle, J, 2005. *In vivo* analysis of mRNA stability using the Tet-Off system in the chicken embryo. *Dev Biol* 284, 292-300.

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

Horne-Badovinac S., Bilder D., 2008. Dynein regulates epithelial polarity and the apical localization of *stardust* mRNA. *PLoS Genet* 4 (1), 8.

Howard, T., Paznekas, W., Green, E., Chiang, L., Ma, N., De Luna, R., Delgado, C., Gonzalez-Ramos, M., Kline, A., Munnich, A., Bonaventure, J., 1997. Mutations of the TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nat. Genet.* 15, 36–41.

Huynh, J.R., St Johnston, D., 2000. The role of BicD, Egl, Orb and the microtubules in the restriction of meiosis to the *Drosophila* oocyte. *Development* 127 (13), 2785-94.

Ip, Y.T., Park, R.E., Kosman, D., Bier, E., Levine, M., 1992. The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev* 6 (9), 1728–1739.

Ip, Y.T., Park, R.E., Kosman, D., Yanzdanbakhsh, K., Levine, M., 1992b. Dorsal-twist interactions establish snail expression in the presumptive mesoderm of the *Drosophila* embryo. *Genes Dev* 6 (8), 1518–1530.

Irvine, S.M. and Martindale, M.Q., 1996. Cellular and molecular mechanisms of segmentation in annelids. *Semin. Cell Dev. Biol.* 7, 593-604

Jambhekar, A., DeRisi, J.L, 2007. Cis-acting determinants of asymmetric, cytoplasmic RNA transport. *RNA* 13 (5), 625–642.

Jansen, R.P., 2001. mRNA localization: message on the move. *Nat. Rev. Mol. Cell Biol* 2, 247-256.

Jansen, R.P., Kiebler, M., 2005. Intracellular RNA sorting, transport, and localization. *Nat Struct Mol Biol* 12 (10), 826-9.

Johnstone, O. Lasko, P., 2001. Translational regulation and RNA localization in *Drosophila* oocytes and embryos. *Annu Rev Genet* 35, 365-406.

Kadesch, T., 1993. Consequences of heteromeric interactions among helixloop-helix proteins. *Cell Growth Differ* 4, 49–55.

Kang D., Pilon M., Weisblat D.A., 2002. Maternal and zygotic expression of a nanos-class gene in the leech *Helobdella robusta*: primordial germ cells arise from segmental mesoderm. *Dev Biol* 245 (1), 28-41.

Karlin-McGinness, M., Serano, T.L., and Cohen, R.S. 1996. Comparative analysis of the kinetics and dynamics of *K10, bicoid,* and *oskar* mRNA localization in the *Drosophila* oocyte. *Dev. Genet.* 19, 238–248.

Kim-Ha J, Kerr K, Macdonald PM. 1995. Translational regulation of *oskar* mRNA by Bruno, an ovarian RNA-binding protein, is essential. *Cell* 81, 403–12.

Kim-Ha, J., J. L. Smith, and P. M. Macdonald, 1991. *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. Cell 66, 23–35.

Kloc, M., N. R. Zearfoss, and L. D. Etkin, 2002. Mechanisms of subcellular mRNA localization. Cell 108, 533–544.

Kwon, S, Abramson, T., Munro, T.T., John, C.M., Kohrmann, M., Schnapp, B.J., 2002. UUCAC- and vera-dependent localization of *VegT* RNA in *Xenopus* oocytes. *Curr Biol* 12 (7), 558-64.

Lantz, V. and Schedl, P. 1994. Multiple cis-acting target sequences are required for orb mRNA localization during *Drosophila* oogenesis. *Mol. Cell. Biol.* 14, 2235–2242.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., Weintraub, H., 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with W12/E47-like proteins in vivo. *Cell* 66, 305–315.

Lee, Y.M., Park, T., Schulz, R.A., Kim, Y., 1997. Twist-mediated activation of the NK-4 homeobox gene in the visceral mesoderm of *Drosophila* requires two distinct clusters of E-box regulatory elements. *J. Biol. Chem* 272 (28), 17531–17541.

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

Leptin, M., Grunewald, B., 1990. Cell shape changes during gastrulation in *Drosophila. Development* 110, 73–84.

Lewis, RA, Kress, TL, Cote, CA, Gautreau, D., Rokop, ME, Mowry, KL, 2004. Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes. *Mech Dev*, 121 (1), 101-9.

Lilly, B., Shao, B., Ranganayakulu, G., Paterson, B., Schulz, R., Olson, E.N., 1995. Requirement of MADS domain transcription factor DMEF2 for muscle formation in *Drosophila. Science* 267, 1404–1407.

Lipshitz, H.D., Smibert, C.A., 2000. Mechanisms of RNA localization and translational regulation. *Curr. Op. Gen. Dev.* 10, 476-488.

Macdonald, P. M., and K. Kerr, 1998. Mutational analysis of an RNA recognition element that mediates localization of *bicoid* mRNA. *Mol. Cell. Biol* 18, 3788–3795.

Mach, J. M., and R. Lehmann, 1997. An Egalitarian-BicaudalD complex is essential for oocyte specification and axis determination in *Drosophila*. *Genes Dev* 11, 423–435.

Manseau LJ, Schupbach T, 1989. Cappuccino and Spire: two unique maternal effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Dev* 3, 1437–52

Master, V.A., Kourakis, M.J., Martindale, M.Q., 1996. Isolation, characterization, and expression of Le-msx, a maternally expressed member of the msx gene family from the glossiphoniid leech, *Helobdella*. *Dev Dyn*. 207 (4), 404-19.

Meng J., Stephenson, E.C., 2002. Oocyte and embryonic cytoskeletal defects caused by mutations in the *Drosophila* swallow gene. *Dev Genes Evol* 212 (5), 239-47.

Mohr, E., Richter, D., 2001. Messenger RNA on the move: implications for cell polarity. *Int. J. Biochem. Cell Biol* 33, 669-679.

Müller, M., Heuck, A., Niessing, D., 2007. Directional mRNA transport in eukaryotes: lessons from yeast. *Cell Mol Life Sci* 64 (2), 171-80.

Murre, C., McCaw, P.S., Baltimore, D., 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell 56*, 777–783.

Nakamura, A., Sato, K., Hanyu-Nakamura, K., 2004. *Drosophila* cup is an eIF4E binding protein that associates with Bruno and regulates *oskar* mRNA translation in oogenesis. *Dev Cell* 6 (1), 69-78.

Navarro, C., H. Puthalakath, J. M. Adams, A. Strasser, and R. Lehmann. 2004. Egalitarian binds dynein light chain to establish oocyte polarity and maintain oocyte fate. *Nat. Cell Biol.* 6, 427–435.

Nelson, C., 2004. Distribution of Hro-two protein in Helobdella leech embryos [thesis]. San Jose (CA): San Jose State University.

Neuman-Silberberg FS, Schüpbach T,,1993. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* 75, 165–74

Nüsslein-Volhard, C. Wieschaus, E. and Kluding, H., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophil melanogaster*: Zygotic loci on the second chromosome. *Rouxs Arch. Dev. Biol.* 193, 267-282.

Olynikov, Y., and Singer, R.H. RNA localization: different zipcodes, same postman? *Cell Biol* 8, 381-394

Pham, A.-D., Mu¨ller, S., Sauer, F., 1999. Mesoderm-determining transcription in *Drosophila* is alleviated by mutations in TAFII60 and TAFII110. *Mech. Dev. 84*, 3–16.

Pokrywka, NJ, Stephenson, EC, 1991. Microtubules mediate the localization of bicoid RNA during *Drosophila* oogenesis. *Development* 113, 55–66.

Quinlan, M.E., Heuser, J.E, Kerkhoff, E., Mullins, R.D., 2005. *Drosophila* Spire is an actin nucleation factor. *Nature* 433 (7024), 382-8.

Quinlan, M.E., Hilger, S., Bedrossian, A., Mullins, R.D., Kerhoff, E., 2007. Regulatory interactions between two actin nucleators, Spire and Cappuccino. *J Cell Biol* 179(1), 117-28.

Riechmann, V., Ephrussi, A., 2004. Par-1 regulates *bicoid* mRNA localisation by phosphorylating Exuperantia. *Development* 131 (23), 5897-907.

Rongo C, Gavis ER, Lehmann R, 1995. Localization of *oskar* RNA regulates oskar translation and requires Oskar protein. *Development* 121, 2737–46

Sandmann, T. Girardot, C. Brehme, M., Tongprasit, W., Stolc, V. Furlong, E.E, 2007. A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev* 21(4), 436-49.

Saunders C, Cohen RS, 1999. The role of oocyte transcription, the 5′ UTR, and translation repression and derepression in *Drosophila gurken* mRNA and protein localization. *Mol. Cell* 3, 43–54

Schmid, M., Jaedicke, A., Du, T.G., Jansen, R.P., 2006. Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. *Curr Biol* 16 (15), 1538-43.

Schnorrer, F., Bohmann, K., Nusslein-Volhard, C.,2000. The molecular motor dynein is involved in targeting *swallow* and *bicoid* RNA to the anterior pole of *Drosophila* oocytes. *Nat Cell Biol* 2 (4), 185-90.

Serano, T. L., and R. S. Cohen. 1995. Gratuitous mRNA localization in the *Drosophila* oocyte. *Development* 121, 3013–3021.

Serano, T.L. and Cohen, R.S. 1995a. A single small stem–loop structure mediates *K10* mRNA localization. *Development* 121, 3809–3818.

Shi, L., Lin, S., Grinberg, Y., Beck, Y., Grozinger, C.M., Robinson, G.E., Lee, T., 2007. Roles of Drosophila Kruppel-homolog 1 in neuronal morphogenesis. *Dev Neurobiol* 67 (12), 1614-26.

Shirokawa, J.M., Courey, A.J., 1997. A direct contact between the Dorsal rel homology domain and Twist may mediate transcriptional synergy. *Mol. Cell Biol* 17 (6), 3345–3355.

Shishido, E., Ono, N., Kojima, T., Saigo, K., 1997. Requirements of DFR1 Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* 124, 2119–2128.

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

Snee MJ, Arn EA, Bullock SL, Macdonald PM, 2005. Recogntion of the *bcd* mRNA localization signal in *Drosophila* embryos and ovaries. *Mol Cell Biol* 25 (4), 1501-10.

Snee, M.J., Harrison, D., Yan, N., Macdonald, P.M., 2007. A late phase of Oskar accumulation is crucial for posterior patterning of the *Drosophila* embryo, and is blocked by ectopic expression of Bruno. *Differentiation* 75 (3), 246.

Sokol, N.S. Ambros, V., 2005. Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev* 19(19), 2343-54.

Song M.H., Huang, F.Z., Chang, G.Y., Weisblat, D.A, 2002. Expression and function of an even-skipped homolog in the leech *Helobdella robusta*. *Development* 129 (15), 3681-92.

Song M.H., Huang, F.Z., Gonsalves, F.C.., Weisblat, D.A, 2004. Cell cycle-dependent expression of a hairy and Enhancer of split (hes) homolog during cleavage and segmentation in leech embryos. *Dev Biol* 269 (1), 183-95.

Soto, J.G., 1994. Hro-twi, a leech homolog of the *Drosophila* morphogen twist, is a maternally inherited factor in the leech embryo, and a mesodermal marker during epiboly [dissertation]. Berkeley (CA): University of California at Berkeley.

Soto, J.G., Nelson, B.H., Weisblat, D.A., 1997. A leech homolog of twist: evidence for its inheritance as a maternal mRNA. *Gene* 199, 31–37.

Spicer, DB, Rhee, J, Cheung, WL, Lasser, AB, 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* 272 (5267) 1476-80.

Spring, J., Yanze, N., Middel, A.M., Stierwald, M., Groger, H., Schmid, V., 2000. The mesoderm specification factor Twist in the life cycle of Jellyfish. *Dev. Biol* 28, 363–375.

Tavares, A.T., Izpisua-Belmonte, J.C., Rodriguez-Leon, J., 2001. Developmental expression of Chick twist and its regulation during limb patterning. *Int. J. Dev. Biol* 45 (5–6), 707–713.

Taylor, M.V., Beatty, K., Hunter, H., Baylies, M., 1995. *Drosophila* MEF2 is regulated by twist and is expressed in both the primordia and differentiated cells of the embryonic somatic, visceral and heart musculature. *Mech. Dev.* 50, 29–41.

Takizawa, P.A., Sil, A., Swedlow, J.R., Herskowitz, I, Vale, R.D., 1997. Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. *Nature* 389 (6646), 90-3.

Theurkauf, WE, Alberts, BM, Jan ,YN, Jongens TA, 1993. A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* 118, 1169–80.

Theurkauf, WE, Hazelrigg, TI, 1998. In vivo analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway. *Development* 125, 3655–66

Thio GL, Ray RP, Barcelo G,Schüpbach T, 2000. Localization of *gurken* RNA in *Drosophila* oogenesis requires elements in the 5′ and 3′ regions of the transcript. *Dev. Biol.* 221, 435–46

Thisse, B., El Messal, M., and Perrin-Schmitt, F., 1987. The twist gend: isolation of a *Drosophila* zygotic gene necessary for the establishment of dorso-ventral pattern. *Nucleic Acids Res.* 15 (8), 3439-3453.

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

Vale, RD, Milligan, RA, 2000. The way things move: looking under the hood of molecular motor proteins. *Science* 288, 88–95.

Wang, S.M., Coljee, V.W., Pignolo, R.J., Rotenberg, M.O., Cristofalo, V.J., Sierra, F., 1997. Cloning of the human twist gene: its expression is retained in adult mesodermally-derived tissues. *Gene* 187, 83–92.

Wang, W., Van Niekerk, E, Willis D.E., Twiss, J.L., 2007. RNA transport and localized protein synthesis in neurological disorders and neural repair. *Dev Neurobiol* 9, 1166-82.

Webster PJ, Liang L, Berg CA, Lasko P, Macdonald PM, 1997. Translational repressor Bruno plays multiple roles in development and is widely conserved. *Genes Dev* 11, 2510–21

Weisblat, D.A., 2007. Asymmetric cell divisions in the early embryo of the leech *Helobdella robusta. Prog Mol Subcell Biol* 45, 70-95.

Wihelm, J.E., Smibert, C.A., 2005. Mechanisms of translational regulation in *Drosophila. Biol Cell* 97 (4), 235-52.

Wihelm, J.E., Vale, RD,1993. RNA on the move: the mRNA localization pathway. *J Cel Biol* 123 (2), 269-74.

Zhou, Y., King, M.L., 1996. Localization of *Xcat*-2 RNA, a putative germ plasma component, to the mitochondrial cloud in *Xenopus* stage I oocytes. *Development* 122, 2947–2953.